

# ***Clinical and Technical Review***

## **Complement Diagnostics**

Haemocompatibility testing  
of medical devices, pharmaceuticals  
and blood products

*always your partner*

# Contents

## Complement Diagnostic – Haemocompatibility testing of medical devices, pharmaceuticals, and blood products.

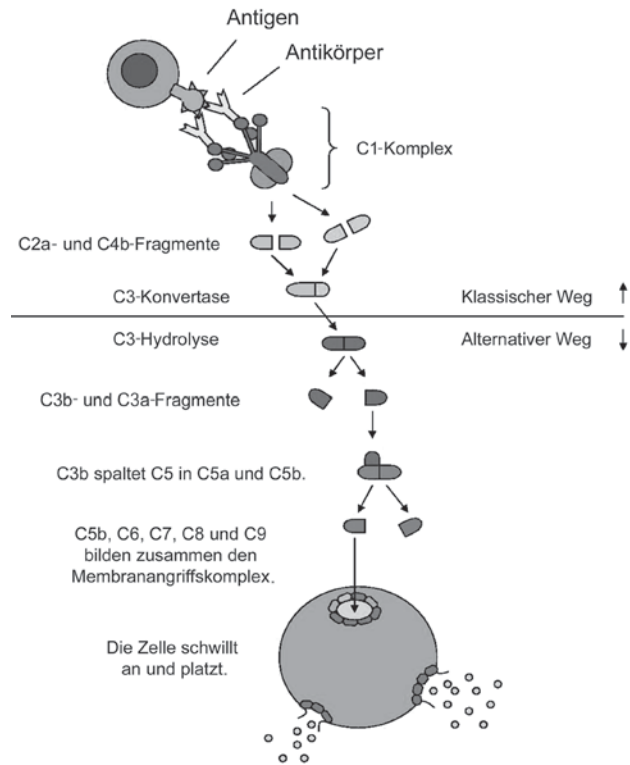
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# 1. The Complement System

The complement system, as part of the innate immune system, helps to eliminate cellular antigens (e.g. bacteria) and leads through several cascade-like activation steps to the lysis of the attacked cell. Excessive activation leads to massive acute inflammatory reactions which, in severe cases, may be life threatening.

In addition to the classical complement-associated disease states, intra- or extracorporeal contact with synthetic medical products, blood products and pharmaceuticals may trigger complement activation.



Quidel® has developed several enzyme-linked immunoassays that allow assessment of complement status and can be used to perform clinical as well as preclinical studies. With these assays, standardized tests can be performed with the aim to develop better biocompatible materials with minimal complement-activation potential.

In terms of developmental physiology, the complement system is the oldest barrier against infections. It was discovered by Jules Bordet in 1894 and in 1920 he was awarded the Nobel Prize for his discovery. The complement system helps to clear immunocomplexes and is a lytic system for removal of pathogenic microorganisms. Essentially, it can be initiated via two different activation cascades, the classical and alternative pathways. Several years ago, a third method of complement activation, the Lectin pathway, was described. All activation pathways lead to the terminal phase of the complement system, via the activation of C3 and C5 through C3- and C5-convertases. The formation of the terminal lysis complex (C5b-9, TCC), referred to as “membrane attack complex” (MAC), causes the formation of a membrane-integrated protein channel and leads to complete lysis of the attacked cell [1].

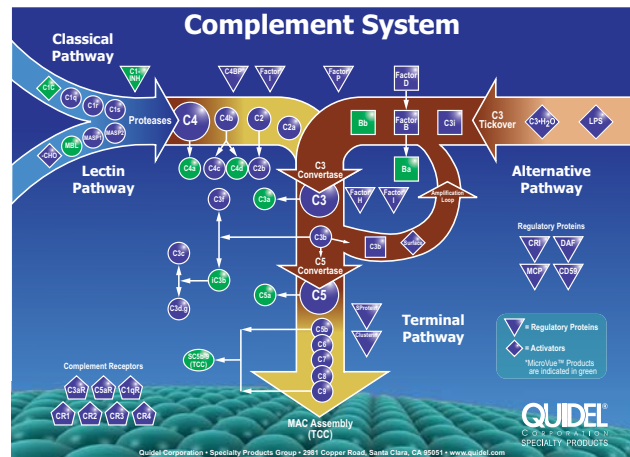


Figure 1: Comprehensive view of the activation pathways of the complement system.

## Activation of the complement system

The complement system is a crucial defense system against invading bacteria [2, 3]. Recognition of foreign antigens opsonised with IgG and IgM by the C1-complex (Classical pathway) and specific sugar moieties by the MBL-MASP2 complex (Lectin pathway), activates the complement system thereby forming the C3 convertase C4b2a. After activation, the amplification loop (Alternative pathway) is initiated forming the second C3-convertase C3bBb [4]. Moreover, the complement system mediates the interaction of bacteria (antigens) with various effector cells (T-cells, B-cells, Dendritic cells, Neutrophils and Macrophages) by opsonizing molecules, such as C4b and C3b. Complement activation is accompanied by the production of two important chemoattractants, C3a and C5a [5]. C3a has anti-microbial activity as well [6]. Due to the C5a gradient, neutrophils (mast cells, lymphocytes, dendritic cells, and mesenchymal stem cells) migrate towards the site of infection and phagocytose the invaders. Inside the neutrophil, anti-microbial peptides disrupt bacterial membranes, interfere with bacterial metabolism, and target bacterial cytoplasmic components [7, 8]. In the last part of the complement cascade, the lytic pathway, the formation of C5b-9 complexes (TCC) forms pores into cell membranes thereby lysing the cells (especially affecting gram-negative bacteria).

After initiation of the complement cascade, the activation is non specific, i.e. molecules can react on any surface which is nearby. So tight regulation by the host is crucial. Several secreted regulators like C1-inhibitor (which inactivates the C1 complex), C4 binding protein (C4BP) factor I, and factor H, accelerate the dissociation of the two C3 convertases and inactivate the C4b and C3b molecules. To prevent activation of complement on host tissue, membrane bound complement regulators, such as MCP (Membrane co-factor protein, CD46), DAF (decay accelerating factor, CD55) and Protectin (CD59), can either accelerate the dissociation of the C3-convertases or prevent integration of the C5b-9 complex.

## Interactions of the complement system

The complement system is not just a humoral immune system, but it may also act as a mediator of the cellular immune cascade. Thus, the activation of leucocytes leads to the activation of the coagulation (fibrinolysis) and contact phase system (kininogen-kinin system), which in turn activates the complement system in a positive feedback loop. This strong positive feedback mechanism may occur as a result of missing feedback from the complement system and lead to pathological inflammatory processes including sepsis and Acute Respiratory Distress Syndrome (ARDS) [11].

Relationships between the initial phases of classical complement activation (C1) and the contact phase system, as well as the key enzyme of the fibrinolysis (plasmin), have been described in vitro as well as in vivo. Plasmin activation can take place via endothelial t-PA release as well as via kallikrein-activated urokinase. Plasmin can activate the first factor of the complement cascade (C1) directly or via the release of the Factor XII cleavage product  $\beta$ -Factor XIIa. For example, in the case of blood contacting biomaterials (a non-endogenous material), complement activation is expected.

## 2. Diagnostics of the complement system

The complement system is a tightly-regulated system and a defect can have tremendous consequences with different pathological outcomes.

### Indications for complement diagnostics are:

- clarification of complement defects
- detection of an activation of the system
- verification of adequate regulation

Complement defects can be primary (hereditary) or secondary (acquired). Hereditary defects make up approx. 10 % of all primary humoral immune deficiencies and are typically recessive autosomal in origin. Consequently, heterozygous carriers usually remain asymptomatic. Secondary deficiencies are caused by complement consumption, reduced synthesis and/or increased catabolism. A lack of components of the classical activation sequence (C1, C4, C2) is often associated with autoimmune processes (SLE and clinical pictures similar to lupus). Recurrent bacterial infections are indicative of defects of the components C3 – C8. Infections with *Neisseria* predominate in the case of deficiency of the late components. Defects are described for nearly all complement and regulator proteins with the exception of factor B.

Investigation of the functional impact of the individual complement factors was significantly facilitated by the occurrence of diseases where individual factors or regulators are missing.

Disease states associated with complement can be attributed to complement defects or complement activation can develop, especially with false pathophysiological reactions, significant cell-destroying properties and may become responsible for massive tissue damage. Increased and decreased values of the different complement factors or their metabolites are used for diagnostic purposes:

Clinical disease states	Associated Complement factor
Systemic lupus erythematoses (SLE) and Lupus nephritis CP: C4d detection	CH50, C3, C4 ↑ C1, C4, C2 and C3, Factor B ↓
Cardiac infarction and apoplexy	Terminal pathway [SC5b-9] and anaphylatoxins, ↑ especially C5a
Vasculitis, glomerulonephritis	Consumption of C3, C4
Angioedemas, Capillary Leak Syndrome	C1-INH
Haemolytic-uraemic syndrome	Factor H
Rheumatoid arthritis	Factor H, C3d, C1q
Transfusion reactions, transplantation/allograft rejection	Classical pathway C4d
Age-related macular degeneration (AMD)	Factor H, C3, C5
Alzheimer	C3, C5, SC5b-9, Factor H, E100
Polytrauma, burns, sepsis	C5a
Acute Respiratory Distress Syndrome (ARDS), multiorgan failure	C5a
Diabetic ketoacidosis (DKA)	C3a and sC5b-9

**Figure 2:**  
clinical diseases associated to complement factors

Disease states associated complement deficiency	
<b>Complement factors</b>	
C1, C4, C2	SLE, syndrome similar to lupus, (recurrent infections)
C3	recurrent serious bacterial infections
C5 – C8, MBL	recurrent bacterial infections (in particular Neisseria infections)
C9	predominantly none
<b>Regulators</b>	
C1 Inhibitor	hereditary angioedema
Factor I, Properdin	recurrent infections
Factor H	haemorrhagic-uraemic syndrome (HUS), MPGN
DAF, C8bp, CD59	paroxysmal nocturnal hemoglobinuria
<b>Receptors</b>	
CR 1	SLE
CR 3, CR 4	recurrent infections
(CD11b/c, CD18)	(Leukocyte adhesion defect, LAD)

**Figure 3:**  
Complement factors associated with clinical diseases

## Complement analytic – methods

Searched-for parameter	Complement measurement methods
<b>Overall function</b>	
Classical pathway	CH50: ELISA, haemolytic titration
Alternative pathway	APH50: ELISA, haemolytic titration
Lectin pathway	haemolytic titration, ELISA
<b>Individual factors</b>	
Protein determination: C1q, C2 – C9	nephelometry, RID, ELISA
Functional determination: C1 – C9	haemolytic titration (depleted sera)
<b>Activation products – all pathways</b>	
C3a, iC3b, C5a, SC5b-9	ELISA
C3d	ELISA, rocket electrophoresis
<b>Activation products – classical pathway</b>	
C1rs C1- Inhibitor	ELISA
C4a, C4d	ELISA
<b>Activation products – alternative pathway</b>	
C3b (Bb) P	ELISA
Ba, Bb	ELISA
<b>Activation terminal sequence</b>	
C5a, SC5b-9	ELISA
<b>Regulators</b>	
C1 inhibitor protein (C1 esterase inhibitor) (C1-INH)	nephelometry, RID
C1 inhibitor function	enzyme inhibition test
Factor H and Factor I protein	ELISA, rocket electrophoresis
Factor H and Factor I function	haemolytic test
Carbopeptidase N	enzyme test with chromogenic substrate
DAF, C8bp, CD59 protein	cytofluorometry
DAF, C8bp, CD59 function	acid hemolysis test
C3-Nephritis factor	immunfixation, haemolytic test
Factor P – Properdin	ELISA
Factor B – C3 Proactivator	ELISA, RID
<b>Circulating immune complexes</b>	
Circulating immune complexes – C1q fragments	CIC-C1q ELISA
Circulating immune complexes – C3 fragments	CIC-RCR ELISA

Figure 4

### 3. Biocompatibility Testing

The term “biocompatibility” describes the quality of materials that are compatible with living tissue (bones, soft tissue, blood). An exact definition in physical and chemical terms does not yet exist. Materials used today should be inert in the body; not decompose, not cause rejection reactions or significantly affect metabolism. Research is being done worldwide to understand biocompatibility and to develop materials that adapt physiologically in the body and allow functional tissue integration.

