Preface

The complement system, first described more than a century ago, was for many years the ugly duckling of the immunology world, but no more. Complement in recent years has blossomed into a fascinating and fast moving field of immediate relevance to clinical scientists in fields as diverse as transplantation biology, virology, and inflammation. Despite its emergence from the shadows, complement retains an unwarranted reputation for being "difficult." This impression derives in large part from the superficially complicated nomenclature, a relic of the long and tortuous process of unraveling the system, of naming components in order of discovery rather than in a systematic manner. Once the barrier of nomenclature has been surmounted, then the true simplicity of the system becomes apparent.

Complement comprises an activation system and a cytolytic system. The former has diverged to focus on complement to distinct targets—bacteria, immune complexes, and others—so that texts now describe three activation pathways, closely related to one another, but each with some unique features. The cytolytic pathway is the same regardless of the activation process and kills cells by creating pores in the membrane. Complement plays an important role in killing bacteria and is essential for the proper handling of immune complexes. Problems occur when complement is activated in an inappropriate manner—the potent inflammation-inducing products of the cascade then cause unwanted tissue damage and destruction.

Complement's renaissance has been driven in large part by the discovery of the complement regulatory molecules and the realization that these molecules and other agents can provide effective anticomplement agents for use in therapy. As newer and better anticomplement agents become available, the requirement for laboratories to assess complement activation in clinical samples and to monitor the effects of anticomplement agents will grow.

Complement Methods and Protocols aims to provide a comprehensive source of up-to-date protocols for the study of the complement system, both for the basic scientist interested in understanding the mechanisms of activation and the clinical scientist wishing to quantify complement activation. In the first

chapter, the complement system is briefly reviewed to set the stage for the methods chapters to follow. The next two chapters describe methods for purifying complement components, using classical chromatography and immunoaffinity approaches, respectively. Chapters 4 to 6 describe methods for the functional analysis of complement components, regulators, enzymes, and complexes, including a detailed description of the generation of the depleted sera essential for complement assays. Methods for measurement of complement activation fragments and complexes deposited on cells, in tissues, or in biological fluids are detailed in Chapters 7 to 10. Chapter 11 provides an overview of screening methods for identifying and assessing complement deficiency and Chapter 12 a detailed account of methods needed to assess deficiency of C1 inhibitor. Other clinically relevant protocols for analysis of complement autoantibodies, immune complexes, and complement allotypes are provided in Chapters 13 to 15. Chapter 16 departs from the main theme of the book to describe protocols for generating gene-deleted mice, included here because of the enormous influence such methods are now having on complement research. The final chapter reviews complement deficiencies in experimental animals, listing the different complement deficiencies defined in animals and the experimental models in which these deficient animals have been examined.

I am grateful to my friends and colleagues who have contributed to this volume for their willingness to make time in their busy schedules. In particular, I wish to thank the members of the Complement Biology Group in Cardiff, many of whom have contributed chapters to this volume and others who have reviewed parts of the manuscript or contributed to the tedious task of assembling the appendices. I promise I won't do it again in a while! Finally, thanks to The Wellcome Trust for their continued and generous support of complement research in Cardiff.

B. Paul Morgan

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Purification of Complement Components, Regulators, and Receptors by Classical Methods

Carmen W. van den Berg

1. Introduction

1.1. Overview

The aim of this chapter is to describe methods for purification of the individual complement (C) components using classical chromatography methods available in most biochemistry laboratories. None of these methods require the large amounts of specific antibodies needed for the popular and rapid immunoaffinity methods to be described in Chapter 3. A further advantage of classical chromatography is that the methods described in this chapter for human C can easily be adapted for purification of C components of other species. Indeed, some of the methods described here were originally developed for the purification of animal components, but proved also to be suitable for human components. One precautionary note when adapting methods to C components of another species is that there is occasionally species incompatibility in that some components will not function in combination with components of another species. Functional assays may, therefore, require modification. It should also be noted that antisera and monoclonal antibodies against a C component frequently do not crossreact with that component from other species.

An important consideration when purifying C components is the freshness of the serum or plasma used as raw material. It is important to start whenever possible with fresh plasma because this will increase the yield of active components. In stored plasma, C components will have undergone proteolysis or other forms of inactivation to varying degrees. C activation and consumption of C components is also a potential problem, particularly if serum is used as source. To prevent C activation, the use of plasma is preferred, and plasma

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should be stored at –70°C in the presence of protease inhibitors. To minimize C activation or degradation of proteins by enzymes in the serum/plasma during the purification procedure, all the purification steps are carried out at 4°C unless mentioned otherwise. Even when rapid methods such as fast protein liquid chromatography (FPLC) are used to minimize the run time, activation can occur if runs are performed at room temperature and it is advisable to run the FPLC in a 4°C cold room. Protease inhibitors should also be included during purification, particularly in the early stages when plasma enzymes are present in abundance. Of the protease inhibitors, NPGB (p-nitro phenyl p'-guanidinobenzoate) is a particularly good inhibitor of C1r and C1s activation; it is dissolved as a 100× concentrated stock solution (100 m*M*) in dimethyl formamide (DMF) and used fresh. Phenyl methyl sulfonyl fluoride (PMSF), a broad-range protease inhibitor, is dissolved as a 100× stock solution (100 m*M*) in isopropanol. Many other protease inhibitors are soluble in water and can be directly dissolved in the required buffer. Diisopropyl fluoro phosphate (DFP) is suggested in some published purification methods, however this agent is highly toxic and can often replaced by PMSF. All the above protease inhibitors are toxic to varying degrees and should be handled with extreme caution. Ethylene diamine tetraacetic acid (EDTA) can also be added to chelate calcium and magnesium in order to prevent C activation and to inhibit activation or degradation by metalloproteases in the serum/plasma, however, some multichain complexes, like C1, require divalent cations and will disassociate in the presence of EDTA.

Plasma and serum to be used for preparation of C components and column fractions at various stages of the purification should be stored at –70°C. However, repeated freeze-thawing will also lead to C activation. C3, C4, and C5 are particularly susceptible to damage or activation by freeze-thaw and fractions containing these components are better stored at 4°C for short periods of time.

1.2. Purification Steps—General Considerations

Most purification methods for C components involve the use of an initial fractionation step to precipitate the protein of interest, followed by standard chromatography using multiple columns. Some methods have been developed that rely on the affinity of the component for its substrate/ligand. The first step in purification usually involves a crude enrichment of the protein required by fractional precipitation. For some components this involves a euglobulin precipitation (i.e., low salt) or salting out using ammonium sulfate or sodium sulfate. More common in recent methods are procedures involving fractionated precipitation using polyethylene glycol (PEG). PEG separates molecules roughly according to molecular weight; the larger molecules precipitating at lower PEG concentrations. PEG is supplied with different average chain

lengths (shown as a suffix indicating average molecular weight) and a longer PEG species (e.g., PEG 6000) will precipitate a given protein at a lower percentage than a shorter PEG (e.g., PEG 3350). It is, thus, extremely important, when following a published protocol, to use the specified PEG species for precipitation. PEG precipitation has advantages over euglobulin or salt precipitation in that it is much faster and generally less denaturing for the protein, but the main advantage is that the dissolved precipitate can be resuspended in a buffer required for the first column step and can be applied directly onto ion-exchange columns without the requirement for prior dialysis to remove salt. Fractionation by PEG precipitation is more efficient and selective when the plasma or serum is first ten times diluted in buffer. However, this is not practical for large volumes of serum. To prevent blockage of columns, redissolved PEG precipitates must always be filtered (0.2 μm) or spun at high speed (10,000*g*, 15 min) to remove any insoluble material.

Columns and column matrices for the various chromatography steps can be obtained from a variety of suppliers including Pharmacia, Bio-Rad, Sigma, and Calbiochem. It is often possible to replace matrices described in the original methods with other media providing the same chemistry for separation and substitute low-resolution columns with high-performance columns. The use of high-resolution, fast-flow columns operating at higher pressures makes purification times shorter and thus reduces the chance of C activation. FPLC columns are particular useful for purification of C components from small amounts of serum and methods described here using large amounts of serum can easily be scaled down to use FPLC columns with increased efficiency and speed of purification, often requiring fewer chromatography steps.

Gel filtration is often used as a final "polishing" step to remove residual contaminants or aggregates and is rarely of use early in a purification protocol. For application onto gel filtration columns, proteins usually have to be concentrated. This can be carried out on an Amicon ultrafiltration system or any one of several other commercially available ultrafiltration systems. Ultrafiltration also permits rapid buffer exchange and can eliminate the need for dialysis. Care should be take to choose a filter with the right molecular weight cutoff for the protein of interest; occasionally, proteins with a reported molecular weight higher than the stated cutoff will still go through the filter; this must be assessed on an individual basis. For most C components, a filter with a cutoff of 30 kDa offers a good compromise. Care should also be taken with some proteins that are sticky and will bind to some filters, resulting in considerable losses during the concentration steps.

Most of the methods described in this chapter are designed to obtain biochemically pure proteins (>90% pure). When all that is required for a particular purpose is a functionally pure protein (a preparation that still contain some

contaminants, but only contaminants that do not interfere with the aim of the experiment for which the protein is being purified) some of the columns can be omitted. When biochemically pure components are required and the yield is not very important, it is best to pool fractions tightly, discarding fractions on the edges of the protein peak which may contain difficult to remove contaminants. Some common contaminants can be removed by inclusion of a specific clean-up step; albumin can be removed by passage over Cibacron Blue Sepharose and immunoglobulin by passage over protein-A or protein-G columns.

Most methods described in this chapter are for the purification of a single component. Methods have been described for the purification of multiple complement component from a single batch of plasma ***(1,2)***. Although these multicomponent methods are economical in usage of raw materials, for most workers they are unnecessary and far too complex. Nevertheless, they do highlight the fact that several components can be obtained from a single pool of plasma without too much difficulty. Fractions obtained during the purification of a specific component that do not contain the component of interest may be frozen at –70°C and used at a later stage to purify other components.

Screening fractions for the component of interest after each of the purification steps is perhaps the most challenging aspect of a purification protocol. This can best be carried out using a functional hemolytic assay. The use of functional assays also ensures that the functionally active component and not an inactive product is being purified. Throughout this chapter, the aim is to suggest the simplest screening methods possible using sera deficient in or specifically depleted of a single component. Sera obtained from patients (or animals) deficient in individual components are now quite widely available; methods for the generation of sera depleted of specific components are described in Chapter 4. Some C deficient and depleted sera can be obtained from commercial sources (*see* Appendix).

2. Materials

2.1. Buffers

1. Phosphate-buffered saline (PBS): supplied in tablet form by Oxoid Ltd.
2. Veronal-buffered saline (VBS^{2+}): supplied in tablet form by Oxoid Ltd.
3. Tris-buffered saline (TBS): 50 m*M* Tris-HCl, 150 m*M* NaCl, pH 7.5.
4. Various specific buffers for different column chemistries, including phosphate-, Tris- and barbitone-based buffers, detailed with the specific method.

2.2. Column Matrices

1. Matrices for gel filtration: Sepharose, Sephadex, and sephacryl matrices, precise type chosen depending on M_r of protein of interest (all from Amersham Pharmacia).

2. Matrices for ion exchange: diethylaminoethyl (DEAE) Sepharose and carboxymethyl (CM) sepharose (Amersham Pharmacia).
3. Prepoured columns for FPLC gel filtration and ion exchange (all from Amersham Pharmacia).

2.3. Starting Materials

1. For plasma proteins, a source of plasma in sufficient quantities for the planned purification. Fresh EDTA plasma is best for most C components, although fresh serum works better for others. Expired plasma (available from Blood Banks) is suitable for purification of some C components, although some degree of proteolysis is often seen.
2. For erythrocyte-expressed C regulators or receptors, a source of erythrocytes in sufficient quantities for the planned purification. Expired erythrocytes are usually adequate. Platelets are often available from blood banks in large quantities and offer an alternative source for membrane C regulators and receptors.

2.4. Equipment

1. Pumps, fraction collectors, and absorbance monitors for standard protein purification system (available from many manufactureres, including Bio-Rad and Amersham, Pharmacia).
2. Automated or semiautomated chromatography systems are also widely available and can simplify preparations.
3. Spectrophotometer capable of reading absorbance in ultraviolet (UV) and visible ranges.
4. A cold room or cold cabinet in which to perform chromatographic steps.
5. Centrifuges: Ranging from benchtop microfuges to large, floor-standing refrigerated centrifuges capable of processing 5–10 L of fluid at once.
6. Ultrafiltration system for concentrating/dialyzing samples between chromatography steps. We use an Amicon ultrafiltration cell (Amicon, Inc.); numerous other systems are available.

3. Methods

3.1. Purification of Classical Pathway Components

3.1.1. Purification of C1

C1; M_r 740 kDa. Heteromeric protein, $C1qC1r_2C1s_2$.
C1q: 450 kDa: 6 chains each of: A: 29 kDa, B: 27 kDa, C: 22 kDa. Serum conc.: 180 µg/mL.
C1r: 85 kDa; activated C1r: 56 kDa and 35 kDa; serum conc.: 100 µg/mL.
C1s: 85 kDa activated C1s: 56 kDa, 27 kDa; serum conc.: 80 µg/mL.

3.1.1.1. Functionally Pure C1:

Functionally pure C1 can be obtained by euglobulin precipitation of serum. The usual starting material for this procedure is fresh citrated plasma (plasma anticoagulated with EDTA or heparin will not do).

1. Fresh citrated plasma is recalcified by addition of $CaCl_2$ from 1 *M* stock to 20 m*M* and allowed to clot for 2 h at room temperature.
2. The clot is removed by centrifugation and the protease inhibitor NPGB added to a final concentration of 1 m*M*.
3. The serum is dialyzed overnight at 4°C against a 10-fold excess of 10 m*M* NaBarbitone buffer pH 7.4 containing $CaCl_2$ (5 m*M*) and NPGB (0.1 m*M*). In order to isolate C1q, dialyze in this step against 5 m*M* EDTA alone and proceed as detailed below.
4. The fine euglobulin precipitate which forms in the serum is harvested by centrifugation at 10,000*g* for 30 min at 4°C and dissolved in VBS containing 5 m*M* Ca^{2+}, 0.1 m*M* NPGB, and 0.1 m*M* PMSF.
5. As an optional extra step, the redissolved euglobulin preparation can be subjected to gel filtration on a Sepharose 6B (or similar) column equilibrated in the above buffer; the C1 elutes early.
6. The "functionally pure" C1 can be is stored in small aliquots at –70°C and should not be subjected to freeze-thaw cycles.

3.1.1.2. "Biochemically Pure" C1

Isolation of "biochemically pure" C1 and its subcomponents is most efficiently achieved using methods based on the affinity of C1/C1q for aggregated IgG ***(3)***.

The isolation of unactivated "precursor" C1 free of "activated" C1 in which C1r and C1s are cleaved is difficult and reliant on the speed of purification and attention to maintaining low temperatures during purification.

1. A human IgG-Sepharose column (55 mL: 3.5 mg IgG/mL Sepharose) is made by incubating human IgG (Sigma) with CNBr-activated sepharose (Pharmacia) according to the manufacturer's instructions.
2. The column matrix is saturated with rabbit IgG by repeated passage of rabbit antihuman IgG antiserum containing 10 m*M* EDTA (approx 50 mL) over the column.
3. The column is washed with PBS containing 1 *M* NaCl to remove unbound protein and equilibrated with VBS.
4. Human serum (100 mL), obtained from citrated plasma as described above, is diluted 1:1 with VBS containing 5 m*M* $CaCl_2$ and 1 m*M* NPGB and applied to the column.
5. The column is washed with three column volumes of the same buffer and C1 eluted in the same buffer containing 1 *M* NaCl. If only C1q is required, load the IgG-column with serum in the presence of 10 m*M* EDTA and elute with 1 *M* NaCl. For purification of separate components of C1, the protocol up to **step 4** is identical to that described above, then as below.
6. The column is washed with three column volumes of VBS containing 5 m*M* $CaCl_2$ and 1 m*M* NPGB.
7. C1r and C1s are then eluted in VBS containing 10 m*M* EDTA and 1 m*M* NPGB.
8. C1q is then eluted in the same buffer containing 1 *M* NaCl.

9. C1q can be further purified by dialysis into 20 m*M* HEPES, 60 m*M* NaCl, 10 m*M* EDTA, pH 7.8, application to a Mono S FPLC column equilibrated in the same buffer and elution with a 20-mL linear NaCl gradient from 0.06 to 1 *M* NaCl ***(4)***.
10. To separate C1r from C1s, the eluted material from **step 5** is dialyzed against 20 m*M* NaPhosphate, 2 m*M* EDTA, pH 7.4, applied to a MonoQ column equilibrated in the same buffer and eluted using a 20-mL linear NaCl gradient from 0–1 *M* ***(5)***. C1r elutes before C1s.

See **Note 1**.

3.1.1.3. Functional Assays for C1

3.1.1.3.1. Hemolysis Method

1. Human serum is depleted of *C1* (NHS-C1) by precipitation with 3.5% PEG 4000 (30 min on ice) and the C1-containing precipitate removed by centrifugation (15 min, 10,000*g*, 4°C) ***(6)***.
2. ShEA (50 µL 2%) are incubated in wells of a microtitre plate with 50 µL NHS-C1 diluted 1/10 in VBS and 50 µL of the test fractions for 30 min at 37°C.
3. Incubate for 30 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
4. Fractions restoring C1 hemolytic activity are pooled for further use.

3.1.1.3.2. Esterolytic Assay: Esterolytic activity: functional activities of C1r and C1s can be measure using the chromogenic substrate ZLNE [N α carbobenzoxy-L-lysine-p-nitrophenyl ester (Sigma)] or S-2314.

1. Place 60-µL aliquots of fractions containing C1r or C1s in the wells of a 96-well plate and add 1 µL stock ZLNE (3 mg/mL in 90%(v/v) acetonitrile in water, made fresh immediately before use).
2. Incubate 30 min 37°C and measure release of p-nitrophenol spectrophotometrically at 340 nm.

3.1.2. Purification of MBL, MASP-1, and MASP-2

Mannose-binding lectin (MBL): M_r 750 kDa; serum conc.: 2 ng–10 µg/mL.
MBL associated serine protease (MASP)-1: M_r 90 kDa; upon activation: 65 and 25 kDa. Serum conc.: 6 µg/mL.
MASP-2: 76 kDa; upon activation: 52 and 31 kDa; serum conc. not known, but much lower than MASP-1.

Purification of MBL is based on carbohydrate affinity chromatography and the sequential use of different sugars to remove contaminating anticarbohydrate antibodies from the MBL preparation ***(7)***.

1. Serum (1 L) is brought to 7% w/v PEG3500, stirred for 2 h at 4°C, and spun for 15 min at 12,000*g*.
2. The PEG precipitate is dissolved in 400-mL TBS containing 0.05% Tween20, 20 m*M* $CaCl_2$, pH 7.8 (TBS-TCa) and mixed for 2 h at 4°C with 50 mL

mannose Sepharose (mannose coupled to Sepharose 4B), equilibrated in the same buffer.
3. The matrix is placed in a column, washed with 500 mL of the same buffer, and eluted with buffer containing no added Ca^{2+} and 10 m*M* EDTA.
4. The eluate is made 40 m*M* with $CaCl_2$, applied to a 3-mL maltose-Sepharose (maltose coupled to Sepharose 4B) column, washed with TBS-TCa buffer and eluted with 100 m*M* GlcNAc (N-acetyl-D-glucosamine) in TBS-TCa.
5. If further purification is required, the MBL-positive pool is dialyzed into 50 m*M* Tris/100-m*M* NaCl, 2-m*M* EDTA, pH 7.8, applied to a MonoQ FPLC column equilibrated in the same buffer and eluted with a 40-mL linear NaCl gradient from 0.1 to 0.6 *M*.
6. MBL-positive fractions are pooled and applied to a Superose 6 FPLC column; positive peak contains MBL and bound MASP-1/2.

See **Note 1**.

3.1.3. Purification of C4

C4: M_r 189 kDa: α: 89 kDa, β: 72 kDa, γ: 32 kDa;
Activated: C4a: 9 kDa, C4α:80 kDa: serum conc. 200–600 µg/mL.

1. Supplement 1 L of citrated plasma with 5 m*M* benzamidine (to precipitate vitamin K-dependent proteases) and 0.5 m*M* PMSF; add solid PEG6000 to a final conc. of 5%, stir for 30 min on ice, spin 15 min 6000*g* 4°C. Add solid PEG6000 to a final conc. of 8%, stir 30 min on ice, and spin 15 min 6000*g* 4°C ***(8)***.
2. Dissolve the pellet in 10 m*M* Tris, 10 m*M* EDTA, 150 m*M* NaCl, pH 8.0, and apply to a Q-Sepharose column (5 cm × 20 cm) equilibrated in the same buffer. Elute with a 1 L linear NaCl gradient from 0.15 to 0.5 *M*.
3. Pool positive fractions, dialyze against 10 m*M* Tris, 10 m*M* EDTA, 150 m*M* NaCl, pH 8.0, and apply to a MonoQ FPLC column equilibrated in the same buffer. Elute with a 30-mL linear NaCl gradient from 0.15 to 0.5 *M*.
4. Pool positive fractions, concentrate in Amicon ultrafiltration cell and gel filter on a Superose 12 FPLC column equilibrated in PBS. Pool and concentrate positive fractions.

3.1.3.1. Functional Assay for C4

1. Generate C4-depleted guinea pig serum (R4) by incubating fresh guinea pig serum (8.5 mL) with 5% NH_4OH (1.5 mL) at 37°C for 45 min. Immediately adjust pH to 7.4 and store in aliquots at –70°C. If available, C4-deficient guinea pig serum can be used in place of R4.
2. To 50 µL 2% ShEA in VBS, add 50 µL C4-depleted serum (1/5 in VBS) and 50 µL of test fraction for C4.
3. Incubate for 30 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
4. Fractions restoring C4 hemolytic activity are pooled for further use.

3.1.3.2. Generation of C4 Fragments

C4a and C4b can be generated by incubation of purified C4 with activated C1s.

1. To purified C4 (1 mg/mL in PBS) add activated C1 (2% w/w) and incubate for 1–2 h at 37°C.
2. Separate C4a and C4b by gel filtration on a Sephadex G-100 Superfine (or similar) column equilibrated in 10 m*M* NaPhosphate, 1 *M* NaCl, and pH 7.2.
3. Generate C4c and C4d by incubation of C4b (1 mg/mL in PBS) with factor I (2% w/w) and C4bp (10% w/w) at 37°C for 1 h.
4. Separate C4c and C4d from uncleaved C4b by gel filtration on a Sephadex G-150 (or similar) column in 10 m*M* NaPhosphate, 1 *M* NaCl, pH 7.2.
5. Generate iC4b by incubation of C4b with 500 m*M* methylamine, pH 8.0 for 1 h at 37°C. Generate $C4_{MA}$ by incubation of C4 with methylamine as above. C4b can be separated from iC4b and $C4_{MA}$ from C4 by loading on a MonoQ column in 20-m*M* Tris, 2 m*M* EDTA, 150 m*M* NaCl, pH 7.4 and eluting with a 20-mL linear NaCl gradient from 0.15 to 0.5 *M*.

*3.1.4. Purification of C2 (**9**)*

C2: M_r: 110 kDa, activated C2a: 74 kDa, C2b: 34 kDa, serum conc. 25 µg/mL.

1. Dialyze 1 L of serum overnight against 5 m*M* $CaCl_2$, 1 m*M* Benzamidine, and spin 30 min 23,000*g*.
2. Add 10 mL 200 m*M* EDTA and 50 mL 400 m*M* NaPhosphate, 40 m*M* EDTA, pH 6.0 to the supernatant, and adjust pH to 6.0. Apply to CM-Sephadex C-50 (8 cm × 10 cm) column equilibrated in 100 m*M* NaPhosphate, 1 m*M* benzamidine, pH 6.0, and elute with a 2-L linear phosphate gradient from 0.1 to 0.25 *M* NaPhosphate, and pH 6.0.
3. To the C2-positive fractions, add $(NH_4)_2SO_4$ to 50% saturation (291 g/L), stir 1 h 4°C, and centrifuge 23,000*g* for 30 min at RT.
4. To the supernatant, add $(NH_4)_2SO_4$ to 75% saturation (159 g/L), stir 1 h at RT and spin 23,000*g*, 90 min at RT.
5. Resuspend pellet in 50 mL, 5 m*M* NaBarbitone, 0.5 m*M* $CaCl_2$, 2 m*M* $MgCl_2$, 40 m*M* NaCl, pH 8.5, and gel filter on Sepharose 4B (or similar) column (4 cm × 30 cm) equilibrated in the same buffer.
6. Pool and concentrate C2-positive fractions.

3.1.4.1. Functional Assay for C2

C2 and fB are the most thermosensitive of the C components and can be specifically depleted from serum by careful heating.

1. Serum (1 mL) is placed in a glass tube preheated in a 56°C water bath and incubated at 56°C for precisely 6 min with constant shaking ***(10)***. C2-depleted serum should be used immediately.