Strictly speaking, biocompatibility must be divided into cytocompatibility (cell and tissue compatibility) and haemocompatibility (blood compatibility). Cytocompatibility is of primary importance for long-term implants. Implant is a summary term for all materials that are inserted into the body to fulfill a substitute function for either a limited period of time or for the entire lifetime. In contrast to a transplant, implants usually consist of non-living material (exceptions include endothelialized stents or biological heart valves). Since many implants not only come into contact with tissue, but also with blood (at least for a certain time during the surgery of the implantation) haemocompatibility also plays a major role. Haemocompatibility alone is relevant for “Extracorporeal circulation”, due to the short one-time period of use.

**Medical products can be designed for very different blood contact times:**

- Short (minutes to hours): extracorporeal circulation (dialysis, heart-lung machine), cannulae, etc.
- Long (days and weeks): artificial lungs, heart support systems, etc.
- Permanent (for life): heart valves, stents, etc.

Blood-contacting product	Blood-contacting material	Number
Catheter	Silicone, polyurethane, PVC, Teflon	200 million
Stents	Stainless steel, styrene isobutylene polymer, magnesium	4 million
Guidewires	Stainless steel, Nitinol	several millions
Dialysis Device	Polyacrylonitrile, polysulfone, cellulose	1.2 million
Oxygenators (HLM)	Hollow fibers (PP, etc.)	~1 million
Pacemaker	Silicone, polyurethane, platinum	300,000
Stents	Dacron, Teflon	200,000
Heart valves	Titanium, carbon, Dacron, fixed natural tissue	200,000
Artificial lungs	Silicone, PP, etc.	20,000
Heart-supporting systems	Polyurethane, etc.	1,000

**Figure 5:**  
Overview of the numbers of widely used medical devices in contact with blood (modified according to Rutner)

### Problems of haemocompatibility

In the provocative article, “The Catastrophe Revisited: Blood Compatibility in the 21st Century”, Buddy Rutner [4] aptly describes the inability of the biomaterial community, despite 50 years of intense research, to agree on an exact definition of the term “blood compatibility” [5].

He proposes 5 hypotheses why progress towards a clear understanding of blood compatibility is so slow:

- Hypothesis 1:** It is impossible to manufacture material that is completely blood compatible.
- Hypothesis 2:** We do not understand the biological processes of blood compatibility.
- Hypothesis 3:** We do not know how to test blood compatibility.
- Hypothesis 4:** Certain materials of natural origin appear to have a better blood compatibility, but we do not know why.
- Hypothesis 5:** In the meantime, better blood compatible materials are available but regulatory requirements and economic conditions prevent implementation in clinical practice.

## Strategies for improving the haemocompatibility of artificial surfaces

“Medical Devices”, used in clinical intra- or extracorporeal applications, are produced from a wide range of plastic materials: polyethylene, polypropylene, polyvinyl chloride (PVC), polyester, polystyrene, polyurethane, silicone, polysulphone, polyamide, polytetrafluoroethylene, etc., and their derivatives. In addition, ceramic components and many metallic materials such as stainless steel, titanium and their alloys are used. Although these products have excellent mechanical and physical properties, originally they were designed for industrial applications and only indirectly for use in the biomedical field [9]. All these materials are characterized by a more or less distinct haemoincompatibility. If they come into contact with blood, this may cause a pathophysiological response of the organism which is similar to a traumatic shock. For example, in cardiopulmonary bypass surgery using a heart-lung machine, the entire blood supply of a patient comes into contact – over a period of, in some cases, several hours – with about 3 m<sup>2</sup> artificial surfaces. The human body responds with a massive activation of humoral and cellular defense mechanisms against the alleged pathogenic “invader” and the sequence of a different cascade system is triggered.

During molecular interaction between biomaterial surface and tissue or blood, a sequence of reactions takes place that is decisive for the boundary layer compatibility of biomaterial. The first biological reaction is the non-specific adsorption of proteins (fibrinogen, C3, etc.), which due to conformation changes can be converted into signal substances or anaphylatoxins, affecting subsequent colonization, or providing the actual matrix for the adhesion of blood and tissue cells and bacteria colonization.

In recent years, medical device manufacturers have increasingly tried to minimize the activation of different cascade reactions by developing surfaces with better haemocompatibility [10]. More than ever, however, there is an urgent need to step up efforts to optimize the preclinical evaluation strategies of blood-contacting “Medical Devices” for the benefit of patients.

## International Standard ISO 10993

Unfortunately, the issue of haemocompatibility of medical products is often neglected. Neither the American Food and Drug Administration (FDA) nor the European Directives or the German Central Authority for Health Protection with Regard to Medicinal Products and Medical Devices (ZLG) have defined any clear qualitative and quantitative guidelines for the blood compatibility of plastic materials. Even the implementing standard ISO 10993-4 (testing for interactions with blood) is relatively unclear and ambiguous.

ISO 10993 covers the following aspects, which are summarized under the heading “Biological evaluation of medical devices”:

- Part 1: Evaluation and testing
- Part 2: Animal welfare requirements
- Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
- Part 4: Selection of tests for interactions with blood
- Part 5: Tests for in-vitro cytotoxicity
- Part 6: Tests for local effects after implantation
- Part 7: Ethylene oxide sterilization residuals
- Part 8: Selection and qualification of reference materials for biological tests
- Part 9: Framework for identification and quantification of potential degradation products
- Part 10: Tests for irritation and sensitization
- Part 11: Tests for systemic toxicity
- Part 12: Sample preparation and reference materials
- Part 13: Identification and quantification of degradation products from polymeric medical devices
- Part 14: Identification and quantification of degradation products from ceramics
- Part 15: Identification and quantification of degradation products from metals and alloys
- Part 16: Toxicokinetic study design for degradation products and leachables
- Part 17: Establishment of allowable limits for leachable substances
- Part 18: Chemical characterization of materials

- Note:** 1. a new Version of the ISO 10993-4 will be implementation in 2012.  
2. The FDA is implementing stronger regulations especially for humanized antibodies, nanoparticles, pharmaceuticals and tissue material.

The implementing standard ISO 10993-4, "Selection of tests for interactions with blood", version 2002 (second edition 2002-10-15), is valid for the field of blood compatibility under which complement activation has to be subsumed.

It applies to the following medical products:

**1. External communicating devices that serve as an indirect blood path include but are not limited to:**

- cannulae
- extension sets
- blood collection devices
- devices for the storage and administration of blood and blood products (e.g. tubing, needles and bags)
- cell savers

**2. External communicating devices in contact with circulating blood include but are not limited to:**

- atherectomy devices
- blood monitors
- catheters
- guidewires
- intravascular endoscopes
- intravascular ultrasound
- intravascular laser systems
- retrograde coronary perfusion catheters
- cardiopulmonary bypass circuitry
- extracorporeal membrane oxygenators
- haemodialysis/haemofiltration equipment
- donor and therapeutic apheresis equipment
- devices for absorption of specific substances from blood
- interventional cardiology and vascular devices
- percutaneous circulatory support systems

**3. Implant devices which are placed largely or entirely within the vascular system.**

**Examples include but are not limited to:**

- annuloplasty rings
- mechanical or tissue heart valves
- prosthetic or tissue vascular grafts
- circulatory support devices (ventricular-assist devices, artificial hearts, intra-aortic balloon pumps)
- inferior vena cava filters
- embolization devices
- endovascular grafts
- implantable defibrillators and cardioverters
- stents
- arteriovenous shunts
- blood monitors
- internal drug delivery catheters
- pacemaker leads
- intravascular membrane oxygenators (artificial lungs)
- leukocyte-removal filters

For the medical products listed above, at least one, and occasionally several, tests from the list below have to be carried out in accordance with ISO 10993-4:

- thrombosis
- coagulation
- platelets
- haematology
- complement system

ISO 10993-4 proposes the following parameters as test methods for the evaluation of the complement activation of medical products: C3a, C5a, Bb, iC3b, C4b, SC5b-9, CH 50, C3 convertase, C5 convertase.

In addition, please see the recommendations of the “American Society for Testing and Materials”:  
ASTM F1984-99, Standard Practice for Testing for Whole Complement Activation in Serum by Solid Materials  
ASTM F2065-00, Standard Practice for Testing for Alternative Pathway Complement Activation in Serum by Solid Materials

Static and dynamic systems should be used as test systems (see Chapters 4 and 5).

## **Pharmaceuticals, blood products, infusion solutions**

Essentially, all intravenously applied pharmaceuticals, such as antibodies (passive and humanized), oligonucleotides (antisense therapy), infusion solutions and blood products (plasma, albumin, coagulation factors, etc.) should be tested with regard to direct complement activation (C3a or SC5b-9). Moreover, the assessment of success for immunosuppressive therapies, plasma exchange treatments and immunoglobulin transfusions (IVIG) are often dependent on a regained homeostasis of the complement system (C3a or C4d). C1-inhibitor concentrate or recombinant C1-inhibitor isolated from donor blood is successfully used in the therapy of hereditary angioedema (HAE).

The products created in the new field of tissue engineering (native tissue and cells; with and without polymer scaffold) need to be tested thoroughly with regard to their complement-activating potential prior to clinical use.

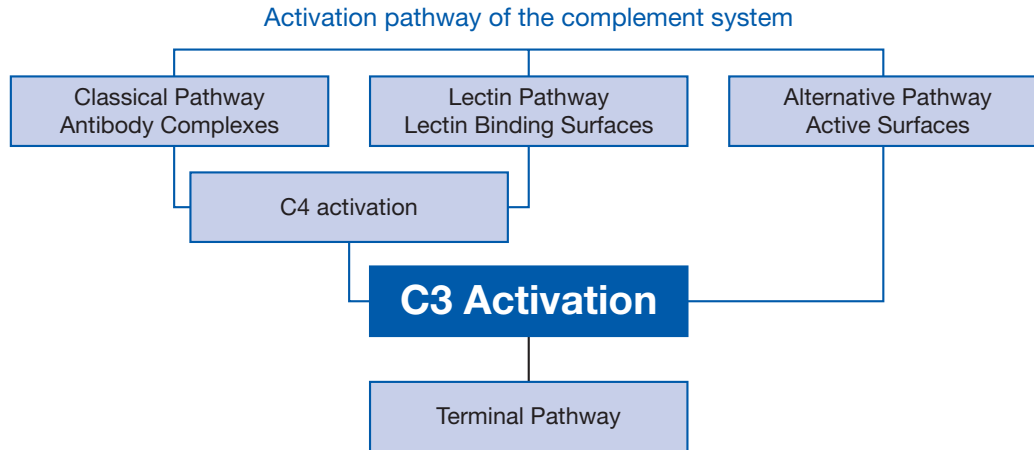
In therapies involving monoclonal antibodies, complement activation is triggered via the C1q binding site on the heavy chain of the mouse IgG. Humanized antibodies were introduced because use of these antibodies minimizes this activation.

Lately, different therapeutics have been developed for activation or inhibition of the complement system. Special complement inhibitors, such as Eculizumab, (a monoclonal antibody against C5) blocks terminal activation. Eculizumab is used against the paroxysmal nocturnal hemoglobinuria (PNH), a rarely acquired and life-threatening disease of haemopoietic stem cells (CD59 defect in the erythrocyte membrane).

Additional peptide-based complement inhibitors (C3, C5) are presently under intense research.

## 4. Measuring of complement activation

As discussed at length in Chapter 1, complement activation helps to clear immunocomplexes and is a lytic system for removal of pathogenic microorganisms. The crucial event in the complement cascade is the enzymatic cleavage of C3 into C3b and C3a, initiated by two C3 convertases (C4b,2a and C3b,Bb), which can be activated by all three complement pathways.



**Figure 6:**

Schematic presentation of the complement cascade. Complement activation through non-self surfaces can take place via the classical, lectin and alternative pathway. The classical and lectin activation pathway is triggered by an Antigen-IgG-C1 complex or sugar-MBL-Masp2 complex respectively, which causes an enzymatic activation of the complement proteins C4 and C2. This leads to the formation of C3 convertase, which splits C3 into the biologically active complement factors C3a and C3b. With the alternative activation pathway, C3 interacts directly with bound C3b creating an amplification loop of the Classical and lectin pathway. The protein fragment Bb (caused by enzymatic cleavage of factor B) binds to bound C3b to generate the C3 convertase of the alternative activation pathway. C3b is part of the C5 convertase, which splits C5 into C5a and C5b. C5b ultimately leads to the formation of the terminal complement complex (TCC). TCC is the summary term for C5b-9 (protein of the plasma membrane) and sC5b-9 (protein in phase).

### Evidence for activation of the system and verification of adequate function

Observations of the development of the complement activation are necessary to detect at risk patients after polytrauma, burns, sepsis, transplantation, etc. Repeated monitoring of activation products helps to assess immunological-inflammatory activity, especially in the control of immune suppressive therapy. All components and regulators should be included in a comprehensive complement diagnostics; this is true both with respect to their plasma concentration and their function within the cascade reaction.