2. In the wells of a 96-well plate, mix 50 µL 2% ShEA in VBS, 50 µL C2-depleted serum (diluted 1/5 in VBS) and 50 µL of fraction to be tested for C2.
3. Incubate for 30 min at 37°C, spin and read the absorbance of the supernatant at 415 nm.
4. Fractions restoring C2 hemolytic activity are pooled for further use.

3.1.5. Purification of C3

C3: M_r 187 kDa; α: 112 kDa, β: 75 kDa; activated: C3a: 9 kDa, C3a': 102 kDa; serum conc. 1.3 mg/mL.

C3 is the most abundant C component in serum and can easily be purified in large quantities.

3.1.5.1. Large-scale Purification of C3 *(11)*

1. To 1 L of plasma, add PMSF (0.5 m*M* final concentration) and PEG4000 to a final concentration of 5% (from a 15% stock in 100 m*M* NaPhosphate, 15 m*M* EDTA, 150 m*M* NaCl, 0.5 m*M* PMSF, pH 7.4); stir 30 min at 4°C.
2. Spin 30 min 10,000*g* at room temperature and make supernatant 12% PEG4000 by addition from a 26% stock in the same buffer, stir 30 min and spin 10,000*g* 30 min at 4°C.
3. Resuspend pellet in 100 mL of the same buffer and pass over a 100-mL lysine-Sepharose column to remove plasminogen.
4. Dilute breakthrough with 5 m*M* EDTA to a conductance of 3 mmho/cm (about 3× dilution), load onto a diethylaminoethyl (DEAE) cellulose column (5 cm × 40 cm) equilibrated in 25 m*M* NaPhosphate, 5 m*M* EDTA, pH 7.0 and elute with a 2-L linear NaCl gradient from 25 m*M* to 300 m*M*. Pool C3-positive fractions.
5. To the C3 pool, add PEG4000 to 16%, stir 30 min, and spin 30 min 10,000*g*, 4°C. Resuspend pellet in 15 mL 100 m*M* NaPhosphate, 5 m*M* EDTA, 50 m*M* aminocaproic acid (EACA), 0.5 m*M* PMSF, 150 m*M* NaCl, pH 7.4, and gel filter on a Sepharose 6B column (2.5 cm × 100 cm).
6. Dialyze against 25 m*M* KPhosphate, 100 m*M* KCl, 50 m*M* EACA (ε amino caproic acid), pH 7.4, load on a Hydroxylapatite column (2 cm × 20 cm) equilibrated in the same buffer, wash with the same buffer and elute first with 25 m*M* KPhosphate, 2 *M* KCl (elutes C5) followed by 125 m*M* KPhosphate, 100 m*M* KCl (elutes C3).
7. Pool and concentrate C3-containing fractions.

3.1.5.2. Small Scale Purification of C3 *(12)*

1. Dilute NHS (1–5 mL) 1/10 in PBS/5 m*M* EDTA/4% PEG6000 and spin 30 min 10,000*g*. Make supernatant 10% with PEG6000 by addition from a 30% PEG6000 stock solution in PBS/EDTA. Spin 30 min at 10,000*g*, 4°C.
2. Dissolve pellet to original volume in 20 m*M* Tris, 5 m*M* EDTA, pH 8.9. Apply to MonoQ column (0.5 mL/run) and elute with a 20-mL linear NaCl gradient from 0 to 500 m*M*.

3. Pool C3-positive fractions and gel filter on a Superose 12 FPLC column equilibrated in PBS to obtain pure C3.
4. Store in aliquots at –70°C.

3.1.5.3. Generation of $C3_{MA}$ for Cofactor Assays

C3 with an inactivated thioester may be required for use in cofactor assays (*see* Chapter 18).

1. To C3 containing fractions off MonoQ column, add 1/10 (v/v) 2 *M* methylamine pH 8.0. Incubate 2 h 37°C.
2. Dialyze against MonoQ start buffer, apply to MonoQ and elute as above. Methylamine-reacted C3 ($C3_{MA}$) elutes a few fractions before native C3 from MonoQ ***(13)***.

3.1.5.4. Functional Assay for C3

1. Generate C3/C4-depleted serum (R3) by incubation of fresh serum with 1/10 (v/v) 2 *M* methylamine pH 8.0 for 2 h at 37°C ***(14)***.
2. Dialyze serum overnight against PBS and store in aliquots at –70°C.
3. In the wells of a 96-well plate mix 50 µL RaE (2% in VBS), zymosan (1 mg/mL final), 50 µL C3/C4-depleted serum diluted 1:10 in VBS and 50 µL of the test fraction.
4. Incubate for 30 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
5. Fractions restoring C3 hemolytic activity are pooled for further use.

3.1.5.5. Generation of Fragments

Generation of fragments of C3 can either be achieved using the C3 convertases of the C system or by enzymatic digestion using non-C enzymes.

3.1.5.5.1. Generation of C3a and C3b Using Cobra Venom Factor (CVF)-Bb

M_r; C3a: 9 kDa, C3b: 178 kDa ***(15)***

1. Generate CVF-Sepharose solid phase by coupling CVF to CNBr-activated Sepharose (1 mg CVF/mL column volume) according to the manufacturer's instructions (for purification of CVF, *see* **Subheading 3.2.4.**). Wash solid phase and incubate with serum (30% suspension of CVF-Sepharose in neat serum) for 30 min at 37°C with constant mixing. A CVF-Bb complex will now be formed on the solid phase.
2. Wash CVF-Bb sepharose in PBS; add purified C3 (2 mg/mL; 1 mL per mL packed solid phase) and incubate 4 h at 37°C with constant stirring. Analyze the degree of conversion by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
3. Separate C3a and C3b in the supernatant by gel filtration on Sephadex G-100 (2 cm × 100 cm), equilibrated in 20 m*M* NaPhosphate, 1 *M* NaCl, pH 7.0. C3b elutes in the breakthrough. Unreacted C3 can be separated from C3b by application on MonoQ, as described for C3 (*see* **Subheading 3.1.5.2.**).

3.1.5.5.2. Generation of iC3b and C3f:

M_r iC3b: 176 kDa, C3f: 2 kDa ***(16)***.

1. Incubate C3b or $C3_{MA}$ (approx 1 mg/mL; generated as described in **Subheading 3.1.5.3.**) with factor I and factor H, both at 2% w/w of C3b/$C3_{MA}$, for 2 h at 37°C. Confirm digestion by running an aliquot on SDS-PAGE.
2. Fractionate mixture by gel filtration on a Sephadex G-100 column as described in **Subheading 3.1.5.1.** iC3b elutes in the breakthrough. For complete removal of fH and fI, apply the iC3b peak on MonoQ as described for C3.

3.1.5.5.3. Generation of C3c and C3dg

M_r C3c: 138 kDa, C3dg: 38 kDa ***(17)***.

1. Incubate C3b or $C3_{MA}$ (approx 1 mg/mL; generated as earlier described) with factor I and CR1, both at 2% w/w of C3, at 37°C for 2 h. If purified CR1 or recombinant soluble CR1 are not available, a human erythrocyte ghost membrane extract can be used as a source of CR1.
2. Determine incubation time necessary to get complete cleavage by removing aliquots at intervals and running on SDS-PAGE.
3. Separate C3c and C3dg by gel filtration on Sephadex G-150 superfine in 10 m*M* NaPhosphate, 1 *M* NaCl, pH 7.4.

3.1.5.5.4. Generation of C3d and C3g

M_r C3d: 34 kDa, C3g: 4 kDa ***(18)***.

1. Incubate C3dg (approx 1 mg/mL; generated as described in **Subheading 3.1.5.5.3.**) with trypsin (1% w/w) for 5 min at 37°C.
2. Stop reaction by adding 1 m*M* PMSF or 5% (w/w) solid-phase soybean trypsin inhibitor (SBTI).
3. Separate by gel filtration on Sephadex G-75 superfine in 10 m*M* NaPhosphate, 1 *M* NaCl, pH 7.4.

3.1.5.5.5. Generation of C3b, C3g, C3c, and C3d Using Trypsin (18)

1. For the simultaneous generation of C3g, C3c, and C3d, incubate C3 (approx 1 mg/mL) with trypsin (1% w/w) for 5 min at 37°C. For the tryptic generation of C3b, incubate C3 with 0.05–0.1% w/w trypsin for 2–3 min at 37°C. Fragments can also be generated using thrombin or plasmin at 2% w/w.
2. Stop with 1 m*M* PMSF or 5% (w/w) solid-phase SBTI.
3. Separate by gel filtration on Sephadex G-100 superfine in PBS.

3.2. Purification of Alternative Pathway Components

3.2.1. Purification of Factor B (fB)

M_r: fB: 93 kDa; Ba: 30 kDa, Bb: 63 kDa; serum conc.200–400 µg/mL ***(19)***.

1. To 200 mL serum at 4°C, add PMSF (0.5 m*M* final), pepstatin A (2 μ*M*), leupeptin (3 μ*M*) and iodoacetamide (2 μ*M*).
2. Slowly add 58 g $(NH_4)_2SO_4$ (50% saturation) and stir 1 h at 4°C, centrifuge 10,000*g*, 30 min, 4°C.
3. Filter supernatant through Whatman no. 1 filter paper and dialyze against 10 vol of 25 m*M* Tris, 0.5 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$ pH 7.4.
4. Add sodium caprylate (binds to albumin and prevents binding of albumin to column) to a final concentration of 25 m*M* and mix for 1 h at 4°C with 70 mL Cibacron Blue F3GA agarose equilibrated in the same buffer.
5. Pack the agarose in a 2 cm × 30 cm column, wash in the same buffer and elute with a 100 mL linear KCl gradient from 0 to 2 *M* KCl in the same buffer.
6. Identify fB-containing fractions, pool and dialyze against 10 m*M* KPhosphate, 5 m*M* EDTA including above-described inhibitors, pH 7.0 (start buffer).
7. Load onto a Hiload S-Sepharose column (1.6 cm × 10 cm) equilibrated in start buffer and elute with 100 mL linear NaCl gradient from 0 to 400 m*M* NaCl in start buffer.
8. Identify and pool the fB-containing fractions and reapply to the reequilibrated Hiload S-Sepharose column using the same conditions.
9. Identify, pool, dialyze and concentrate the fB-containing fractions, and store in aliquots at –20°C.

3.2.1.1. Functional Assay for fB

1. Generate fB-depleted serum by incubating 1–2 mL serum in a glass tube under continuous shaking at exactly 50°C for 20 min. This procedure also inactivates C2 and partially inactivates C6 and C7.
2. In the wells of a 96-well plate mix 2% RaE in APB (50 μL) with fB-depleted serum (1:5 in APB, 50 μL) and fractions to be tested for fB (50 μL). Incubate for 30 min at 37°C, spin and read the absorbance of the supernatant at 415 nm.
3. Fractions restoring fB hemolytic activity are pooled for further use.

3.2.2. Purification of Factor D (fD)

M_r: fD: 24 kDa; serum conc. 1–2 μg/mL.

Plasma concentration of fD is very low and alternative sources have often been used, notably, urine from patients with renal tubular dysfunction or peritoneal dialysis fluid. The following method has been developed to purify factor D from plasma ***(20)***.

1. Serum (2 L) is dialyzed overnight at 4°C against 40 L of 5 m*M* EDTA pH 5.4.
2. The euglobulin precipitate is removed by centrifugation at 10,000*g* for 30 min at 4°C. To the supernatant, add 400 mL 0.5 *M* NaPhosphate, pH 6.0 to bring conductivity to 12–14 ms.
3. Apply to a CM-Sephadex C-50 column (8 cm × 12 cm) equilibrated in 200 m*M* NaPhosphate, pH 6.0. Elute with a 1-L linear NaCl gradient from 0 to 2 *M* NaCl in 100 m*M* NaPhosphate, pH 6.0.

4. Identify fD-containing fractions, pool, add $(NH_4)_2SO_4$ to 50% saturation (291 g/L), stir 2 h, 4°C, and centrifuge 10,000*g*, 1 h, 4°C.
5. Make supernatant 70% saturated with $(NH_4)_2SO_4$ by adding a further 125 g/L, stir 2 h at RT and spin 10,000*g*, 1 h.
6. Dissolve the pellet in 40 mL 0.1 *M* Tris, 2 m*M* EDTA pH 8.0; gel filter on a Sephadex G-75 column (8 cm × 90 cm) equilibrated in the same buffer. Identify and pool fD-containing fractions.
7. Apply to CM-cellulose 32 column (1.5 cm × 20 cm) equilibrated in 230 m*M* NaAc, pH 5.2 and elute with a 250-mL linear NaCl gradient from 0 to 300 m*M*.
8. Pool fD-containing fractions, concentrate, dialyze against 10 m*M* Tris, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 150 m*M* NaCl, apply to a concanavalin A-Sepharose column (1 cm × 12 cm) equilibrated in the same buffer and collect breakthrough. Low-molecular weight contaminants can also be removed by passage over heparin-Sepharose.
9. Pool, concentrate by ultrafiltration using a 10-kDa filter, and store in aliquots at –70°C.

3.2.2.1. Functional Assay for fD

1. Generate fD-depleted serum by gel filtration of small volumes of serum on Sephadex G-75 (fD is separated because of its small size; it, alone of the C components, is significantly retarded on the column).
2. The column void fractions, containing the bulk of the serum proteins minus fD, are pooled and stored in aliquots at –70°C.
3. In the wells of a 96-well plate, mix RaE (50 µL of a 2% suspension in APB) with 50 µL of fD depleted serum diluted 1:5 in APB and 50 µL of the fraction to be tested for fD activity. Incubate for 30 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
4. Fractions restoring fD hemolytic activity are pooled for further use.

3.2.3. Purification of Properdin

M_r: properdin: 54 kDa, 5 µg/mL; ***(21)***.

The serum concentration of properdin is low and it strongly associates with activated C3; it is thus important to prevent C3 activation during purification or properdin will be lost. Properdin is present in plasma in the form of oligomers, mainly dimers (P_2), trimers (P_3), and tertramers (P_4). Methods have been described for the separation of the different properdin oligomers in plasma based upon gel filtration or cation exchange ***(22,23)***.

1. Plasma (1 L) is adjusted to pH 6.0 by addition of dilute HCl and 1 m*M* PMSF and dialyzed overnight at 4°C against 10 vol of 5 m*M* EDTA, pH 5.4.
2. The euglobulin precipitate is removed by centrifugation at 10,000*g* for 30 min, dissolved in 100 mL 20 m*M* NaPhosphate, 2 m*M* EDTA , pH 6.0 and applied to a CM-Sephadex C50 column (2.5 cm × 40 cm) equilibrated in the same buffer. Elute using a 2-L linear NaCl gradient from 0 to 1 *M* NaCl in 20 m*M* NaPhosphate, 2 m*M* EDTA, pH 6.0.

3. Identify properdin-containing fractions, pool, dialyze into 20 m*M* NaPhosphate, 10 m*M* NaCl, 2 m*M* EDTA, pH 6.0, apply to a CM-cellulose CM32 column (2.5 cm × 40 cm) and elute using a 2-L linear NaCl gradient from 0 to 1 *M* NaCl in 20 m*M* NaPhosphate, 2 m*M* EDTA, pH 6.0.
4. Identify properdin-containing fractions, concentrate and gel filter on a Superdex HR200 column (2 cm × 100 cm) equilibrated in 50 m*M* Tris, 2 m*M* EDTA, 500 m*M* NaCl pH 7.0.
5. Pool, concentrate, and store in aliquots at –70°C.

3.2.3.1. Functional Assay

The functional assay is based on the ability of Properdin to stabilize the C3 convertase. Numerous complex assays have been proposed, but a simple hemolytic assay is adequate for testing column fractions.

1. In wells of a 96-well plate, mix 2% RaE in APB (50 µL) with various dilutions of serum (1:20; 1:10; 1:5 in APB, 50 µL) and fractions to be tested for properdin (50 µL).
2. Incubate for 30 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Identify fractions enhancing AP activity, pool, and concentrate.

3.2.4. Purification of Cobra Venom Factor (CVF)

CVF: M_r: 149 kDa; α: 69 kDa; β: 49 kDa; γ: 32 kDa ***(24)***.

CVF is a C3-like molecule found in the venom of several different species of cobra. The purification method described is based on that described by Vogel and Müller-Eberhard ***(24)***.

See **Note 2**.

1. Freeze-dried cobra venom (1 g; harvested from the cobra *Naja naja kaouthia*; available from Sigma in some parts of the world) is dissolved in 5 m*M* NaPhosphate, pH 7.2 (start buffer) and applied to a DEAE Sepharose fastflow column (5 cm × 14 cm) equilibrated in the same buffer.
2. After washing, the column is eluted with a 600-mL linear NaCl gradient from 0 to 400 m*M* in start buffer. CVF-containing fractions are identified, pooled, and dialyzed against 10 m*M* NaPhosphate, pH 5.0.
3. The dialyzed pool is applied to a CM-Sepharose fastflow column (2.6 cm × 30 cm) equilibrated in the same buffer and eluted with a 500-mL linear NaCl gradient from 0 to 600 m*M* in start buffer. Identify and pool CVF-containing fractions. Dialyze against 10 m*M* NaPhosphate, pH 5.0. Apply the CVF pool to an Affigel blue column (1.5 cm × 13 cm) equilibrated in the same buffer, wash in the same buffer, and elute bound CVF in 10 m*M* NaPhosphate, pH 6.5. Residual CVF and the contaminating PLA2 can be eluted from the Affigel column with 20 m*M* $(NH_4)_2SO_4$, pH 10.5.

4. Pool CVF-containing fractions, dialyze against VBS, concentrate to approx 1 mg/mL in an Amicon ultrafiltration cell using a 30-kDa cutoff membrane and store in aliquots at –70°C. Thawed aliquots remain active for several weeks when stored at 4°C.

3.2.4.1. Functional Assay for CVF

CVF from *Naja naja kaouthia* can be detected in a reactive lysis assay utilizing the ability of CVF from this source to form a C3/C5 convertase and activate membrane attack complex (MAC) assembly on a target cell.

1. In the wells of a 96-well plate, mix GpE (50 µL of a 2% suspension in VBS) with serum (50 µL of a 1/10 dilution in VBS) and 50 µL of the test fraction (neat and diluted 1:10 and 1:100 in VBS). Incubate 30 min at 37°C.
2. Control for PLA_2 contamination by parallel testing of fractions for lysis of GpE in the absence of serum (PLA_2 can cause hemolysis independent of serum).
3. Spin and read the absorbance of the supernatant at 415 nm.
4. Fractions inducing serum-dependent hemolysis of target GpE are pooled for further use.

3.2.4.2. Alternative Functional Assay for CVF

CVF from the cobra *Naja haje* does not form a C5 convertase and must be detected using an inhibition assay.

1. In the wells of a 96-well plate, mix 50 µL of the test fraction with 50 µL human serum (1/20 in VBS) and incubate for 30 min at 37°C.
2. Add 50 µL ShEA (2% in VBS) and incubate for a further 30 min at 37°C.
3. Spin and read the absorbance of the supernatant at 415 nm. Fractions containing CVF show less lysis because of consumption of C in the first incubation.

3.3. Purification of Terminal Pathway Components

3.3.1. Purification of C5

M_r: C5: 192 kDa, α: 118 kDa, β: 74 kDa; activated: C5a: 11 kDa; C3α': 107 kDa; serum conc. 75 µg/mL ***(11)***.

C5 copurifies with C3 through the procedure described in **Subheading 3.1.5.**

C5 is separated from C3 by Hydroxyapatite chromatography (**step 6** in the large-scale isolation of C3, **Subheading 3.1.5.**).

1. Identify C5-containing fractions off Hydroxyapaptite column from C3 preparation. Pool, dialyze against 20 m*M* Tris, 1 m*M* EDTA, 2.5 m*M* NaCl, pH 8.0, and apply to Q-Sepharose column (2 cm × 20 cm).
2. Wash with the above buffer and elute with a 500-mL linear NaCl gradient from 0 to 0.5 *M* in the same buffer.

3. Identify positive fractions, pool, concentrate, and gel filter on a Superdex HR200 column (2 cm × 90 cm) equlibrated and run in PBS.
4. Identify and pool positive fractions, concentrate and store at –70°C.

3.3.1.1. Functional Assay for C5:

C5 can be measured in a functional assay using C5-deficient mouse serum as a source of other C components. C5-deficient mice are readily available (*see* Chapter 20 for C5-deficient mouse strains) and the serum can be obtained commercially.

1. In the wells of a 96-well plate, mix antibody-sensitized rabbit E (50 µL of 2% in VBS) with 50 µL C5-deficient mouse serum (1/5 in VBS) and 50 µL of the test fraction.
2. Incubate 60 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Pool C5-containing fractions for further stages.

3.3.2. Purification of C6

M_r: C6: 108 kDa, serum conc. 70 µg/mL ***(26)***.

1. To 2 L of serum at 4°C, add ammonium sulfate (213 g/L; 37.5% saturation), 1 m*M* PMSF and 1 m*M* benzamidine; stir 1 h at 4°C, and spin 10,000*g* for 30 min at 4°C.
2. Retain pellet for purification of C7 (**Subheading 3.3.3.**). Add ammonium sulfate to the supernatant (80 g/L; final saturation 50%), stir 1 h 4°C, and spin 30 min 10,000*g* at 4°C.
3. Dissolve precipitate in 100 mL 10 m*M* NaPhosphate, 1 m*M* PMSF, 1 m*M* Benzamidine, 2 m*M* EDTA, pH 6.2, and dialyze against the same buffer.
4. Apply to a phosphocellulose column (4 cm × 80 cm), wash and elute with a 2-L linear NaCl gradient from 0 to 1 *M*.
5. Identify C6-containing fractions, pool, concentrate, and dialyze into 10 m*M* NaPhosphate, 1 m*M* PMSF, 1 m*M* Benzamidine, 2 m*M* EDTA, 25% glycerol, pH 7.8.
6. Load on a Q-Sepharose column (2.5 cm × 80 cm) equilibrated in the same buffer, wash and elute with a 2-L linear NaCl gradient from 0 to 500 m*M*.
7. Identify and pool C6-containing fractions, concentrate and gel filter on a Sephadex G200 column (2 cm × 90 cm) equilibrated in VBS.
8. Identify and pool positive fractions, concentrate and store at –70°C.

3.3.2.1. Functional Assay for C6

C6 can be measured in a functional assay using C6-deficient rabbit or rat serum (*see* Chapter 20) or C6-depleted serum obtained by passage of NHS over an anti-C6 immunoaffinity column (Chapter 4).

1. In the wells of a 96-well plate, mix ShEA (50 µL of 2% in VBS) with 50 µL C6-deficient rat serum (1/5 in VBS) and 50 µL of the test fraction.

2. Incubate 60 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Pool C6-containing fractions for further stages.

3.3.3. Purification of C7

M_r: C7: 121 kDa; serum conc. 60 µg/mL ***(26)***.

1. Resuspend the 37.5% ammonium sulfate pellet obtained in stage 1 of the C6 preparation (**Subheading 3.3.2.**) in 150 mL NaPhosphate, pH 6.0, and dialyze against the same buffer.
2. Apply to a Phosphocellulose column (4 cm × 80 cm) equilibrated in the same buffer, wash, and elute with a 2-L linear NaCl gradient from 0 to 1 *M* in the same buffer. Identify C7-containing fractions, pool, and dialyze against 10 m*M* NaPhosphate pH 7.0.
3. Apply to a QAE-Sephadex column (2.5 cm × 40 cm) equilibrated in 10 m*M* NaPhosphate, 25% glycerol, pH 7.0, wash, and elute with a 2-L linear NaCl gradient from 0 to 500 m*M*.
4. Identify and pool C7-containing fractions, concentrate and gel filter on a Sephadex G-200 column (2 cm × 90 cm) equilibrated in VBS.
5. Identify and pool positive fractions, concentrate and store at –70°C.

3.3.3.1. Functional Assay for C7

C7 can be measured in a functional assay using C7-deficient serum or C7-depleted serum obtained by passage over an anti-C7 immunoaffinity column (Chapter 4).

1. In the wells of a 96-well plate, mix antibody-sensitized sheep E (50 µL of 2% in VBS) with 50 µL C7-depleted serum (1/5 in VBS) and 50 µL of a dilution of the test fraction.
2. Incubate 60 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Pool C7-containing fractions for further stages.

3.3.4. Purification of C8

M_r: C8: 150 kDa; nonreduced: α-γ: 86 kDa β: 64 kDa; reduced: α: 64 kDa; β: 64 kDa; γ: 22 kDa. Serum conc. 80 µg/mL ***(27)***.

1. To 0.5 L plasma or serum at 4°C, add PMSF and benzamidine both to 1 m*M* final concentration and $BaCl_2$ to 40 m*M*; mix 15 min at 4°C and centrifuge at 6000*g* for 15 min at 4°C.
2. To the supernatant at 4°C, add PEG4000 from a 20% stock solution to a final concentration of 5%, stir for 30 min, spin 6000*g* 15 min 4°C.
3. Make supernatant 10% PEG4000 by addition of solid PEG4000, stir 30 min, and spin 15 min 6000*g*, 4°C. Remove and retain supernatant for purification of C9 (**Subheading 3.3.5.**). Dissolve pellet in 25 m*M* imidazole, 150 m*M* NaCl, 1 m*M* benzamide, 1 m*M* PMSF pH 6.1, and remove insoluble material by centrifugation for 15 min at 6000*g*, 4°C.