Modern complement diagnostics, which safely identifies a defect or an activation of the system, requires a step-by-step approach. The following parameters can be used for analysis:

For an initial examination, we recommend assessing the total haemolysis activity of the classical pathway (CH50), the alternative pathway (APH50) and the detection of the complement activation product C3d.

- CH50: ELISA or titration of the total haemolytic activity of the complement system, classical pathway.
- APH50: ELISA or titration of the total haemolytic activity of the complement system, alternative pathway.
- C3d: ELISA or rocket immune electrophoresis - detection of the C3 cleavage product C3dg/C3d.  
Parameters for the activation of the classical and the alternative pathway of the C-activation.

## Systems for testing complement activation

Several in vitro or ex vivo test models may be used for preclinical assessment of new components (artificial devices) regarding their clinical usability for blood contact. The quality of the blood components used (whole blood, platelet-rich plasma, plasma, serum) and the choice of suitable anticoagulants (Citrate, EDTA, heparin, hirudin) are of decisive importance for all these models. Therefore, the following elimination criteria must be observed when selecting test individuals: no smokers and no intake of medication 14 days prior to blood donation (especially haemostasis-influencing pharmaceuticals such as acetylsalicylic acid (ASA), oral contraceptives, non-steroidal antiphlogistics, etc). Equally important are gentle blood drawing and rapid processing. Dynamic models using fresh human whole blood should be used for testing complete haemocompatibility, including complement activation.

### Blood, plasma, serum extraction and heparinization

To test heart-lung machines, etc., a large amount of blood is needed. For other tests a defined serum that has been prepared and tested accordingly should be used. If no fresh whole blood is available, frozen NHS (normal human serum) should be used (e.g. A113 or A112). This serum should be specially harvested to preserve the complement factors and tested for infectious diseases, complement activation and CH50. **EDTA plasma or recalcificated plasma are not suitable.**

**Note:** NHS must be placed on ice at all times prior to activation.

Heparin (low dose) or hirudin are to be used for plasma or whole blood. After testing, EDTA must be added to the plasma/blood samples to rule out any further in vitro activation.

### Use of fresh blood

#### Important criteria for blood donors:

- perfect health, no infectious diseases
- no known factor deficiency
- no coagulation disorder
- no medication (especially haemostasis-influencing pharmaceuticals)

#### Important criteria for blood collection:

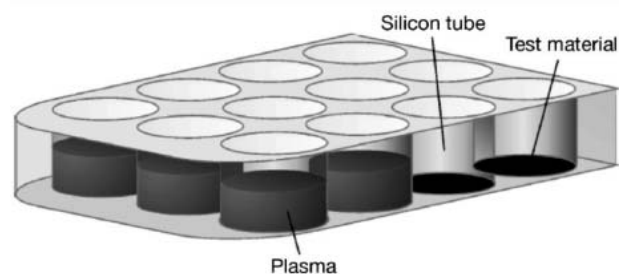
- skin disinfection
- brief congestion
- gentle puncture of the large cubital vein on the arm by an injection cannula (e.g. Venisystems Butterfly-M9)
- use of pre-prepared neutral monovettes (Heparin low dose, Hirudin, etc.)
- rejection of the first 3 ml blood

## Static models with serum

The materials to be tested will be incubated with serum (e.g. NHS = normal human serum, defined and complement- tested standard serum) for a defined period of time. Once the individual complement factors have been identified, a conclusion on the overall complement activation and on the respective pathway can be made. Serum is preferably used not just for testing biomaterials, but also for testing pharmaceuticals, solutions, blood products, etc.

### Example of a static test

Round platelets made of metal, ceramics or polymer plastic are to be tested.



**Figure 7:**

*Schematic presentation on 12-well plate. The front wells include plasma or serum. The walls of the wells are lined with silicone tube sections. Plasma or serum only comes into contact with the silicone tube and the test material at the bottom of the well.*

## Dynamic models with fresh whole blood

### Chandler loop model

In an in vitro “closed loop” model (modified Chandler loop), 30 ml heparinized (1 IE/ml Heparin) fresh human blood is recirculated (20 U/min, 37 °C) in circular closed tube sections. This test arrangement can be employed through various modifications; for testing new coatings, new pharmaceuticals (anticoagulants, inhibitors, etc.), stents, etc. This model can be used to quantify activation products directly via traditional ELISAs (C3a, C5a, SC5b-9) and the adsorbed plasma proteins can be detected directly on the surface using a newly developed modified ELISA technique (e.g. C3a). For this purpose, the tube sections to be examined will be rinsed directly after completion of the Chandler circulation, fixed (4 % para-formaldehyde in PBS [w/v], pH 7.4), cut into 2 cm long pieces and frozen. The tube sections will then be closed on one side and used directly as large “ELISA well”. Using appropriate antibodies, different plasma proteins can be detected directly on the test surface (e.g. C3a). Using incubation times of different length in the Chandler loop model, the kinetics of the adsorption behavior of plasma proteins can be determined. In addition, the soluble complement activation markers C3a, C5a, SC5b-9, etc. can be measured in the systemic blood.

### Heart-lung machine model

In this rather complex model, fresh human blood is recirculated in a short-circuited system, using oxygenation, defined tube length and a build-up of an arterial counter pressure (i.e. the same conditions as for a patient during cardiopulmonary bypass surgery). The activation potential of foreign surfaces with respect to the different parameters of haemostaseological systems can be checked, without endogenous counterbalances trying to compensate for these effects [9]. Concentrations of C3a, C5a, SC5b-9, etc. can be determined from the blood drawn at different times and thus the kinetics of the complement activation can be quantified.

### Flow cytometric examinations

With the aid of the flow cytometer (FACS), Gemmell [10] identified adsorbed complement proteins on polymer surfaces. Instead of cells, microparticles which resembled the solid blood components in form and size were analyzed through flow cytometry. After incubation of the microparticles in serum, proteins adsorbed on the surface were marked using fluorescent dyes. The fluorescence intensity, in addition to other parameters, could be detected in the flow cytometer. Compared with standardized fluorescent particles, Gemmell obtained information on the absolute amount of adherent complement proteins and their composition after various incubation times.



## 5. In Vitro Testing for Biocompatibility

The complement system is activated by three different routes, the Classical, Lectin and Alternative Pathways. Activation of any of these pathways results in cleavage of C3 protein, a crucial molecule in the complement cascade. The final pathway of the complement system, the Terminal Pathway, is subsequently activated by C3 activation. Each pathway can be discriminated by unique proteins, which can be cleaved into specific complement fragments (see figure 8). These fragments express unique neo-epitopes that are detectable by specific monoclonal antibodies. Exposure of serum (complement source) to a test sample (biomaterial or therapeutic) results in production of these fragments, which can be characterized quantitatively under standard conditions. The steps below will facilitate investigation of complement activation and identification of the exact underlying route of activation.

### Step 1. Test for C3 cleavage

Complement component 3 (C3) is the pivotal protein of the complement system. The two C3 convertases, C4b2a (classical and lectin pathway) and C3bBb (alternative pathway) cleave C3 into C3a and C3b. This step exposes a reactive thioester group within the C3b molecule, which can result in C3b deposition on surfaces. C3a is an important anaphylatoxin and can be detected in the fluid phase.

**Thus, C3 activation can be tested by detecting surface bound C3b and/or fluid phase C3a.**

C3: Special cases

In some cases, C3 cleavage products may be masked in the complement sample. For example, C3a may be adsorbed on to specific biomaterials like polyacrylonitrile (PAN) and C3b may adhere to the surface of reactive materials like cellulose acetate (e.g. hemodialysis circuits). Therefore it is recommended not only to test for both C3a and C3b, but to also test for SC5b-9 and C5a (see step 2).

### Step 2. SC5b-9 and C5a: Confirming results

C5 cleavage and subsequent formation of Terminal Complement Complexes (TCC) is evidence of activation of the common terminal pathway in response to complement activation. There are several reasons to test for Terminal Pathway activation

1. Biomaterials which do not activate C3 should be confirmed (see C3: special cases)
2. To check the extent of complement activation when C3 cleavage (step 1) is positive

SC5b-9 and C5a are unique fragments of the Terminal pathway. After activation of C3, the two C5-convertases, C4b3aC3b (Classical and Lectin pathways) and C3bBbC3b (Alternative pathway) cleave C5 into C5a (the most potent anaphylatoxin) and C5b. C5b binds Component C6 and C7, which in turn bind C8 and C9, thereby forming the lytic C5b-9 complex. The soluble, non-lytic complex, SC5b-9, is also formed and can be detected fluid phase together with C5a.

### Step 3. Understanding the process: test for C4d and Bb

If prior studies are positive and if the method of activation needs to be defined (for example surface characterization) testing can be done for C4d and Bb. C4d is a fragment of C4b formed through activation of the Classical and Lectin pathway after cleavage of C4. C4b is deposited on a reactive surface; C4d may be released by action of complement regulatory proteins in the serum. Bb, a fragment of the alternative pathway, can be detected when Factor B is cleaved into Bb and Ba. Bb is indirectly bound to a surface via binding to C3b. Testing for these fragments will provide insight into chemical modifications or other bioengineered approaches for circumventing the problem.

## Summary

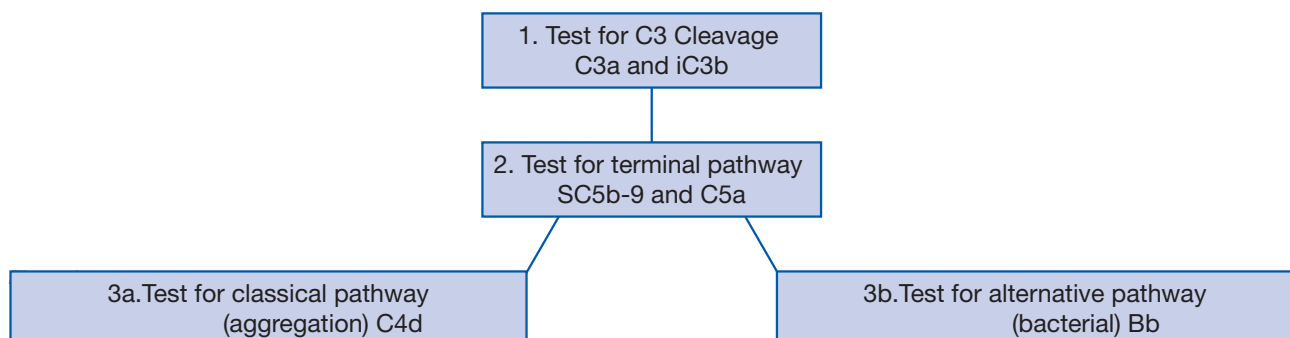


Figure 9

## Algorithm for complement biocompatibility testing

For a proper design of experiments, there are three elements to consider:

1. Complement source
2. Controls
3. Standardization

### 1. Complement source

Human serum is the best complement source to test. However, the serum has to be fresh, frozen serum with low existing levels of activation fragments. **EDTA, Heparin and re-calcified plasma are not suitable.** Quidel provides human serum, which is standardized and collected specially to preserve active complement (product No A113 5.0 ml or A112 2.5 ml). This human serum has been tested for infectious diseases, for complement activation markers, and for the CH50. After testing, specimen stabilizer (Quidel A9576) must be added to the plasma/blood samples to prevent any further complement activation.

### 2. Controls

An adequate number of controls and reference materials must be used for the respective experimental test system. Most kits include internal controls and standards. Complement activation takes place on the boundary layer of the blood-material contact. Therefore, the relation between the size of the test surface and the volume used (serum, plasma, blood) is an absolutely essential parameter for the standardization of the test system.

In addition to the controls provided by the kit, the following experimental controls (CONTROL) must be considered:

#### CONTROL 1 Background: Serum source only

Measures the impact of the system alone on complement

#### CONTROL 2 Positive control: Serum + activator

Confirms complement activity and demonstrates strong positive signal

#### CONTROL 3 Negative control: Serum + inhibitor (EDTA)

Negative controls shows any direct interference in the assay

#### CONTROL 4 Buffer control (NEG): Buffer which contains the therapeutics

Confirms buffer does not interfere

**Activator** – Since the complete sample handling is never entirely possible without artificial activations, the background activation can be quantified by using control samples and then compared to the test samples. Based on the experimental condition, one can decide which activator to use to function as a positive control.

- HAGG: Heat Aggregated Gamma globulin (Quidel A114)  
Activator of the classical pathway – C4d, however also C3a will be activated (application therapeutic antibodies, passive gamma globulin therapies)
- Zymosan: yeast lipopolysaccharide  
Activator of the alternative pathway - iC3b – C3a - C5a - SC5b-9 - Bb (application antisense oligonucleotides, charged biomaterial surfaces.)
- CVF: Cobra Venom Factor (Quidel A600) Alternative pathway: iC3b – C3a - C5a - SC5b-9 - Bb  
Acts directly on C3 and is an extremely potent complement activator to fully activate the complement system.
- Certain biomaterials (e.g. Cuprophan™)  
A Membrane that can activate. Maybe used as activator for material comparison - should only be used if the surface area of the test material is similar.

**Note** that for inhibition experiments, titrating an activator is challenging to find the right balance between activation and inhibition using CVF. In this case, HAGG is a better option.

**Temperature** has an impact on complement activation. So to control for temperature related activators created by the system (e.g. “nephritic” factors), keep one sample of each control (CONT 1-4) on ice.

### 3. Material or therapeutic

It is important to standardize for size & surface area respectively standardize for volume/concentration of therapeutic/ biomaterial.

## 5.1 Experimental Design

### Experimental Set up with controls only

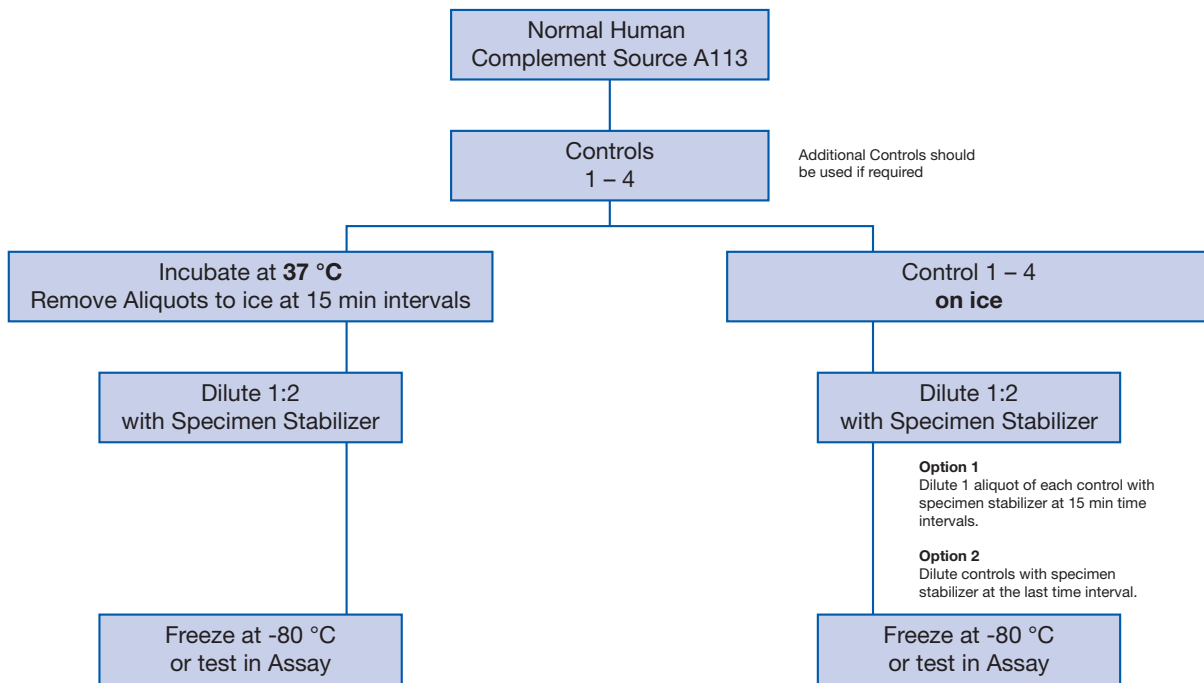


Figure 10

### Experimental Set up with Test material

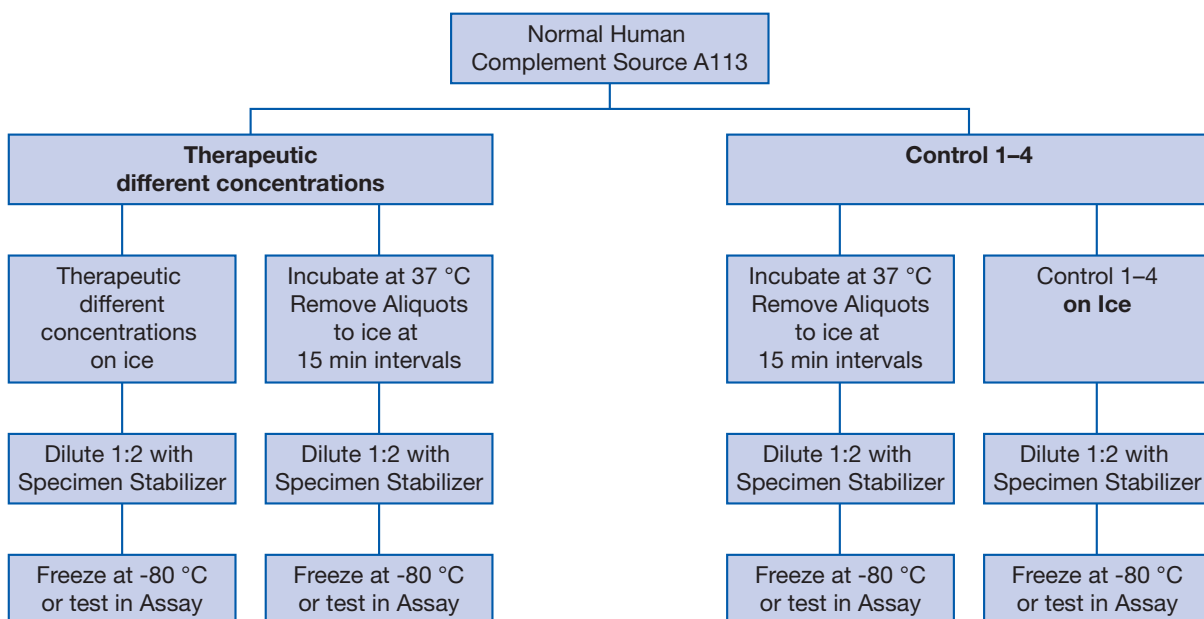


Figure 11

## 5.2 Experimental Set Up

See also [Algorithm for complement biocompatibility testing](#)  
[Page 16](#)

### Complement source

- Neat normal human complement serum (A113)
- EDTA, Heparin and recalcified plasma are not suitable

### Therapeutics

- Neat and dilutions 1:2 to 1:32

### Controls

Activator HAGG (A114) for positive control 2:

- 10 µl for 1 ml (normal human complement serum) = Complete activation after 30 Minutes at 37 °C

Activator CVF (A600) for positive control 2:

- App. 8 Units for 1 ml (normal human complement serum) = Complete activation after 30 – 90 Minutes at 37 °C

Activator Zymosan for positive control 2:

- 1 mg Zymosan for 1 ml (normal human complement serum) = Complete activation after 60 Minutes at 37°C

Inhibitor for negative Control 3:

- EDTA 10 mmol final concentration

**One aliquot of all controls on ice = Background activation**

### Incubation

- At 37 °C
- Move aliquots to ice every 15 minutes and dilute 1:2 with sample stabilizer (to block complement reaction)

### Timepoints

- 15 – 30 – 45 – 60 – 120 – 240 minutes

## Assay's (Testkits)

### Assay:

- Dilute Aliquots according to protocol – Sample is already diluted 1:2 with stabilizer
- e.g. dilution protocol 1:100 = dilute Aliquot 1:50
- Calculate concentration, include dilution factor
- Run controls included in the Kit
- Run additional external controls A115

## Refining the Protocol

- Determining the proper therapeutic concentration
- Impact on activator titration
- Optimizing the incubation parameters – Time, temperature
- Optimizing dilution profiles for activators and non activators –Impact of assay type
- Additional Controls

## Interpreting results – controls

### **CONTROL 1 Background: Serum source only**

Measures the impact of the system alone on complement

- Control 1 – on ice = Value as QC Sheet
- Control 1 – 37 °C = Background activation due to Temperature = 2 – 3 times higher value as QC Sheet

### **CONTROL 2 Positive control: Serum + activator in the experimental protocol**

Confirms complement activity and demonstrates strong positive signal

- Control 2 – on ice = Background = app. value as QC Sheet
- Control 2 – 37 °C = Total activation = app. 10 times higher value as QC Sheet

### **CONTROL 3 Negative control: Serum + inhibitor (EDTA)**

Negative controls shows any direct interference in the assay

- Control 3 – on ice = Value as QC Sheet
- Control 3 – 37 °C = Should be negative/low – **Value as QC Sheet**

### **CONTROL 4 Buffer control (NEG): Buffer which contains the therapeutics**

Confirms buffer (therapeutics) does not interfere

- Control 4 – on ice = app. Value as QC Sheet
- Control 4 – 37 °C = Should be app. value as QC Sheet. Influence of buffer only

**Protocols for the set up of assays for the measurement of complement activation of different biomaterials are available from **TECO**medical see also appendix**

## In vivo / In vitro Testing

- In vitro testing is very sensitive to small changes in complement activation
- Assays show high sensitive to the complement factors
- A Complement reaction In vitro – Complement activation value – does not mean that In vivo a Complement reaction/activation will take place
- **In-vitro-testing of therapeutics in Serum of defined patient groups**
  - Blood collection (patients without therapy)
  - Store serum at -80 °C
  - Use these serum as complement source for testing
  - Add therapeutic in different concentration to the Serum from the individual patients
  - Follow protocol Flow Chart for Rapid Assessment Page 17
  - Test for complement factors C3a/iC3b/SC5b-9/C5a/C4d/Bb
- **In-vivo-testing in primates**
  - Collect serum samples from the primates and test for complement factors C3a/iC3b/SC5b-9/C5a/C4d/Bb
  - Administer therapeutic to primates (possibly different concentrations)  
Collect serum at different time points and measure complement activation directly in serum –C3a/iC3b/SC5b-9/C5a/C4d/Bb

## Sample types and methods in Preclinical/Clinical Testing

Sample types:

- Serum from humans and primates
- Cell culture – human or primate cells
- Tissue from humans and primates (Antibodies IHC)

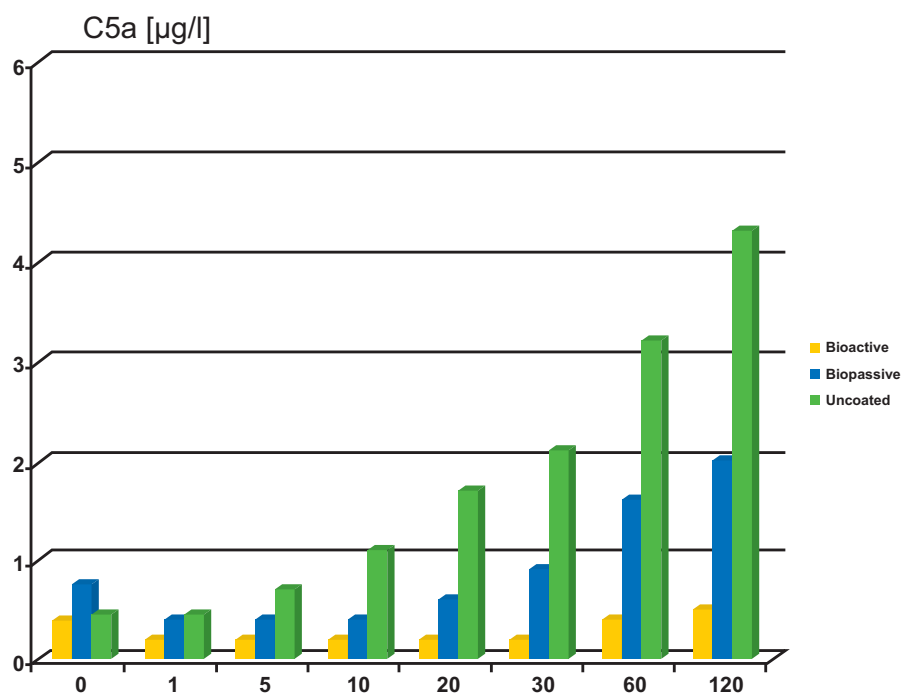
Methods:

- ELISA's
- Antibody based:
  - Western blot
  - RID
  - FACS
  - IHC

## Applications

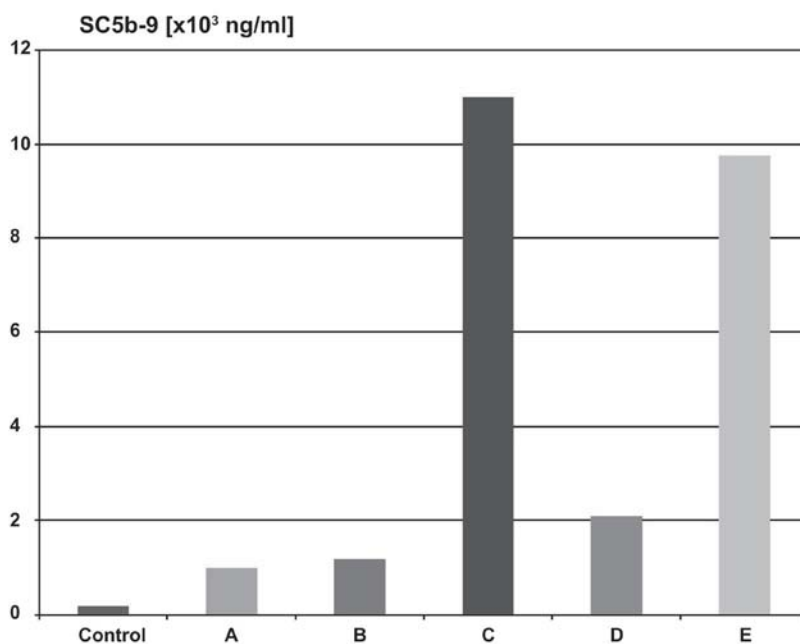
- Gamma Globulin Therapeutics – C3a and C4d
- Humanized Antibody Therapeutics – C3a and SC5b-9
- Metal Implants – C3a and SC5b-9
- Liposome Mediated Activation Methylation of the phosphate oxygen moiety of phospholipid-methoxy(polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production, Moghimi et al, The FASEB Journal, Vol. 20 December 2006
- Oligonucleotides (Anti sense) Therapeutics Aptamers Influence the Hemostatic System by Activating the Intrinsic Coagulation Pathway in an In Vitro Chandler-Loop Model, Paul, Wendel et al, Clinical Application Thromb Hemost, December 2008

## Examples of complement analyses



**Figure 12:**

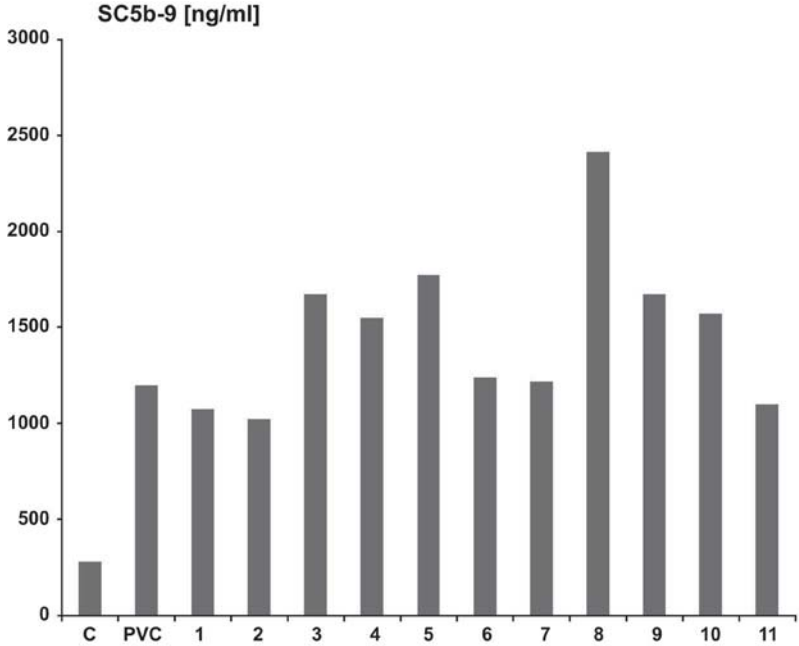
Chronological sequence (0 – 120 min) of the complement activation quantified by means of ELISA C5a in a heart-lung machine model with two different surface modifications of oxygenators versus an uncoated control group [11].



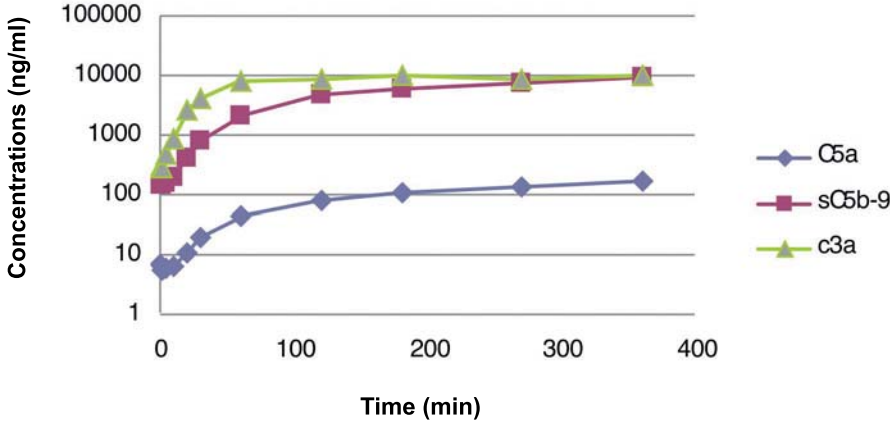
**Figure 13:**

Activation potential (SC5b-9) of different surface coatings on stainless steel (stent material), tested for 1 hour in a Chandler Loop model with fresh human blood.

**Examples of complement analyses**



**Figure 14:** Activation potential (SC5b-9) of different surface structures and modifications on titanium (dental implant) versus reference (PVC), tested for 1 hour in a Chandler Loop model with fresh human blood.



**Figure 15:** Complement Activation C3a, C5a and SC5b-9 of Biomaterial in a Chandler Loop Model.

## 6. Measurement of Complement activation in non-primate sera by a hemolytic assay – CxH50

In complement research, the availability of methods to directly assess complement activation in non-human models is poorly addressed. So, to study complement in non-human models, detection of complement activation must be done indirectly via hemolytic assays such as CH50. The traditional CH50 is a measure of total complement activation through the ability of the complement system to lyse sensitized sheep red blood cells. The CH50 reflects the ability of the complement in a sample to activate; the more activation, the higher the CH50 signal. However this signal is not a direct measurement of complement activation.

### CH50 drawbacks as a technique for measuring complement activation:

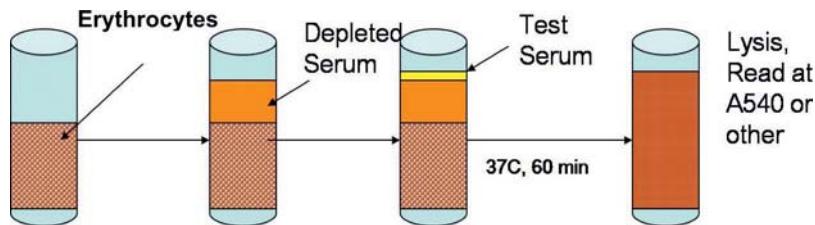
- Measures total (C1-C9) complement activation.
- Technique is very sensitive to serum matrix irregularities (such as antibodies, improper handling, etc.), which may lead to erroneous results. This sensitivity is less with direct measurements.
- Normalization of the serum is required. A decrease in any of the complement proteins due to normal fluctuation manifests as a low CH50 and poor complement activation. This complicates the use of non-human models where levels of complement proteins may be very different from human.

### CxH50

The traditional hemolytic assay can be made more specific assay by focusing on a single protein. This assay, called CxH50, detects the functional activity of a specific complement component (for example, C3H50 for C3 function). The method utilizes specifically complement depleted sera as a source of complement proteins. A test specimen can reconstitute the specifically depleted protein in the test matrix, thereby restoring the complement activity. Thus, all lysis of the erythrocyte (minus background lysis of depleted serum and erythrocytes) is a function of the specific complement protein from the test specimen. Studies have shown that non-human serum can serve to replace the depleted protein and reconstitute complement function.

The CxH50 is available in many test systems such as:

- C2 (C2H50)
- C3 (C3H50)
- C4 (C4H50)
- C5 (C5H50)
- C1q (C1qH50)



**Figure 16:**

The CxH50 assay. A depleted sera (e.g. C3) is used as the major complement source, missing one complement protein. Adding a test sample will provide the missing protein, thereby restoring the complement activity which can be measured by the lysis of erythrocytes.

### Example: C3H50 assay

By reconstituting the lytic potential of a C3 depleted human serum with C3 from a non-human species, the assay will provide a sensitive and specific method for measuring complement activation, by C3 consumption, in non-primate species for which direct capture assays do not exist. This method has some major advantages:

- Focus on a single complement protein improves the specificity of the hemolytic assay
- More consistent lysis with greater reproducibility by using proteins from a standard characterized source (the terminal pathway of non-human sera can vary greatly).
- The assay also relies upon human classical pathway activation by erythrocytes, which is well characterized and documented, in contrast to non-human classical pathway activation. For example, complement from rats and mice specifically have exceedingly low affinity for erythrocytes.

The following experiments were adapted from DeSautel et al 1999 and were performed in conjunction with Quidel Corp.

## 6.1 Experimental Design CxH50

### Example C3H50Assay

#### MATERIALS REQUIRED

- Normal Rat Serum (NRS, GS7)
  - Complement Activated Rat Serum
  - C3 Depleted Human Serum (QUIDEL Corp. Item A508)
  - Ea (sensitized sheep erythrocytes  $6.5 \times 10^7$  cells/mL) prepared using standard techniques, in gelatin veronal buffer or Hepes 20 mM with 20 mM CaCl and 20 mM MgCl (GVB++)
  - Human Complement Standard (QUIDEL Item A100)
- **Step 1:** Define optimal dilution of normal Animal Serum
- Dilution of normal non treated Animal Serum 1:10 – 1:10<sup>6</sup>
  - Dilution of normal Human Complement Standard 1:10 – 1:10<sup>6</sup>
  - Add GVB++ to all Tubes
  - Pipette the diluted Samples into the GVB++ Buffer
  - Add to 1 Sample of each dilution 10 $\mu$ l C3 depleted Serum
  - 1 Sample of each dilution without C3 depleted Serum
  - Add to all Samples 120  $\mu$ l Ea
  - Total Assay Volume 500  $\mu$ l
  - Positive Control: 100 % lyses 120  $\mu$ l of erythrocytes and 380  $\mu$ l dest. Water

Serum should be kept frozen at  $-70^\circ\text{C}$  until use  
All test tubes should be kept on ice during pipetting and should be transferred all together to  $37^\circ\text{C}$  for incubation

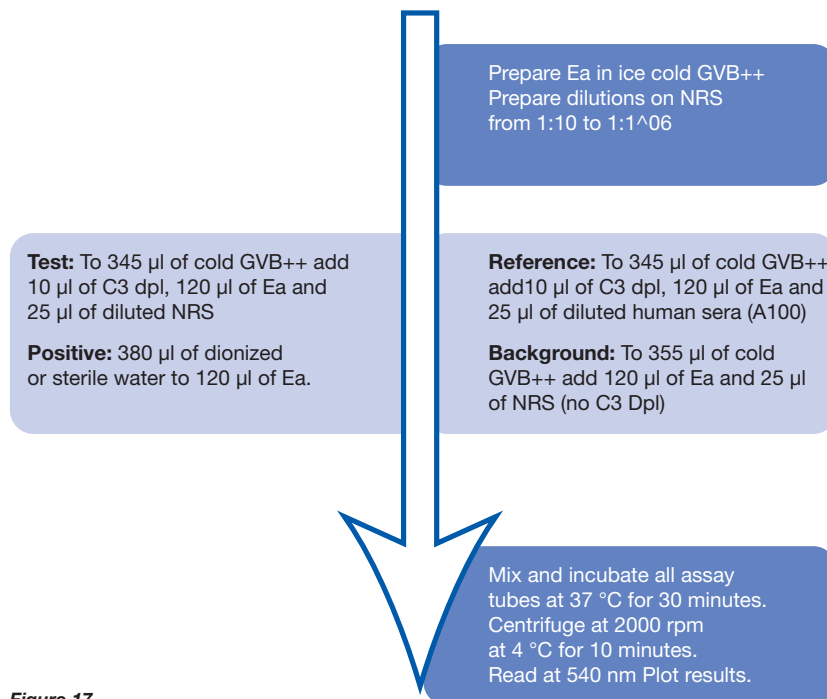


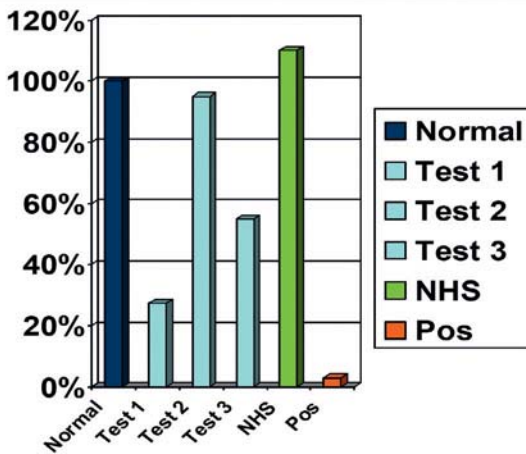
Figure 17

## C3H50 Assay

- **Step 2:** Serum from treated experimental Animals
  - Use optimal dilution as identified in Step 1
  - Control: Serum from non treated experimental Animal
  - Control: Serum form experimental Animal treated with CVF
- Option
  - compare directly samples (diluted) before and after treatment

## Determination of C3H50

- To determine C3H50 in test sera, samples are run v. a. normal unactivated control (100 %) at the optimal dilution.
- Results can expressed as a % of the normal.
- A100 is an assay control.
- CVF activate NRS is a positive control (strong activation, low Ch50).
- Alternatively, standard curve could be constructed from multiple dilutions around the optimal.