4. Add solid $(NH_4)_2SO_4$ to 37.5% saturation (213 g/L) at 4°C. After 30 min stirring, spin at 6000*g* for 15 min at 4°C, discard pellet, and adjust supernatant to 50% saturation $(NH_4)_2SO_4$ (80 g/L) , stir further for 1 h, and spin 6000*g*, 15 min 4°C. Discard supernatant, solubilize the pellet in 100 mL 70 m*M* NaPhosphate, 50 m*M* NaCl, 1 m*M* benzamidine HCl, 1 m*M* PMSF, pH 6.1 (CM buffer), and dialyze against the same buffer.
5. Apply to a CM-Sephacel column (5.5 cm × 25 cm) equilibrated in CM buffer. Wash and elute with a 600-mL linear NaCl gradient from 50 to 500 m*M* in CM buffer. Identify and pool positive fractions, concentrate, and dialyze against 25 m*M* Tris, 70 m*M* NaCl, 1 m*M* benzamidine, pH 8.0 (Q buffer).
6. Apply to a Q-Sepharose column (3 cm × 34 cm) equilibrated in Q buffer. Wash and elute with a 600-mL linear NaCl gradient from 70 to 220 m*M* in Q buffer. Identify and pool positive fractions and concentrate using a 30-kDa cutoff filter.
7. Apply to a Sephacryl S-300 gel filtration column (3.5 cm × 52 cm) equilibrated in 25 m*M* Imidazole, 150 m*M* NaCl, 0.02% NaAzide, pH 7.2. Identify and pool positive fractions, concentrate, add 20% (v/v) glycerol (to prevent aggregation which occurs on freezing) and store in aliquots at –20°C.

3.3.4.1. Functional Assay for C8

C8 can be measured in a functional assay using C8-deficient serum or C8-depleted serum obtained by passage over an anti-C8 immunoaffinity column (Chapter 4).

1. In the wells of a 96-well plate, mix ShEA (50 µL of 2% in VBS) with 50 µL C8-depleted serum (1/5 in VBS) and 50 µL of the test fraction.
2. Incubate 60 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Pool C8-containing fractions for further stages.

3.3.5. Purification of C9

M_r: C9: 66 kDa; serum conc. 60 µg/mL ***(27)***.

1. To the 10% PEG4000 supernatant obtained in **Subheading 2.5.4.** (**step 3**), add solid PEG4000 to a final concentration of 25%, stir 30 min, and spin 15 min 6000*g*, 4°C.
2. Discard supernatant, dissolve pellet in 10 m*M* NaPhosphate, 10 m*M* EDTA, 90 m*M* NaCl, 1 m*M* PMSF pH 7.4 (DE buffer), and centrifuge 15 min, 6000*g*, 4°C to remove insoluble material.
3. Apply supernatant to a DEAE-Sephacel column (5 cm × 10 cm) equilibrated in DE buffer, wash, and elute with a 1-L linear NaCl gradient from 90 m*M* to 500 m*M* in DE buffer. Identify and pool C9-containing fractions (*see* **Note 3**).
4. Apply directly to a Hydroxylapatite column (3.5 cm × 22 cm) equilibrated in 50 m*M* KPhosphate, 100 m*M* NaCl, pH 8.3, wash, and elute with a 1-L linear KPhosphate gradient from 50 mM to 300 m*M*.

5. Identify, pool, and concentrate C9-containing fractions and apply to a Sepharose CL-6B gel filtration column (1.5 cm × 46 cm) equilibrated and run in VBS. Identify, pool, and concentrate positive fractions and store at –70°C.

3.3.5.1. Functional Assay for C9

C9 can be measured in a functional assay using C9-depleted serum obtained by passage over an anti-C9 immunoaffinity column (Chapter 4).

1. In the wells of a 96-well plate, mix antibody-sensitized sheep E (50 µL of 2% in VBS) with 50 µL C9-depleted serum (1/5 in VBS) and 50 µL of a dilution of the test fraction.
2. Incubate 15 min at 37°C, spin, and read the absorbance of the supernatant at 415 nm.
3. Pool C9-containing fractions for further stages.

3.3.6. Purification of the C5b6 Complex

M_r: C5b6: 328 kDa ***(2)*** (*See* **Note 3**).

1. Incubate 1 L of C7-depleted serum with 10 mg/mL zymosan overnight at room temperature to generate C5b6.
2. Remove zymosan by centrifugation and dialyze serum against 20 m*M* NaPhosphate pH 5.4.
3. Spin 30 min at 10,000*g* and dissolve the euglobulin precipitate in 50 mL 10 m*M* NaPhosphate, 5 m*M* NaCl, 25% glycerol, pH 7.0 (DE buffer).
4. Apply to DEAE Sephacel column (5 × 30) equilibrated in DE buffer. Elute with 5-L linear gradient from 0 to 500 m*M* NaCl in DE buffer. Pool and concentrate C5b6-containing fractions and apply to Sephadex-G-200 (2 cm × 100 cm) equilibrated in 10 m*M* NaPhosphate, 500 m*M* NaCl, pH 7.0. Pool C5b6-containing fractions, concentrate and store at –70°C.

3.3.6.1. Functional Assay

Numerous functional assays have been used, all relying on the principle of reactive lysis. One simple protocol is to measure lysis of GpE in agarose.

1. Warm 5 mL of a 1.5% suspension of GpE in PBS/10 m*M* EDTA to 40°C and mix with 5 mL of a 2% solution of agarose (in PBS/10 m*M* EDTA) also at 40°C.
2. Immediately pour mixture into a Petri dish or onto an appropriate glass plate and allow to set.
3. Punch sets of holes in agarose, each set comprising a central hole surrounded by six holes all equidistant from center.
4. Place NHS (10 µL) in central hole and fractions to be tested (10 µL) in surrounding holes.
5. Incubate at RT for 4–8 h, look for lines of hemolysis (clearing) between central well and wells containing positive fractions.
6. Pool positive fractions and concentrate for next step.

3.4. Purification of the Anaphylatoxins: C3a, C4a, *and* C5a

1. To fresh serum, add the protease inhibitors 6-aminohexanoic acid (1 *M*) and either 2-mercaptomethyl-5-guanidinopentanoic acid (1 m*M*) or 2-mercaptomethyl-3-guanidinoethylthiopropranoic acid (1 m*M*) ***(28)***.
2. Add zymosan at 20 mg/L to activate AP and generate C3a and C5a and, after 10 min at 37°C, add aggregated IgG at 0.5 mg/mL (prepare by heating IgG at 10 mg/mL in PBS at 63°C, 20 min) to activate CP and generate C4a. Continue incubation for a further 30 min at 37°C.
3. Add 10 *M* HCl to a final concentration of 1 *M* and cool on ice for 1 h.
4. Remove precipitate by centrifugation and dialyze supernatant against 100 m*M* Ammonium formate, pH 5.0 (use dialysis tubing with 3.5 kDa cutoff), and concentrate to 1/5 of original volume in an ultrafiltration cell (5 kDa cutoff).
5. Apply to Biogel P-60 gel-filtration column (2 cm × 100 cm) equilibrated in 100 m*M* ammonium formate, pH 5.0, identify positive fractions, pool and concentrate.
6. Apply to a SP-Sephadex column (1.6 × 30) equilibrated in the same buffer. Wash with 100 m*M* ammonium formate pH 7.0 to elute contaminating proteins. Elute bound protein with a 650 mL linear gradient to 800 m*M* ammonium formate pH 7.0. Anaphylatoxins elute in the order: C5a, C4a, C3a.
7. Apply C5a containing fractions to CM-Sephadex C-25 (0.6 cm × 14 cm) equilibrated in 0.1 *M* ammonium formate pH 7.0 and elute with equilibration buffer.
8. Dialyze C4a fractions against 150 m*M* ammonium formate pH 5.5 and apply to CM-cellulose (CM52; 1.6 × 15) equilibrated in the same buffer and elute with 400 mL linear gradient to 350 m*M* ammonium formate.
9. Dialyze C3a fractions against 150 m*M* ammonium formate pH 7.0 and apply to CM-cellulose (CM52; 1.5 × 27) equilibrated in the same buffer and elute with 600 mL linear gradient to 450 m*M* ammonium formate (*see* **Note 4**).

3.4.1. Functional Assays

Numerous assays for the activities of the anaphylatoxins have been used, including smooth muscle contraction, neutrophil chemotaxis assays, and neutrophil oxidase assays. Such methods are beyond the scope of this chapter. For identification of C3a and C5a in column fractions the simplest approach is to use specific antisera in immunochemical assays.

3.5. Purification of Complement Regulators

A variety of functional assays can be used to measure the inhibitory activity of the fluid phase and membrane bound regulators. A large number of regulators have cofactor activity for factor I (C4bp, fH, CR1, MCP). The method to measure cofactor activity is fairly general for all inhibitors and is described later and in more detail in Chapter 18. Some membrane bound regulators (DAF, CD59) have the ability to reincorporate into a membrane and so confer C resistance to the target cell, however, all these inhibitors require a different target

cell and the methods will be described with the respective purification methods. Fluid phase regulators are generally purified from serum. For the membrane bound regulators and receptors, it is important to choose the right cell type. Erythrocytes are the most convenient source of C regulators because they can easily be obtained in large quantities, are enucleate, and do not contain large amounts of proteolytic enzymes. Other cell types that can be obtained in relatively large quantities are platelets, neutrophils, tonsils, and spleen cells. An alternative is to use a cell line expressing the regulator or receptor of interest, but this will usually require a large volume of cultured cells. Membrane bound C regulatory proteins are either attached to the membrane via a transmembrane anchor (TM), like CR1 and MCP, or a glycosyl phosphatidylinositol (GPI) anchor (DAF, CD59). The first step in purification of a C regulator is membrane extraction, to be described in **Subheading 3.5.7.** Erythrocytes are the best source of DAF, CD59 and CR1 (MCP is not expressed on human erythrocytes), whereas platelets or an appropriate cell line can be used for purification of MCP.

3.5.1. Purification of C1-inh

C1-inh: M_r 110 kDa, serum conc. 200 µg/mL ***(29)***.
Small scale purification of C1-inh can be carried out as follows:

1. Dilute 5 mL serum to 50 mL with 15% PEG6000 in PBS/5 m*M* EDTA, spin 30 min 10,000*g*, 4°C, and retain supernatant.
2. Increase PEG concentration in the supernatant to 25% by addition of solid PEG6000. Stir 30 min and spin 10,000*g*, 4°C, 30 min.
3. Dissolve precipitate in 5 mL 20 m*M* Tris, pH 7.0, and apply to a Heparin Sepharose column (1.6 × 8) equilibrated in the same buffer. Elute with 60 mL of a linear gradient from 0 to 1 *M* NaCl.
4. Pool C1-inh-containing fractions, dilute four times in 20 m*M* Tris, pH 7.0 and apply to a MonoQ column equilibrated in the same buffer. Elute with a 20-mL linear gradient to 500 m*M* NaCl.
5. Identify C1-inh-containing fractions, pool, concentrate and fractionate on Superose 12 in PBS. Concentrate C1-inh pool and store in aliquots at –70°C.

3.5.1.1. Functional Assay for C1-inh

The functional assay of C-inh is based on the inhibition of C1s esterolytic activity (*see* **Subheading 3.1.1.**).

1. In wells of a 96-well plate, mix C1-inh containing fractions with C1s (1 µg/well) in a total volume of 100 µL and incubate for 30 min at 37°C.
2. To each well, add chromogenic substrate specific for C1s (*see* **Subheading 3.1.1.**)
3. Incubate 30 min 37°C and measure absorbance of the cleaved chromogenic substrate.
4. Pool fractions that inhibit C1s esterolytic activity.

*3.5.2. Purification of C4 Binding Protein (*C4bp*)*

C4bp: M_r 450–550 kDa, reduced: 72 kDa. 6–7 α chains: 70 kDa, 0–1 β chain: 45 kDa, serum conc. 250 µg/mL ***(30)***.