**Figure 18:**  
Samples 1 and 3 show high values of complement activation.

## Conclusion

- C3H50 represents a much more specific method for measurement of complement activation.
  - Unlike the standard CH50 procedure which measures total levels of complement activation, C3H50 is specific for C3 cleavage and function.
  - C3H50 is specific for C3 alone. Variations in levels of tangential complement proteins will not impact assay results to a significant degree.
- The benefits of this assay for research in complement activation in non-human species are two-fold:
  - It is specific for a single key complement protein, in this case C3, and is therefore a sensitive assay for complement activation.
  - It relies on a characterized and highly functional standard reagent (C3 depleted serum) as a complement source and not a sera with an unknown complement lytic potential (species serum).
- A modification of this protocol has been used to measure complement activation in rats and mice. Similarly, other human depleted sera (including C1q, C5, C4, C2 and Factor B) have been used to determine pathway specific complement activation in other species. Optimal may change.

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## 8. Complement and Biocompatibility products

### 8.1 Complement ELISA Kits

#### CIC-C1q MicroVue™ Quidel®

Quantification of circulating immune complexes

CE/FDA

**Cat. No.:** A001  
**Tests:** 96  
**Method:** ELISA  
**Range:** 0 – 31 µg Eq/ml  
**Sensitivity:** 1.0 µg Eq/ml  
**Incubation time:** 2 hours  
**Sample volume:** 100 µl (diluted 1:50)  
**Sample type:** Serum and plasma  
**Sample preparation:** All specimens should be collected aseptically and prepared using standard techniques for clinical laboratory testing. Do not heat-inactivate the specimens. Sample may be stored at 2 – 8 °C for up to 7 days. For longer periods store below -20 °C.

**Reference values:** One hundred six (106) sera were collected from normal, asymptomatic subjects. The average CIC concentration was 2.1 µg Eq/mL (S.D.=1.9)

**Specificity:** Complement-CICs are bound to immobilized to C1q-Protein  
**Species:** Human

#### Intended use:

The CIC-C1q Assay is designed for the detection of circulating immune complexes (CIC) in human serum or plasma. CIC have been measured in a variety of conditions: infections, autoimmune disorders, trauma and neoplastic proliferative diseases. In addition the measurement of CIC can be important for the evaluation of certain forms of rheumatoid arthritis and for monitoring the effectiveness of therapy.

#### CIC Control

**Cat. No.:** A013  
1 Set (2 levels)

## CIC-C3d MicroVue™ Quidel®

Raji-Cell-Replacement

### Quantification of circulating immune complexes with C3 Activation Fragments

CE/FDA

**Cat. No.:** A002  
**Tests:** 96  
**Method:** ELISA  
**Range:** 5 – 48 µg Eq/ml  
**Sensitivity:** 4 µg Eq/ml  
**Incubation time:** 2 hours  
**Sample volume:** 100 µl (diluted 1:50)  
**Sample type:** Serum and plasma  
**Sample preparation:** All specimens should be collected aseptically. Do not heat-inactivate the specimens. Samples may be stored on ice for up to 6 hours. For longer periods store below -70 °C.

**Reference values:** Normal ≤ 15 µg Eq/ml  
Abnormal ≥ 20 µg Eq/ml

**Specificity:** Immobilized monoclonal anti human C3 fragments capture C3d containing immune complexes.  
**Species:** Human

#### Intended use:

The CIC-RCR Assay is designed for the detection of circulating immune complexes (CIC) in human serum or plasma. CIC have been measured in a variety of conditions: infections, autoimmune disorders, trauma and neoplastic proliferative diseases. In addition the measurement of CIC can be important for the evaluation of certain forms of rheumatoid arthritis and for monitoring the effectiveness of therapy.

Functional C1 Inhibitor Proteins

CE/FDA

**Cat. No.:** A003  
**Tests:** 96  
**Method:** **ELISA**  
**Range:** Concentrations of Functional C1-INH are expressed as Mean Percentage. Range 35 – 100 %.  
**Sensitivity:** 4 µg Eq/ml  
**Incubation time:** 2 hours  
**Sample volume:** 100 µl (diluted 1:101)  
**Sample type:** Serum and plasma  
**Sample preparation:** Specimens should be collected aseptically and prepared. EDTA plasma sample may be held at room temperature (15 – 30 °C) for up to 24 hours. Serum sample should not be stored at room temperature for longer than 6 hours. If exceeded, the plasma or serum must be stored frozen (-20 °C or below). Avoid freezing and thawing of the sample.

**Reference values:** Concentrations ≥ 68 % Mean Normal is considered normal.

**Specificity:** C1-INH-Reactant, binds specific to functional active C1-INH.  
**Species:** Human

**Intended use:**

The C1-Inhibitor assay measures the amount of functional C1 inhibitor protein (C1-INH) in human plasma or serum. This protease inhibitor has enzyme regulating functions. A deficiency of functionally active C1-INH may lead to life-threatening angioedema. Two major forms of C1-INH deficiency have been reported: the congenital form, termed hereditary angioedema (HAE), and the acquired form, which is associated with a variety of diseases including lymphoid malignancies. Hereditary angioedema is characterized by transient but recurrent attacks of nonpruritic swelling of various tissues throughout the body.

**Quantification of circulating immune complexes with C3 Activation Fragments**



<b>Cat. No.:</b>	A006
<b>Tests:</b>	96
<b>Method:</b>	<b>ELISA</b>
<b>Range:</b>	0.2 – 2.0 µg/ml
<b>Incubation time:</b>	1.5 hours
<b>Sample volume:</b>	100 µl (dilute for optimal absorption values)
<b>Sample type:</b>	Serum and plasma
<b>Sample preparation:</b>	The proper collection and storage of specimens is essential, since C3 is highly susceptible to proteolysis and hydrolysis. Serum or EDTA plasma specimens should be collected aseptically using standard techniques. They should be tested immediately or stored at 4 °C or on ice until assayed. This should not exceed four hours. For longer-term storage, freeze at -70 °C within two hours after collection.
<b>Specificity:</b>	Anti-iC3b monoclonal anti body specifically binds iC3B and not to C3.
<b>Species:</b>	Human

**Intended use:**

The iC3b enzyme immunoassay measures the amount of the iC3b fragment in human plasma or serum, as well as in other biological fluids, experimental samples or mixtures.

The levels of iC3b can be significantly elevated in the serum and plasma of some patients with complex-associated diseases such as rheumatoid arthritis and systemic lupus erythmatosus. iC3b levels may also be elevated in body fluids from other patients with infections, burns, myocardial infarctions, glomerulonephritis, and acute respiratory distress syndrome.

**Bb fragments of Factor B of the Alternative Complement Pathway**

CE/FDA

**Cat. No.:** A027  
**Tests:** 96  
**Method:** **ELISA**  
**Range:** 0.06 – 0.65 µg/ml  
**Sensitivity:** 0.033 µg/ml  
**Incubation time:** 1.5 hours  
**Sample volume:** 100 µl (dilute 1:10 for plasma, 1:20 for serum)  
**Sample type:** Serum and plasma  
**Sample preparation:** Specimens should be tested immediately or stored at 4 °C or on ice until assayed. This should not exceed four hours. For longer-term storage, freeze at -70°C within two hours after collection.

**Reference values:** 0.49 – 1.42 µg/ml in Plasma ( $\pm 2$  SD)  
0.80 – 6.26 µg/ml in Serum ( $\pm 2$  SD)

**Specificity:** Monoclonal mouse-antibody, specifically binds Bb.  
**Species:** Human, cynomolgus macaque, baboon, rhesus macaque

**Intended use:**

The Bb fragment enzyme immunoassay measures the amount of the complement fragment of Bb, an activation fragment of Factor B in human plasma or serum, as well as in other biological fluids, experimental samples or mixtures. This measurement allows a quantitative assessment of the extent of activation of the alternative pathway of complement in the test sample. Measurement of alternate pathway activation aids in the diagnosis of several kidney diseases (e.g. chronic Glomerulonephritis, lupus nephritis), as well as several skin diseases (e.g. dermatitis herpetiformis and pemphigus vulgaris). Other diseases in which activation of the alternate pathway of complement has been observed include rheumatoid arthritis, sickle cell anemia, and gram-negative bacterial infections.

**Quantification of C4d-containing fragments of activated C4 of the Classical Complement Pathway**

CE/FDA

<b>Cat. No.:</b>	A008
<b>Tests:</b>	96
<b>Method:</b>	<b>ELISA</b>
<b>Range:</b>	0.025 – 0.25 µg/ml
<b>Sensitivity:</b>	0.022 µg/ml
<b>Incubation time:</b>	1.5 hours
<b>Sample volume:</b>	100 µl (dilute 1:70 for normal Samples)
<b>Sample type:</b>	Serum, EDTA plasma or other biological fluids
<b>Sample preparation:</b>	The proper collection and storage of specimens is essential, since C4d is highly susceptible to proteolysis. Serum or EDTA plasma specimens should be collected aseptically using standard techniques. They should be tested immediately or stored at 4 °C or on ice until assayed. This should not exceed four hours. For longer-term storage freeze at -70 °C within two hours after collection.
<b>Reference values:</b>	EDTA Plasma 0.7 µg – 6.3 µg/ml ( $\pm 2SD$ ) Serum 1.2 µg – 8.0 µg/ml ( $\pm 2SD$ )
<b>Specificity:</b>	Monoclonal mouse-antibody, specifically binds C4d.
<b>Species:</b>	Human, cynomolgus macaque, baboon, rhesus macaque

**Intended use:**

The C4d fragment enzyme immunoassay measures the amount of the C4d-containing activation fragments of C4 (C4b, iC4b, and C4d) present in human serum, EDTA plasma and other biological or experimental samples.

The levels of C4d, when normalized for the presence of endogenous C4, can be significantly elevated in plasma specimens obtained from some patients with rheumatoid arthritis, hereditary angioedema, systemic lupus erythematosus and other illnesses. C4d may also be elevated in body fluids and plasma samples obtained from patients in which classical complement pathway activation is known to occur, e.g. from patients with a variety of humoral autoimmune diseases, septicemia, thermal injury, multiple organ trauma, myocardial infarction, hereditary angioedema, glomerulonephritis and acute respiratory distress syndrome.

## SC5b-9 Plus MicroVue™ Quidel®

### Quantification of the SC5b-9 complex

CE

**Cat. No.:** A029  
**Tests:** 96  
**Method:** **ELISA**  
**Range:** 10 – 170 ng/ml  
**Sensitivity:** 8.8 ng/ml  
**Incubation time:** 2 hours  
**Sample volume:** 100 µl (dilute 1:10 for plasma- and 1:40 for serum)  
**Sample type:** Serum, EDTA plasma, spinal fluid or other biological fluids.  
**Sample preparation:** The proper collection and storage of specimens is essential since SC5b-9 may be generated in improperly handled specimens. Serum or EDTA plasma specimens should be collected aseptically using standard techniques. They should be tested immediately or stored at 4 °C or on ice until assayed. This should not exceed four hours. For longer-term storage freeze at -70 °C. Plasma concentrations better reflect in vivo concentrations in comparison to serum concentrations.

**Reference values:** Serum 334 – 1672 ng/ml  
EDTA Plasma 127 – 303 ng/ml

**Specificity:** Monoclonal mouse-antibody, specifically binds SC5b-9.  
**Species:** Human, cynomolgus macaque, baboon, rhesus macaque

#### **Intended use:**

The SC5b-9 enzyme immunoassay measures the amount of SC5b-9 present in human plasma, serum and other biological or experimental samples.

The Terminal Complement Complex (TCC, SC5b-9) is generated by the assembly of C5 through C9 as a consequence of activation of the complement system by either the classical, lectin or alternative pathway. The membrane attack complex (MAC), a form of TCC, is a stable complex that mediates the irreversible target cell membrane damage associated with complement activation.

## C3a Plus MicroVue™ Quidel®

### Quantification of the C3a fragment

CE

<b>Cat. No.:</b>	A032
<b>Tests:</b>	96
<b>Method:</b>	<b>ELISA</b>
<b>Range:</b>	0.05 – 5 ng/ml
<b>Sensitivity:</b>	0.023 ng/ml
<b>Incubation time:</b>	2 hours 15 minutes
<b>Sample volume:</b>	100 µl (dilute 1:200 for plasma, 1:5000 for serum)
<b>Sample type:</b>	Human plasma, spinal fluid or other biological fluids
<b>Sample preparation:</b>	Sample collection is critical. Care must be taken to avoid C3a generation in the sample. For plasma, blood samples should be collected with disodium EDTA as anticoagulant and should be centrifuged at 2000xg at 2 – 8 °C. The entire operation must be completed immediately. Samples should be prepared and assayed immediately or stored on ice for up to 4 hours. For longer-term storage freeze at -70 °C with stabilizing solution.
<b>Reference values:</b>	Serum 71.0 – 589.2 ng/ml EDTA Plasma 33.5 – 268.1 ng/ml
<b>Specificity:</b>	Monoclonal mouse-antibody, specifically binds C3a-desArg.
<b>Species:</b>	Human, cynomolgus macaque, baboon, rhesus macaque, pig

#### Intended use:

The C3a enzyme immunoassay measures the amount of C3a-desArg in human EDTA plasma, serum and other research samples.

Under normal conditions, activation of the classical, alternative or lectin complement pathways results in the formation of a C3 convertase multi-molecular enzyme capable of cleaving C3 to C3a and C3b. C3a is a low molecular weight (approximately 9 kD) protein fragment of 77 amino acids. C3a is rapidly metabolized by the serum enzyme, carboxypeptidase N, to the more stable, 76 amino acid form, C3a des-Arg. The quantitation of C3a des-Arg therefore provides a reliable measurement of the level of complement activation in the test sample.

Measurement of terminal complement pathway activation in experimental samples



<b>Cat. No.:</b>	A025
<b>Tests:</b>	96
<b>Method:</b>	<b>ELISA</b>
<b>Range:</b>	0.1 – 1 ng/ml
<b>Sensitivity:</b>	0.050 ng/ml
<b>Incubation time:</b>	2 hours 15 minutes
<b>Sample volume:</b>	100 µl (dilute 1:20 for plasma, 1:50 for serum)
<b>Sample type:</b>	Serum, EDTA- and citrated plasma, spinal fluid or other biological fluids
<b>Sample preparation:</b>	The proper collection, storage and shipment of specimens are essential. Test immediately or stored up to 4 hours at 2 – 8 °C or on ice. For longer-term storage the samples should kept frozen at -70 °C with stabilizing solution (Cat. No. A9576), dilute samples 1:1 with stabilizing solution.
<b>Reference values:</b>	EDTA Plasma 0.37 – 74.33 ng/ml Serum 13.31 – 179.23 ng/ml
<b>Specificity:</b>	C5a and C5a des-Arg
<b>Species:</b>	Human

**Intended use:**

C5a is generated as a result of cleavage of the terminal complement protein C5, during activation of the complement system via the classical, alternative or lectin pathway. C5a is a low molecular weight (approximately 9 kD) protein fragment of 74 amino acids. C5a is rapidly metabolized by the serum enzyme carboxypeptidase to more stable, less active, 73 amino acid form, C5a des-Arg.

Research has associated elevated levels of fluid phase and adsorbed C5a with hemo-incompatibility of some biomaterials, particularly in extracorporeal circuits. Levels of C5a have also been associated with pathogenesis of a variety of disease states, including myocardial infarction, stroke, as well as vascular leak syndrome and associated kidney injury. The role of C5a in the pathogenesis of malaria and other infectious diseases, as well as sepsis, is likewise well documented.

**Total classical complement pathway activity**

CE/FDA

<b>Cat. No.:</b>	A018
<b>Tests:</b>	96
<b>Method:</b>	<b>ELISA</b>
<b>Range:</b>	Appr. 0 – 300 U Eq/ml
<b>Incubation time:</b>	3.5 hours
<b>Sample volume:</b>	14 µl (dilute 1:200)
<b>Sample type:</b>	Serum ONLY. Plasma CANNOT be used.
<b>Sample preparation:</b>	The proper collection, storage and shipment of specimens are essential, since complement may be activated in improperly handled specimens. Assay immediately or keep on ice for testing within four hours. For longer-term storage, freeze at -70 °C. Avoid repeated freezing and thawing.
<b>Reference values:</b>	133 ± 54 U Eq/ml
<b>Specificity:</b>	The monoclonal antibody specific to terminal complement complexes arising as the result of the activation step in the test.
<b>Species:</b>	Human, cynomolgus macaque

**Intended use:**

The binding of C1q component of C1 to immune complexes triggers the classical complement pathway. This activation results in a cascade of enzymatic and non-enzymatic reactions, culminating in the formation of terminal complement complexes (TCC). Under standard conditions, the level of TCC that can be generated in serum is a quantitative expression of the serum's total classical complement activity. The MicroVue CH50 Eq EIA is designed to measure the total classical complement pathway activity in human serum samples. The measurement of CH50 allows detection of deficiencies of one or more complement components (C1 through C9).

## Quantification of the Complement Ba fragment



**Cat. No.:** A034  
**Tests:** 96  
**Method:** ELISA  
**Range:** 0.05 – 2.1 ng/ml  
**Sensitivity:** ULOQ: 3.239 ng/mL  
 LLOQ: 0.033 ng/mL  
 LOD: 0.011 ng/mL  
**Incubation time:** 3 hours  
**Sample volume:** 100 µl (dilute 1:1000 for EDTA plasma, 1:2000 for serum, 1:15 for urine)  
**Sample type:** EDTA Plasma, serum, urine  
**Sample preparation:** The Ba fragment of Factor B is susceptible to proteolysis. For optimal plasma results, K2 EDTA should be used. Collect blood sample and centrifuge immediately at 2 – 8°C. Assay immediately, do not store longer than 2 hours at 2 – 8°C. For longer storage -70°C.

**Urine:** Collect preservative-free first Morning void (FMV) or second morning void (SMV) before 10:00 am. Store sample refrigerated (2 – 8°C) for less than 1 day, or freeze the sample at -70°C for longer storage. Maximum 5 freeze and thaw cycles.

## Reference values:

Sample	n	Mean	Range µg
EDTA Plasma	35	658	226 – 2153
Serum	29	1642	436 – 3362
Urine	16	7.7	0.6 – 27.0

**Specificity:** Monoclonal mouse-antibody, specifically to capture the Ba fragment.  
**Species:** Human, African green monkey, cynomol gus monkey, rhesus monkey, canine

## Intended use:

By quantifying the amount of Ba, the extent of alternative pathway activation at the time of sample collection can be determined. Activation of the alternative pathway has been associated with a variety of disease states including SLE, chronic glomerulonephritis, rheumatoid arthritis, sickle cell anemia and gram negative bacterial infections. The activation of the alternative complement pathway can be triggered by a variety of substances including microbial polysaccharides or lipids, gram-negative bacterial lipopolysaccharides, surface determinants present on some viruses, parasites, virally infected mammalian cells, and cancer cells. In autoimmune diseases, the alternative complement pathway may contribute directly to tissue damage. Alternative complement pathway activation may also be an indicator of hemo-incompatibility of biomaterials.

## Application

- kidney diseases
  - chronic glomerulonephritis
  - lupus nephritis
- skin diseases
  - dermatitis herpetiformis
  - pemphigus vulgaris
- age-related macular degeneration
- fetal loss in at risk pregnancy
- rheumatoid arthritis
- sickle cell anemia

## 8.1.1 Crossreactivity of complement fragment ELISA's to primate samples

### Summary of Results:

Species crossreactivity of various primate plasma and sera were assessed in the Quidel® MicroVue™ Complement Product EIA's as well as the Quidel® MicroVue™ CH50 Eq EIA kit. Of the assays tested, C3a, Bb, C4d, SC5b-9 and CH50 all demonstrated a high degree of species crossreactivity with baboon and old world monkey samples. Results for normal and activated sera were comparable to human control samples.

### Experiment I:

Samples were tested for the ability to block activated human complement binding to the neo-antigen specific monoclonal capture antibodies via ELISA inhibition assay. The capture monoclonals are the same antibodies used in the Quidel® MicroVue™ Complement Product assays. Positive blocking ability was defined as greater than 80 % reduction in signal vs. a standard dilution of activated human serum giving endpoint signals (OD 405) between 1.2 and 1.4 units in the assay. Results are shown in Table I.

	Baboon	Cynomologus Macaque	Rhesus	lemur	Marmoset	Squirrel Monkey	African Green Monkey	Canine	Porcine
BA A034	-	+	+	-	-	-	+	+	-
Bb (neo) A252	+	+	+	-	-	-	-	N/T	N/T
iC3b (neo) A209	N/T	-	-	-	-	-	-	-	-
SC5b-9 (neo) A239	+	+	+	-	-	-	-	-	-
C4d (neo) A251	+	+	+	-	-	-	-	-	-
C3a (neo) A015	+	+	+	-	-	-	-	N/T	+
C5a (neo) A025	-	-	-	-	-	-	-	-	-

**Figure 19:**  
EIA Inhibition Results

### Experiment II:

Species crossreactivity in the ELISA assays was shown using various complement activating substances (Cobra Venom Factor, Zymosan and Heat Aggregated Gamma Globulin). These products activate specific proteins and complement pathways. Normal results are shown in Table II. Positive cross-reactivity was defined as a tenfold or greater increase in fragment levels after activation. This threshold was based upon comparable human responses. Of the assays tested, clear cross-reactivity was shown for cynomologus, baboon and rhesus monkey samples in Bb, C4d, C3a, SC5b-9 and CH50 EIA kits. No specific reaction could be demonstrated using marmoset sera or any non-human sera in the IC3b EIA.

	Bb (µg/ml)	C4D (µg/ml)	iC3b (µg/ml)	C3a (ng/ml)	SC5b-9 (ng/ml)	CH50 (U Eq/ml)
Human (N = 5)	1.5 ± 0.975	4.1251 ± 2.55	51.0 ± 1.5	1209 ± 177	237 ± 28	110.3 ± 6.3
Cynomologous Macaque (N = 10)	0.83 ± 0.45	4.065 ± 0.82	NR	879 ± 110	241 ± 28	72.9 ± 13
Baboon (N = 12)	0.75 ± 0.43	3.75 ± 1.5	NR	900 ± 177	165 ± 57.5	NT
Marmoset (N = 8)	NR	4.41 ± 1.8	NR	NR	NR	NR

**Figure 20:**  
Crossreactivity of selected primate sera with the CSP assay kits

## Selected References

- [1] Birmingham D, Lee HA et al. (1999)  
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- [2] Henry SP, Giclas PC et al. (1997)  
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- [3] Katopodis AG, Warner RG et al. (2002)  
**Complement analysis in the 21st century.**  
Mol. Immunol., Sep, 44 (16): 3838 – 49 . Review
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Rother K and Till GO (eds), Springer Verlag Berlin Heidelberg, pp. 522 – 547.
- [5] Loss M, Vangerow B, et al. (2000)  
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Circ Res 91, 907 – 914.
- [7] Mollnes TE (1997)  
**Analysis of in vivo complement activation. In: Complement and Complement Receptors.**  
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- [8] Undar A, Eichststaedt HC et al. (2002)  
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## 9. Complement antibodies and reagents

### Depleted or Deficient Sera

Except for the C3-dpl, C3/C4-dpl and the C4-deficient guinea pig sera, a specific complement protein has been removed immunochemically from each depleted human serum reagent. Depleted sera are well suited for the detection and quantitation of hemolytically active complement proteins. Except for the specifically depleted component, the classical and alternative pathways are intact. 1.0 ml per vial.