1. Collect blood in 10 times concentrated buffered solution to obtain final conc. of 5 m*M* NaPhosphate, 10 m*M* benzamidine, 10 m*M* EDTA and 0.1 m*M* PMSF, pH 7.4, and separate cells from plasma by centrifugation.
2. To 500 mL buffered plasma, add 250 mL 15% (w/v) PEG4000 (in 5 m*M* NaPhosphate, 10 m*M* EDTA, 0.1 m*M* PMSF, 75 m*M* NaCl, pH 7.4) to achieve a final PEG4000 concentration of 5%, centrifuge at 10,000*g* 30 min.
3. Resuspend precipitate in 20 mL 50 m*M* NaPhosphate, 1 *M* NaCl, 5 m*M* EDTA, 5 m*M* EACA, 0.5 m*M* PMSF, pH 6.7 (GF buffer).
4. Apply to Sepharose 6B gelfiltration column (5 cm × 50 cm) equilibrated and run in GF buffer. Identify and pool C4bp-containing fractions.
5. To pooled fractions add 0.5 vol of 22.5% (w/v) PEG4000 in 50 m*M* Tris, 75 m*M* NaCl, 0.5 m*M* $CaCl_2$, pH 8.0, and stir 30 min. Centrifuge 10,000*g* 30 min and resuspend pellet in 80 mL of 50 m*M* Tris, 75 m*M* NaCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* PMSF, pH 8.0 (HS buffer).
6. Apply to heparin Sepharose column (2.5 × 70) equilibrated in HS buffer. Elute with 1-L linear gradient 0 to 0.6 *M* NaCl in HS buffer.
7. Add 0.5 vol of 22.5% (w/v) PEG4000 in 50 m*M* Tris, 20 m*M* NaCl, 0.5 m*M* $CaCl_2$, 0.5 m*M* PMSF, pH 8.0. Centrifuge 10,000*g* 30 min and resuspend pellet in 70 mL 50 m*M* Tris, 20 m*M* NaCl, 0.5 m*M* $CaCl_2$ (DE buffer).
8. Apply to DE-52 anion exchange column (2.5 cm × 30 cm) equilibrated in DE buffer and elute with 500-mL linear gradient 0 to 0.4 *M* NaCl in DE buffer.
9. Concentrate by ultrafiltration and apply to an affinity column consisting of C4 coupled to Sepharose (5 mg C4 coupled to 5 mL CNBr-activated sepharose) equilibrated in 10 m*M* Tris, 15 m*M* NaCl, 0.1 m*M* $CaCl_2$, pH 8.1.
10. Elute bound C4bp in 50 m*M* NaAc, 300 mM NaCl, pH 6.5, dialyze into VBS, concentrate and store at –70°C.

3.5.2.1. Functional Assay for C4bp

Functional assay is based on the capacity of C4bp to act as cofactor for factor I in the cleavage of iC3b. In the assay, methylamine treated human C3 ($C3_{MA}$) is used as a substrate.

1. Dialyze fractions containing cofactor (factor H, CR1, or MCP) into PBS/0.1% NP40.
2. Mix with 200 µg methylamine treated human C3 (*see* **Subheading 3.1.5.**) and 20 µg human factor I and incubate 2 h or overnight at 37°C.
3. Run on 10% SDS-PAGE under reducing conditions, blot onto nitrocellulose and probe blots with anti-C3. Cofactor activity is revealed by the disappearance of the α-chain and the appearance of the 43 and 47 kDa α-fragments.
4. To measure cofactor activity of C4bp, methylamine treated human C4 ($C4_{MA}$) is used as a substrate, but the assay is otherwise similar.

3.5.3. Purification of Factor I

fI: 2 chains: M_r 50 Kda, 38 kDa, serum concentration 30–50 µg/mL ***(31)***.

1. Add 1 m*M* PMSF to 500 mL of fresh plasma and pass over a lysine Sepharose column (5 cm × 15 cm) equilibrated in 100 m*M* KPhosphate, 150 m*M* NaCl, 15 m*M* EDTA, pH 7.0. Collect breakthrough.
2. Dialyze against 20 m*M* Tris, 60 m*M* NaCl, 1 m*M* PMSF, pH 7.8, and load onto QAE-Sephadex column (7 cm × 36 cm) equilibrated in the same buffer. Elute with a 4-L linear gradient of 60–360 m*M* NaCl.
3. Pool and make 60% saturated with $(NH_4)_2SO_4$ (36 g/100 mL), stir 2 h, 4°C, and spin 20 min, 8000*g*, 4°C.
4. Dissolve precipitate in 60 mL 50 m*M* NaPhosphate, 200 m*M* NaCl, 1 m*M* PMSF, pH 7 and pass over a wheat germ lectin column (Pharmacia; 4 cm × 7.5 cm) equilibrated in the same buffer. Elute with buffer containing 100 mg/mL N-acetyl-D-glucosamine.
5. Dialyze against 25 m*M* KPhosphate, 1 m*M* PMSF, pH 7.5, and load onto Hydroxylapatite column (3.2 cm × 15 cm) equilibrated in the same buffer. Elute with 600 mL gradient to 200 m*M* KPhosphate
6. Concentrate by addition of 40 g/mL $(NH_4)_2SO_4$, stir 2 h, 4°C, and spin 20 min, 8000*g*, 4°C.
7. Dissolve precipitate in 5 mL 25 m*M* Tris, 150 m*M* NaCl, pH 7.0, and pass over Sephacryl-200 gel filtration column (2 cm × 90 cm) equilibrated in the same buffer. Store at 4°C

3.5.3.1. Functional Assay for fI

Cofactor assay using $C3_{MA}$ and appropriate cofactor (e.g., erythrocyte cell extract as source of CR1 or purified MCP, CR1, or fH) essentially as described in **Subheading 3.5.2.**

3.5.4. Purification of Factor H

FH: M_r 170 kDa, serum concentration: 200–600 µg/mL; 20 SCR ***(32)***.

1. Bring 1 L of serum to 5% PEG4000 by addition of 250 mL of 25% (w/v) PEG4000 in 50 m*M* NaPhosphate, 150 m*M* NaCl, 15 m*M* EDTA, pH 7.4, stir for 30 min, and spin 30 min 8000*g*.
2. Bring supernatant to 12.5% PEG4000 by addition of 190 mL of 50% (w/v) PEG4000 in the same buffer, stir 30 min, and spin 8000*g*, 30 min. Dissolve precipitate in 300 mL 50 m*M* NaPhosphate, 15 m*M* EDTA, 150 m*M* NaCl pH 7.0.
3. Pass over lysine-Sepharose column (5 cm × 15 cm) and dialyze against 25 m*M* KPhosphate, 5 m*M* EDTA, pH 7.0 (DE buffer).
4. Load on DEAE-Sephadex column (5 cm × 50 cm) equilibrated in DE buffer and elute with 4 L of a linear gradient 0 to 500 m*M* NaCl in DE buffer.
5. Pool fI-containing fractions and bring to 14% (w/v final conc.) PEG4000 by addition of appropriate volume of 50% stock solution, stir 30 min, and spin 8000*g*, 30 min.

Resuspend precipitate in 20 mL 100 m*M* KPhosphate, 5 m*M* EDTA, 150 m*M* EDTA, pH 7.4.

6. Apply to Sepharose 6B gelfiltration column (5 cm × 90 cm) equilibrated in the same buffer and dialyze against 10 m*M* KPhosphate, pH 6.8 (HA buffer).
7. Apply to Hydroxyapatite column (1.5 cm × 20 cm) equilibrated in HA buffer. Elute with 500 mL of a linear gradient 0 to 200 m*M* KPhosph in HA buffer. Dialyze against 10 m*M* Tris, 1 m*M* EDTA, 25 m*M* NaCl, pH 8.4 (DE buffer).
8. Apply to DEAE-Sepharose CL-6B column (2 cm × 25 cm) equilibrated in DE buffer and elute with 1.4 L linear gradient 25–400 m*M* NaCl in DE buffer.

3.5.4.1. Functional Assay for Factor H

Cofactor assay essentially as described in **Subheading 3.5.2.**

3.5.5. Purification of S-protein

S-protein: M_r:89 kDa, reduced 84 kDa and 69 and 15 kDa fragments. Serum concentration 0.35–0.5 mg/mL. Alternative name: vitronectin ***(33)***.

1. To 1 L of fresh citrated plasma add the following inhibitors: 10 m*M* benzamidine, 1 m*M* PMSF, 0.5 m*M* NPGB. Add slowly while stirring, 80 mL of 1 *M* $BaCl_2$, stir for 1 h, and spin 15 min 6000*g* 4°C.
2. To the supernatant, add PEG4000 (from a 50% stock solution in 10 m*M* Tris, 150 m*M* NaCl, pH 7.4) to a final concentration of 9%. Stir for 1 h and spin 6000*g*, 15 min, 4°C.
3. To the supernatant, add solid PEG4000 to give a final concentration of 20%. Stir for 1 h and spin 6000*g*, 15 min 4°C. Dissolve pellet in 20 m*M* NaPhosphate, 2 m*M* EDTA, 2 m*M* benzamidine, 1 m*M* reduced glutathione, 1 m*M* PMSF, pH 7.0 (DE buffer), and spin at 6000g, 15 min 4°C.
4. Load onto DEAE-Sephacel column (2.9 cm × 40 cm) equilibrated in DE buffer. Wash with DE buffer containing 25 m*M* NaCl, elute with a 2-L linear gradient from 25 to 300 m*M* NaCl. S-protein coelutes with ceruloplasmin (blue fractions off column) and just before C9, S-protein eluting after C9 is usually aggregated.
5. Pool positive fractions and add solid $(NH_4)_2SO_4$ to give 70% final saturation. Stir for 1 h and spin 10,000*g*, 20 min 4°C. Dissolve precipitate in 50 m*M* Tris, 150 m*M* NaCl, 2 m*M* benzamidine, 1 m*M* glutathione pH 7.4, and dialyze against the same buffer.
6. Apply to Blue-Sepharose column (2.5 cm × 25 cm) equilibrated in the same buffer and elute with 1 *M* NaCl.
7. Pool positive fractions and concentrate by ultrafiltration using a YM10 membrane.
8. Apply to a Sephacryl S-200 gel filtration column (2.8 cm × 105 cm) equilibrated in 50 m*M* Tris 150 m*M* NaCl, 2 m*M* benzamidine, 1 m*M* glutathione pH 7.4.
9. Pool positive fractions and apply to an antihuman albumin immunoaffinity column equilibrated in the same buffer, collect breakthrough.
10. Apply to an anti-C9 immunoaffinity column and collect breakthrough.
11. Concentrate to 1 mg/mL and store in small aliquots at –70°C (*see* **Note 5**).

3.5.6. Purification of Clusterin

Clusterin: M_r approx 80 kDa nonreduced, two chains (α and β) at 35–40 kDa reduced, 0.2–0.4 mg/mL in serum ***(34)***. Methods for clusterin purification all involve the use of immunoaffinity chromatography, which is described in Chapter 4.

3.5.7. General Procedures for Membrane Extraction

3.5.7.1. Erythrocyte Ghost Preparation

1. Wash 4 U (approx 1 L) of packed erythrocytes three times with PBS to remove plasma and "buffy coat" containing white cells.
2. Lyse packed cells in 10 vol of ice-cold 5 m*M* NaPhosphate, 2 m*M* EDTA. Additional protease inhibitors (1 m*M* benzamidine, 1 m*M* PMSF, 0.02% sodium azide) are required for some proteins. Stir overnight at 4°C (if the lysis mix warms up, ghosts will reseal and include hemoglobin, resulting in less clean ghosts.)
3. Wash and concentrate ghosts using a pellicon ultrafiltration system (membrane 300 kDa cutoff) and/or spin 13,000*g*, 30 min, 4°C until supernatant is almost free of hemoglobin and ghost pellet appears faintly pink. Note that the ghost pellet is very easily disturbed and great care must be taken in removing wash buffer to avoid loss of ghosts.

3.5.7.2. Membrane Extraction

GPI-anchored DAF and CD59 can be extracted using N-butanol or 1% NP40. Butanol extraction yields cleaner preparations and is best for subsequent classical chromatography purification. Butanol extraction is not appropriate for TM anchored CRP or CR. Extraction with NP40 is simpler, can be used for TM proteins and gives better yields making it most appropriate for subsequent affinity chromatography.

3.5.7.2.1. Butanol Extraction

1. To 1 part of packed ghosts, add three parts of PBS and one part of N-butanol. Stir for 30 min at RT, and spin 10 min, 2500*g*.
2. Remove the lower aqueous phase and dialyze against the starting buffer for the first purification step. Change dialysis buffer several times to ensure removal of all N-butanol.

3.5.7.2.2. NP40 Extraction

1. Take one part of ghosts, add four parts of PBS and add NP40 to 1% final concentration. Stir 30 min at RT.
2. Spin to remove undissolved material 15 min, 10,000*g* and use supernatant for further purification.

3.5.7.3. Membrane Extraction of Platelets or Cell Lines

For extraction of membrane bound proteins from nucleated cells it is necessary to add protease inhibitors to prevent degradation of the protein of interest.

1. Wash cells (platelets, leukocytes, or appropriate cell line) three times in PBS to remove serum proteins.
2. Lyse cells in ice cold lysis buffer (20 m*M* Tris pH 8.2, 140 m*M* NaCl, 2 m*M* EDTA, 1% NP40, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 1 m*M* PMSF) at 1–5 × 10^7cells/mL for 30 min on ice. To increase yield, other detergents like digitonin can be used, however, these are much more expensive than NP40.
3. Spin to remove undissolved material 15 min 10,000*g* and use the supernatant for further purification.

3.5.8. Purification of Membrane Cofactor Protein (MCP/CD46)

MCP: M_r 50–80, single-chain, TM anchor, 4 SCR ***(35)***.

A major problem with the purification of MCP is its tendency to stick nonspecifically to various surfaces, including column matrices and filters. A standard chromatography method to purify MCP is carried out as follows.

1. Dialyze a 1% NP40 extract from 20 mL of packed cells (~ 3×10^{10} cells, e.g., U937 or platelets) against 20 m*M* Na-acetate, 0.1% NP40, pH 5.0. Remove precipitate by centrifugation 15 min 10,000*g*.
2. Apply to a chromatofocusing column (2 cm × 50 cm) equilibrated in the same buffer, pH 4.75. Elute the column with a linear gradient (20–250 m*M*) acetate buffer (pH 4.3) in 10% (vol/vol) polybuffer 74. Pool and dialyze positive fractions against 10 m*M* Tris, 0.05% NP40, pH 7.5.
3. Apply to a 50-mL high-resolution Hydroxyapatite column, equilibrated in the same buffer. Elute the column with a linear KPhosphate gradient from 0 to 250 m*M* pH 7.5 containing 0.05% NP40. Pool positive fractions.
4. Load onto a $C3_{MA}$-sepharose column (20 mL sepharose to which 20 mg C3MA (generated and purified as described in **Subheading 3.1.5.**) has been immobilized, equilibrated in 0.05% NP40, pH 7.2. Wash with 20 m*M* NaPhosphate 1 m*M* PMSF, 0.05% NP40, pH 7.0. Elute with the same buffer containing 0.5 *M* NaCl. Dialyze against the same buffer containing 20 m*M* NaCl.
5. Load onto a MonoQ column in equilibrated in the same buffer and elute with a linear NaCl gradient from 20 to 500 m*M*.

3.5.8.1. Functional Assay

Cofactor assay essentially as described in **Subheading 3.5.2.**

3.5.9. Purification of Decay Accelerating Factor (DAF/CD55)

DAF: M_r 70 kDa, single-chain, GPI anchored, 4 SCR ***(36)***.

1. Dialyze N-butanol extract of erythrocytes into 20 m*M* Tris, 40 m*M* NaCl, pH 8.0 (DE buffer), and apply to a DEAE Sepharose column (5 cm × 30 cm) equilibrated in DE buffer. Wash in DE buffer containing 0.1% NP40 and elute with a linear

gradient 40–500 m*M* NaCl in DE buffer with NP40. Concentrate active fractions and dialyze against 20 m*M* NaPhosphate, 0.1% NP40, pH 7.4 (HA buffer).
2. Apply to Hydroxyapatite column (5 cm × 20 cm) equilibrated in HA buffer. Wash and elute with 1 L linear Na/KPhosphate gradient in HA buffer. Pool active fractions.
3. Add NaCl to 300 m*M* and apply to phenyl-Sepharose (1.5 × 6.5) column equilibrated in 40 m*M* NaPhosphate, 300 m*M* NaCl, 0.1% NP40, pH 7.4. Elute with 160 mL inverse linear gradient to from 300 to 40 m*M* NaPhosphate in 50 m*M* NaCl, 1% NP40, pH 9.5.
4. Dialyze pooled active fractions against 5 m*M* NaPhosphate, 25 m*M* NaCl 0.1% NP40, pH 7.5, apply to a trypan blue-Sepharose column (2 cm × 5 cm) and elute with a 100-mL NaCl gradient 25 to 325 m*M* in the same buffer.
5. Identify active fractions, pool, dialyze against PBS-CHAPS (0.05%), concentrate by ultrafiltration and store at 4°C with 0.01% NaN3 or in small aliquots at –20°C.

3.5.9.1. Functional Assay

DAF has the capacity to reincorporate into a membrane through its GPI anchor and so confer resistance to C. The ideal target cells for measuring DAF activity are sheep erythrocytes. No interference of contaminating CD59 will be observed because of the high level of expression of functional sheep CD59 that masks the activity of any addition human CD59 added ***(37,38)***.

1. Incubate column fractions with 2% ShEA for 30 min at 37°C (NB fractions have to be dialyzed into 0.05% CHAPS before assay as NP40 will lyse the cells).
2. Wash cells three times in VBS and resuspend in VBS at 2%.
3. In the wells of a 96-well plate, incubate with dilutions of human serum (1/50–1/100) for 30 min at 37°C.
4. Measure absorbance at 412 nm in supernatant and plot percent lysis at each serum dose (*see* **Note 5**).

3.5.10. Purification of CD59

CD59: M_r 18–20 kDa, single-chain, GPI-anchored ***(37)***.

3.5.10.1. Purification by Anion Exchange and Hydrophobic Interaction:

1. Dialyze butanol extract derived from 1 U (250 mL) of human erythrocytes against 20 m*M* Tris, 0.05% CHAPS, pH 8.4 (Q buffer), and apply to a Q-Sepharose fast-flow column (1.6 cm × 15 cm) equilibrated in the Q buffer. Elute with 600 mL of a linear gradient to 0 to 1 *M* NaCl. Pool positive fractions and dialyze against 20 m*M* NaPhosphate, 0.05% CHAPS, pH 7.4 (Q buffer).
2. Apply to MonoQ column equilibrated in Q buffer. Elute with 30 mL of a linear gradient to 0.5 *M* NaCl. Bring to 1.7 *M* by addition of solid ammonium sulphate.
3. Apply to phenyl superose column equilibrated with 40 m*M* NaPhosphate, containing 1.7 *M* ammonium sulphate, 0.05% CHAPS, and pH 7.4. Elute with a

30-mL inverse linear ammonium sulphate gradient from 1.7 *M* to 0 in 40 m*M* NaPhosphate, 1% CHAPS, pH 7.4.
4. Pool active fractions and dialyze into PBS/0.05% CHAPS.

3.5.10.2. Purification by Preparative SDS-PAGE

An alternative method for purification of CD59 from erythrocytes is preparative SDS-PAGE. Proteins can be highly purified from a relatively crude membrane preparation in a single step ***(39)***. CD59 is remarkably resistant to denaturation and hence this method is suitable to purify functionally active CD59.

1. Dialyze butanol extract from 100 mL packed erythrocytes against PBS/0.1% NP40. Concentrate to 2.5 mL in an Amicon ultrafiltration cell using a 100-kDa cutoff membrane (CD59 has a M_r of 18–20 kDa on SDS-PAGE, but in NP40 resides in a high-molecular weight micelle).
2. Mix 1:1 with SDS-PAGE nonreducing sample buffer and load on a 15% SDS-PAGE tube gel (30 mL gel volume; Prep cell SDS-PAGE apparatus, Bio-Rad).
3. Run at 40 mA constant current and elute at 1 mL/min collecting 5 mL fractions. Dialyze fractions two times against PBS/0.05% CHAPS to remove SDS and assay for functional activity.
4. Pool and dialyze active fractions against PBS/0.05% CHAPS, concentrate in Amicon cell using a 10-kDa membrane and run on a Superose 12 column equilibrated in the same buffer. Pool active fractions and store at 4°C.

3.5.10.3. Functional Assay

This assay is based on the ability of CD59 to reincorporate into a cell through its GPI-anchor. Guinea pig erythrocyte (GPE) are the ideal target cells. Chicken E are an acceptable alternative but sheep E, pig E, or rabbit E are unsuitable, due to the high level of endogenous CD59 on these cells ***(37,38)***. Fractions to be tested have to be in CHAPS because other detergents will lyse the erythrocytes. E can be incubated with up to 0.3% CHAPS without lysis.

1. In a 96-well plate, incubate 50 µL of a 2% suspension of GPE in PBS/0.05% CHAPS with 50 µL of column fraction, 30 min 37°C.
2. Wash cells twice in VBS to remove unincorporated proteins.
3. Incubate with 100 µL human serum 1/10–1/20 (serum dose chosen to cause near-100% lysis in absence of inhibitor) and CVF (1 µg/mL final conc.) 30 min 37°C.
4. Measure absorbance at 412 nm in supernatants. Identify fractions causing inhibition of E, pool and take to next step (*see* **Note 5**).

3.6. Purification of C Receptors

The first step in purification of a C receptor is to obtain a membrane extract from an appropriate cell type as described in **Subheading 3.5.7.** The appropri-

ate cell source for a C receptor is determined by expression levels. There are no functional assays available for the majority of complement receptors other than binding of the relevant ligand. The purification methods described in this chapter are based on the affinity of the receptors for their ligands, ensuring the purification of the desired component. All C receptors have transmembrane anchors. Specific receptors have been described for C1q, fragments of C4, fragments of C3, and fragments of C5. Purification protocols for the majority of these have been described, most studied have been receptors for fragments of C3 (+/– the equivalent fragments in C4). Purification of CR1, CR2, CR3, and CR4 can all be performed by affinity chromatography using the appropriate C3 fragments as ligand. Differential elution of C receptors that have affinity for the same ligand can be achieved by removal of bivalent cations. For example, CR3 and CR4 elute from an iC3b column upon removal of bivalent cations, while CR1 and CR2 are eluted with 130 and 300 m*M* NaCl, respectively ***(48)***. Purification of C3a receptor has not been described. The structure of the C3aR is very similar to that of C5aR and purification can probably be carried out using a similar protocol and specific assays (*see* below).

3.6.1. Purification of Receptors for C1q

At least three different receptors for C1q have been identified so far ***(40–42)***. The two methods outlined here describe, respectively, the purification of the first putative C1qR identified, the so-called collectin receptor ***(43)***, and the more recently described, but much better characterized phagocytic C1q receptor C1qRp ***(41)***.

3.6.1.1. Isolation of the Collectin Receptor (cC1qR, M_r 55–70 kDa) ***(40,44,45)***

1. Extract Raji cells (4×10^{10} cells) or tonsil cells with 1% NP40 in 10 m*M* NaPhosphate, 0.15 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 100 µg/mL SBTI, 5 m*M* iodoacetamide, 2.5 m*M* PMSF, 20 µ*M* 1,10-phenanthroline, 5 µg/mL pepstatin, pH 7.4. Incubate 30 min on ice, spin 30 min 13000*g*, 4°C, and take supernatant.
2. Apply cell lysate to C1q-Sepharose column (3 mL; 3 mg C1q bound per mL Sepharose CL-4B; C1q purified as described in **Subheading 3.1.1.**) equilibrated in 10 m*M* NaPhosphate, pH 7.4, containing 0.1% emulphogene BC720 and the above enzyme inhibitors. Elute with 1 *M* NaCl and dialyze protein-containing fractions against 10 m*M* NaPhosphate, 0.1% emulphogene BC720, 7.4.
3. Apply to MonoQ column equilibrated in the same buffer and elute with 50 mL linear gradient from 0 to 1 *M* NaCl. Identify positive fractions and concentrate by ultrafiltration.
4. Fractionate on a Superose 12 gel filtration column equilibrated in 50 m*M* NaPhosphate, 2 m*M* EDTA, 150 m*M* NaCl, 0.1% emulphogene BC720, pH 7.4. Identify positive fractions, concentrate by ultrafiltration, and store at 4°C.

3.6.1.2. Isolation of the Phagocytic C1qR (C1qRp; M_r 126 kDa) ***(41)***

1. C1q, purified as described in **Subheading 3.1.1.**, is subjected to limited tryptic digestion and the collagenous tails separated from globular heads by gel filtration on Superose 12.
2. C1q collagenous tails (2 mg) are immobilized on 3 mL sepharose 4B (using CNBr-activated sepharose).
3. Extract U937 cells (4×10^{10} cells) or monocytes cells with 1% NP40 in 10 m*M* NaPhosphate, 0.15 m*M* $CaCl_2$, 0.5 m*M* $MgCl_2$, 100 µg/mL SBTI, 5 m*M* iodoacetamide, 2.5 m*M* PMSF, 20 µ*M* 1,10-phenanthroline, 5 µg/mL pepstatin, pH 7.4. Incubate 30 min on ice, spin 30 min 13,000*g*, 4°C, and take supernatant.
5. Apply cell lysate to C1q(tail)-Sepharose column equilibrated in 10 m*M* NaPhosphate, pH 7.4. 0.05% NP40 and the above enzyme inhibitors. Elute with 1 *M* NaCl in the above buffer and dialyze protein-containing fractions against PBS, 0.05% NP40 (*see* **Note 6**).

3.6.1.3. Functional Assay

Column fractions are best assayed by immunochemical methods using antibodies specific for the various receptors The collectin receptor (cC1qR) has been assayed by monitoring inhibition of C1q activity for lysis of ShEA ***(44)***.

1. Incubate column fractions with 50 ng C1q, 60 min 37°C.
2. Add 10 µL C1q depleted serum (containing 20 m*M* $CaCl_2$; generated by passage over IgG column) and 50 µL 2% sensitized ShE in a total volume of 150 µL, incubate 30 min at 37°C and measure lysis.

3.6.2. Purification of C5aR *(CD88)*

C5aR*:* M_r: 42 kDa, seven transmembrane domains ***(46,47)***.
The purification of the C5aR is carried out based on its affinity for C5a.

1. Mix one volume of packed neutrophils (6×10^9 cells) with 1 vol 50 m*M* Hepes, 2% digitonin, 0.1 m*M* PMSF, 10 µg/mL chymostatin , 10 µg/mL leupeptin, pH 7.2, incubate on ice for 1 min and remove insoluble material by centrifugation (200,000*g*, 7 min).
2. Coupling of C5a to commercial resins has proven to be very inefficient. The following procedure has been used for coupling of C5a to solid phase.
   a. Add 120 µL tetrahydrophthalic anhydride (3.65 mg/mL in dioxane) to 12 mL C5a (1 mg/mL in 0.1 *M* Hepes, pH 8.0) and incubate 5 h RT.
   b. Add 86 µL maleic anhydride (78.4 mg/mL in dioxane) and incubate overnight.
   c. Adjust pH to 5.7 and incubate overnight.
   d. Adjust pH to 8.0 and concentrate on an Amicon YM5 membrane to 4 mg/mL.
   e. Add to 2 mL Affigel 10 and incubate overnight at 4°C.

f. Wash gel with 100 m*M* Hepes pH 8.0, block with 1 *M* glycine, 4 h, wash with 100 m*M* Hepes, pH 3.2, and incubate 72 h, 37°C.

Store in 0.1 *M* Hepes, pH 7.2.

3. Incubate neutrophil extract with 2 mL C5a-affigel, equilibrated in 100 m*M* Hepes, 0.1% digitonin, 5 m*M* $MgCl_2$, pH 7.2, and incubate 48 h with mixing.
4. Wash with buffer, containing 1 *M* NaCl and with 100 m*M* Hepes, 0.1% digitonin, pH 7.2. Elute with 50 m*M* formic acid, 200 m*M* KSCN, 0.1% digitonin, pH 4.0, and desalt immediately on 10 mL Bio-Rad P-10 column in 100 m*M* Hepes, 0.1% digitonin, 5 m*M* $MgCl_2$, pH 7.2. Eluted material consists of three components: C5aR and the α and β subunit of the associated G-protein.

3.6.3. Purification of Complement Receptor 1 (CR1/CD35)

CR1: M_r: nonred, 190 kDa, red, 240 kDa, 30 SCR, TM-anchor.

Erythrocytes have only a low level of expression of CR1, but because of the ease with which they can be obtained and because membrane extracts are relatively easy to prepare, erythrocytes are the preferred source of CR1 ***(49)***.

1. Dialyze a 1% NP40 extract obtained from 20 U (5 L) of human erythrocytes against 0.1% emulphogene BC720 (Sigma), 1 m*M* benzamidine, pH 7.0, and 5 vol, 5 m*M* NaPhosphate, 0.5 m*M* EDTA, 5 m*M* benzamidine, pH 7.8 (DE buffer).
2. Apply to DEAE-Sephacel column (5 cm × 85 cm) equilibrated in DE buffer. Elute with 8 L of a linear gradient from 0 to 200 m*M* KCl. Identify positive fractions and dialyze against 10 m*M* KPhosphate, 0.5 m*M* EDTA, 5 m*M* benzamidine, 0.1% emulphogene, pH 7.0 (start buffer).
3. Apply to $C3_{MA}$-Sepharose 4B column (1.5 cm × 20 cm); 7.5 mg $C3_{MA}$ coupled to CNBr-Sepharose 4B $C3_{MA}$ prepared as described in **Subheading 3.1.5.** Elute with 300 mL linear gradient 0 to 350 m*M* NaCl in start buffer.
4. Pool positive fractions, dialyze into start buffer, reapply to $C3_{MA}$-Sepharose column and elute as above.
5. Pool positive fractions, concentrate and store at –20°C.

3.6.3.1. Functional Assay

Assay using cofactor activity with $C3_{MA}$ as substrate as described in **Subheading 3.5.2.**

3.6.4. Purification of Complement Receptor 2 (CR2/CD21)

CR2: M_r: nonred, 110–120 kDa; reduced 145 kDa, SCR 15 or 16; TM-anchor ***(50)***.

1. Extract tonsil lymphocytes (2×10^{10}, approx 40 tonsil pairs) at 10^8 cells/mL with 3% (w/v) Brij96 in 150 m*M* NaCl, 2 m*M* PMSF, 15 min , 4°C, spin 10,000*g* 5 min, and add 0.1% Triton X-100 to supernatant. Dialyze against 10 m*M* NaPhosphate, 1% NP40, 2 m*M* PMSF, pH 7.4.

2. Apply to C3d,g-Sepharose column (1.4 cm × 7 cm; 2.4 mg C3d,g/mL Sepharose) and wash sequentially with 30 mL 10 m*M* NaPhosphate, 1% NP40, pH 7.4 containing 80 m*M* NaCl, 130 m*M* NaCl, and 190 m*M* NaCl, respectively. CR2 elutes with 130 m*M* NaCl. Pool and dialyze against 10 m*M* NaPhosphate, 1% NP40, pH 7.4
3. Apply to DEAE-Sephacel column (2 cm × 7 cm) equilibrated in the same buffer. Elute with 20 mL of 150 m*M* NaCl.

3.6.5. Purification of Complement Receptor CR3 (CD11b/CD18 Heterodimer)

CR3: 2 chain, TM: CD11b: 165 kDa; CD18: 95 kDa ***(51)***.

CR3 can be purified from neutrophils or an appropriate cell line. All buffers contain 1 μg/mL each of the following protease inhibitors: antipain, benzamidine, chymostatin, leupeptin, aprotinin, and pepstatin. Also contain 2 m*M* PMSF, 5 m*M* pefablock.

1. Extract cells at 2–5 × 10^7/mL in 20 m*M* Hepes, 2.5% nOGP (Sigma), 50 m*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, pH 7.4 for 10 min on ice. Spin 12,000*g*, 10 min.
2. Add supernatant to 100 μL C3bi-beads (*see* **Note 6**) in the same buffer.
3. Incubate the cell lysate with C3bi-beads for 3 min at RT and wash with lysis buffer. Elute with 2.5% nOGP, 20 m*M* Hepes, 10 m*M* EDTA, 50 m*M* NaCl pH 7.4 at RT.

3.6.6. Purification of Complement Receptor CR4 (CD11c/CD18 Heterodimer)

CR4: 2 chain, TM: CD11c: 150 kDa; CD18: 95 kDa ***(48)***.

1. Extract neutrophils or spleen cells with 1% NP40 in the presence of protease inhibitors as above.
2. Pass extract over iC3b-Sepharose column (2 mL; 2.2 mg iC3b/mL Sepharose), equilibrated with 1% NP40 in 10 m*M* NaPhosphate, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, pH 7.4 and elute with 10 m*M* NaPhosphate, 2 m*M* EDTA, pH 7.4 (elutes CR3 and CR4); elute other iC3b binding proteins with 500 m*M* NaCl.
3. Further separation of CR3 and CR4 can be achieved using MAb specific for CD11b and CD11c, respectively.

4. Notes

1. C1 can be reconstituted by incubation of purified C1q, C1r, and C1s (molar ratio 1:2:2) in VBS containing 5 m*M* $CaCl_2$. If activated C1r and C1s are required, prior to **step 5** above the column is incubated for 30 min at 37°C in the absence of NPGB and then eluted in VBS containing 10 m*M* EDTA. To separate MBL from MASP-1/2, dialyze against 100 m*M* NaAc, 200 m*M* NaCl, 5 m*M* EDTA pH 5.0, and gel filter on Sephacryl S-300 (1 cm × 45 cm) equilibrated in the same buffer.

No methods have been described to separate MASP-1 from MASP-2. Purification can be followed on SDS-PAGE with CBB staining or western blotting. MASP activity can be measured using the same esterolytic assay used for C1r and C1s, but substituting N-CBZ-L-leu (Sigma) as substrate. Because methylamine also inactivates C4, alternative pathway (AP) activation is required for measuring functional activity. Zymosan is added to drive AP activation and enhance the sensitivity of the assay.

2. CVF is a useful tool in C research because it can form with human factor B a C3/C5 convertase (CVF.Bb) that is resistant to inactivation by factor I and H, giving it a very long half-life (7 h compared to 1.5 min for C3bBb). The CVF.Bb complex can, therefore, cause rapid and uncontrolled activation of C that depletes C activity both in vitro and in vivo. Although not a C component, protocols for purification of CVF are for this reason included here. Whereas CVF from the cobra *Naja naja kaouthia* forms a convertase capable of cleaving both C3 and C5, that from *Naja haje* can only form a C3 convertase. The raw material for purifying CVF is freeze-dried cobra venom; this must be handled with extreme care because of the presence of cardiotoxins and neurotoxins which can be fatal if introduced into the blood stream. Toxins in waste products generated during purification should be neutralized with 4 *M* NaOH. Some cobra venoms contain more than one molecule that has an effect on the complement system ***(25)***, so care has to be taken to choose an appropriate screening method. Acquiring crude cobra venom has recently become almost impossible in many European countries because the CITES Treaty regulating trade in endangered species has severely restricted harvesting and supply of cobra venom.
3. The position of elution of human C9 from DEAE sephacel can be predicted by inspection of the fractions; C9 begins to elute just after the ceruloplasmin peak that gives a blue color to the fractions. It is only necessary to assay fractions from the beginning of the blue peak to some 20 mL after disappearance of the blue color. To purify C5b6 with good yield from serum, acute phase serum (from patients with recent episodes of infections, sports injuries, surgery, or childbirth) can be used because it contains elevated levels of C5 and C6 but not of C7 (not an acute phase reactant). However, acute phase serum may be difficult to obtain; a simple alternative is to use serum depleted of C7 by passage over an anti-C7 column.
4. For the isolation of the anaphylatoxins in their active state from serum it is essential to block the serum exopeptidase carboxypeptidase N completely. In order to achieve this, the inhibitor 6-aminohexanoic acid is used at 1 *M*, together with 1 m*M* 2-mercaptomethyl-5-guanidinopentanoic acid or 2-mercaptomethyl-3-guanidinoethylthiopropranoic acid ***(28)***. An alternative approach to generate C3a and C5a and to circumvent the problems with inactivation by carboxypeptidases is to use purified C3 or C5 and induce cleavage on CVF-Sepharose as described in **Subheading 3.1.5.** The fragments can then be separated by gel filtration. Recombinant C5a can be purchased from Sigma.
5. S-protein in column fractions is best assayed by dot blot or in ELISA using anti-S-protein antiserum. Numerous functional assays for S-protein have been

described, involving either inhibition of C reactive lysis (**Subheading 3.3.6.**) or induction of fibroblast adhesion and spreading. S protein is a "sticky" protein with a tendency to dimerise when impure. The purification should be carried out as quickly as possible and glutathione added to prevent dimerization. Once pure, S-protein is quite stable. Inhibition of lysis may not be sufficiently sensitive for identification of DAF-containing fractions; inhibition of C3b deposition on ShEA can be used instead, although this is technically demanding and requires a source of C8-depleted serum to avoid E lysis. Specific inhibition of C8 and C9 by CD59 can be measured by incorporation into GPE bearing C5b-7 or C5b-8 sites and developing the assay using purified C8/C9 or human serum diluted in PBS/5 m*M* EDTA as a source of C8 and C9.

6. Isolation of gC1qR with M_r 33 kDa can be achieved based on the affinity of this receptor for the globular heads of C1q ***(42)***. An affinity column is made with globular heads of C1q, generated by trypsin digestion as described in **Subheading 3.6.1.** and a cell extract from Raji cells applied. C3b -beads are generated by incubating 100 µL Sepharose CL4B-200 (Sigma) with 1 mL of human serum for 1 h at 37°C. Wash five times in PBS. Sepharose activates C and allows C3b deposition on the matrix. Alternatively, couple C3bi or $C3_{MA}$ to CNBr-Sepharose-4B (2 mg/mL).

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