	Catalog No.
C2 Depleted	A500
C5 Depleted	A501
C6 Depleted	A502
C7 Depleted	A503
C8 Depleted	A504
C9 Depleted	A505
Factor B Depleted	A506
C4 Depleted (Guinea Pig)	A507
C3 Depleted	A508
C1Q Depleted	A509
Factor P Depleted	A512
C3/C4 Depleted	A521
IgG Depleted	

### Complement Reagents

	Catalog No.
CVF, Cobra Venom Factor	A600 (1.0 ml)
Human Complement Standard	A100 (1.0 ml)
Normal Guinea Pig Serum Complement	A119 (1.0 ml)
Normal Human Serum Complement	A112 (2.5 ml)
Normal Human Serum Complement	A113 (5.0 ml)
Complement Activator (HAGG)	A114 (0.2 ml)
Complement Sample Panel	A115 (10 x 0.025 ml)
CVF Activated-NHS	A116 (0.1 ml)
CIC Sample Panel	A117 (5 Samples)
Specimen Stabilizing Solution	A9576 (25 ml)

### Purified Proteins

Each complement protein has been tested for functional purity in standard hemolytic assays and for biochemical purity by SDS-polyacrylamide gel electrophoresis. The concentration of each complement protein, except for Factor D and C3a is approximately 1.0 mg/ml. Volume: 250 µl per vial.

	Catalog No.
C1q	A400 (1.0 ml)
C3	A401
C4	A402
C5	A403
C6	A404
C7	A405
C8	A406
C9	A407
Factor B	A408
Factor D	A409
Factor H	A410
Factor I	A411
Factor P	A412
C3b	A413 (50 µl)
C3a	A414 (100 µl)

### Chicken Polyclonal Antibodies (IgY)

Each polyclonal IgY antibody has been isolated from chicken egg yolks and tested for purity by SDS-polyacrylamide gel electrophoresis. Specificity and activity were assessed by EIA. The protein concentration of each is approximately 1 mg/ml in phosphate buffered saline. Volume: 100 µl per vial.

	Catalog No.
Anti Human C3d	A800
Anti Human C4b	A801
Anti Human SC5b-9	A802

## Monoclonal antibodies

Each monoclonal antibody has been purified from mouse ascites fluid and tested for purity by SDS- polyacrylamide gel electrophoresis. The protein concentration of each is 1 mg/ml in borate buffered saline with 0.02 % sodium azide. Volume: 100 µl per vial.

	Catalog No.
C1q	A201
C3a	A203
C3c	A205
C3d	A207
iC3b (Neoantigen)	A209
C4c	A211
C4d	A213
C4 Binding Protein	A215
C5	A217
C6	A219
C7	A221
C8	A249
C9	A223
Factor B (Ba ) C3 Proactivator	A225
Factor B (Bb) C3 Proactivator	A227
Factor H #1	A229
Factor I #1	A247
Factor I #2	A231
Factor P #1 (Properdin)	A233
Factor P #2 (Properdin)	A235
S Protein (Vitronectin)	A237
SC5b-9 (TCC-Neoantigen)	A239
Clusterin (SP40, 40 und APO J)	A241
C3d (Neoantigen)	A250
C4d (Neoantigen)	A251
Bb (Neoantigen)	A252 0.5 ml
C4d (Neoantigen)	A253
Factor H #2	A254
Factor H #3	A255

## Biotinylated Monoclonal Antibodies

### Biotin labeled Monoclonal Antibodies

Each monoclonal antibody has been purified from ascites fluid and labeled with biotin. The protein concentration of each is 0.2 mg/ml. Volume: 250 µl per vial.

	Catalog No.
C1q	A700
C3c	A701
C3d	A702
C4c	A703
C4d	A704
C5	A705
C6	A706
C7	A707
C8	A708
C9	A709
iC3b (neoantigen)	A710
SC5b-9 (neoantigen)	A711
Factor Bb	A712

## Goat Polyclonal Antisera

Quidel's complement antisera are raised in goats and are quality controlled for specificity by immunochemical analysis. Each contains 0.02 % sodium azide. Volume: 2.0 ml per vial.

	Catalog No.
C1 Inhibitor	A300
C1q	A301
C1s	A302
C2 (Ig Fraction)	A303 1.0 ml
C3	A304
C4	A305
C5	A306
C6	A307
C7	A308
C8	A309
C9	A310
Factor B	A311
Factor H	A312
Factor I	A313

## 10. Complement Specific Antisera: Application Overview

### Preparation

Hyperimmune antisera were raised in goats by standard immunization procedures utilizing highly pure complement antigens isolated from freshly drawn normal human plasma. Antiserum absorption, if required, was accomplished by solid phase adsorption procedures. The antisera were judged to be monospecific when tested at various concentrations against freshly drawn normal human serum containing 10 mM EDTA by double immunodiffusion, one and two dimensional immunoelectrophoresis and rocket immunoelectrophoresis. Antisera were dilapidated and contain 0.02 % sodium azide as a preservative.

### Applications

Quidel's complement antisera have been used in a variety of techniques including double immunodiffusion, quantitative radial immunodiffusion (RID), rocket immunoelectrophoresis, western blotting, immunohistochemistry and ELISA. It is important to note that these products are whole antiserum and therefore may require appropriate whole serum controls in some applications.

### Double Immunodiffusion

Optimal dilutions vary by application. In general, Quidel's antisera will provide clear precipitin lines at a 1:8 or greater dilution under the following conditions:

- Agarose: 1.5 % agarose in 25mM barbital buffer (pH 8.6), 125 mM NaCl, 10 mM EDTA
- Well size: 4 mm in diameter
- Sample: 18  $\mu$ l of undiluted NHS.
- Pattern developed for 24 hours at room temperature.

Antigen or antibody excess may yield "fuzzy" precipitin lines.

### Quantitative Radial Immunodiffusion

Recommended antiserum concentrations are between 0.5 and 3.0  $\mu$ l/cm<sup>2</sup> under the following conditions.

- Agarose: 1.5 % agarose in 25 mM barbital buffer (pH 8.6), 125 mM NaCl, 10 mM EDTA
- Well size: 3mm in diameter
- Sample size: 10  $\mu$ l of each serum dilution
- Patterns developed for 48 to 96 hours in a moisture chamber at RT

If a greater range of NHS dilutions are required for a particular application, the amount of antiserum ( $\mu$ l/cm<sup>2</sup>) should be decreased by half. Precipitin rings should be visualized after staining.

Item	Antigen	Input of antiserum	Optimal dilution
A300	C1 Inhibitor	1.5 $\mu$ l/cm <sup>2</sup>	1:2
A301	C1q	0.8 $\mu$ l/cm <sup>2</sup>	1:2
A302	C1s	2.5 $\mu$ l/cm <sup>2</sup>	1:2
A303	C2	N/T	N/T
A304	C3	0.8 $\mu$ l/cm <sup>2</sup>	1:4
A305	C4	2.5 $\mu$ l/cm <sup>2</sup>	1:4
A306	C5	1.0 $\mu$ l/cm <sup>2</sup>	1:2
A307	C6	1.2 $\mu$ l/cm <sup>2</sup>	1:2
A308	C7	3.0 $\mu$ l/cm <sup>2</sup>	1:2
A309	C8	3.0 $\mu$ l/cm <sup>2</sup>	1:2
A310	C9	1.5 $\mu$ l/cm <sup>2</sup>	1:2
A311	Factor B	0.8 $\mu$ l/cm <sup>2</sup>	1:2
A312	Factor H	1.0 $\mu$ l/cm <sup>2</sup>	1:2
A313	Factor I	1.5 $\mu$ l/cm <sup>2</sup>	1:2

**Figure 21:**

*Specific results for RID*

## Immunohistochemistry

The optimal dilutions of antibodies vary with the specific tissue type and conditions of the experiment. In general, polyclonal and monoclonal antisera from QUIDEL have optimal dilutions between 1:1000 and 1:50000 in histological applications. We strongly suggest that, when optimizing the IHC experiment, this range is bracketed to ensure detection of the proper optimal dilution.

Several techniques for histological evaluation of complement deposition have been published using QUIDEL's mono-clonal antibodies to complement antigens [1 – 4]. The following protocol (adapted from Rogers, et al.) describes one method that has provided excellent results in several studies. Experimental procedures should take into consideration the type of sample, conditions under which the sample was obtained, the specific antibodies and secondary reagents and other lab-specific phenomena. This protocol is provided for reference use only.

## Tissue Preservation

Tissue samples were fixed in 4 % (wt/vol) paraformaldehyde (0.1 M sodium phosphate buffer, pH 7.4) for 24 hours and then cryoprotected in 2 % DMSO/10 % glycerol followed by 2 % DMSO/20 % glycerol for 48 hours each. Samples were sectioned on a freezing microtome at 20  $\mu$ m and stored at -20 °C in a solution of 33 % (vol/vol) glycerol, 33 % polyethylene glycol and 33 % phosphate buffer (0.1M, pH 7.4).

## Histology

Sections were washed 6 x for 15 minutes each in TBS (0.01 M Tris-HCl, pH 7.6/0.09 M NaCl). Endogenous peroxidase was blocked by incubation with 0.3 % H<sub>2</sub>O<sub>2</sub> in 50 % methanol (vol/vol) in TBS followed by 3 x 15 minute washings with TBS/0.5 % Triton X 100. Samples were blocked with 3 % BSA for one hour. Sections were incubated overnight at 4 °C with the optimal dilution of primary antibody (optimal was determined by incubating serial dilutions of antibody from 1:500 to 1:128000 on a strong positive control tissue). Sections were then washed 3 x with TBS/0.05 % Triton X-100 and incubated with biotin conjugated secondary antibody as per the manufacturer's instructions. The bound secondary antibody was allowed to react with DAB (diaminobenzidine) as in standard ABC/DAB immunohistochemistry.

## Controls

Suggested negative controls include omission of the primary antibody, pre-absorption with the purified complement antigen and standard negative tissue. Suggested positive controls include disease state kidney samples (glomerulonephritis) and Alzheimer's brain.

## ELISA

Optimal dilutions for these antisera are greater than 1:50,000 in an antigen capture ELISA. Specific optimal dilutions vary by lot and assay type.

## General Notes

In the case of the QUIDEL polyclonal antisera (A300's), blocking with an appropriate dilution of normal goat sera is suggested.

## Selected References

- [1] Rogers, J., Cooper, N. et al. (1992)  
**Complement Activation by  $\beta$ -amyloid in Alzheimer disease.**  
PNAS 89:10016-10020
- [2] Qian, Z. Wasowska, B.A., Baldwin, W., et al. (1999)  
**C6 produced by macrophages contributes to cardiac allograft rejection.**  
Amer. J. Path 155:4
- [3] Baldwin, W. et al. (1999)  
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- [4] Murphy, B, Kirszbaum, L, et al. (1998)  
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## 10.1 Product Information Monoclonal Antibodies

Item Number	Description (murine anti human)	Epitope description	Isotype
A201	C1q	Binds to globular head of C1q	IgG1k
A203	C3a	Binds C3 and C3a	IgG1k
A205	C3c	Binds C3 and C3c	IgG1k
A207	C3d	Binds C3 and C3d containing fragments	IgG1k
A209	iC3b	Neo antigen, binds only iC3b	IgG2bk
A211	C4c	Binds C4 and C4c containing fragments	IgG1k
A213	C4d	Binds C4 and C4d containing fragments	IgG1k
A215	C4 Binding Protein		IgG2bk
A217	C5	Inhibits lysis of Sheep EA, does not recognize MAC bound to membrane	IgG1k
A219	C6	Recognizes MAC	IgG1k
A221	C7	Inhibits lysis of Sheep EA, does not recognize MAC bound to membrane	IgG1k
A249	C8	Recognizes MAC	IgG2ak
A223	C9	Does not inhibit lysis of sheep EA, does not recognize MAC	IgG2bk
A225	Factor B (Ba) C3 Proactivator	Inhibits function of Factor B; Binds both nascent Factor B and the Ba cleavage product	IgG1k
A227	Factor B (Bb) C3 Proactivator	Inhibits function of Factor B; Binds both nascent Factor B and the Bb cleavage product	IgG1k
A229	Factor H #1	Does not inhibit the function, Factor H like Protein is not detected	IgG1k
A247	Factor I #1	Does not inhibit the function	IgG1k
A231	Factor I #2	Does not inhibit function of Factor I	IgG1k
A233	Factor P #1 (Properdin)	Blocks Function	IgG1k
A235	Factor P #2 (Properdin)	Does not inhibit function of Factor P	IgG1k
A237	S Protein (Vitronectin)	Binds free S protein (Vitronectin) as well as SC5b-9	IgG1k
A239	SC5B-9	Directed against a neo antigen on poly C9 and denatured C9	IgG2ak
A241	Clusterin	Clusterin is also called Apo J and SP40,40	IgG1k
A250	C3d (Neoantigen)	Binds to C3d containing activation products of C3 Only	
A251	C4d (Neoantigen)	Binds C4d activation products of C4 only	
A252	Bb (Neoantigen)	Binds the Bb Sub Unit Only	
A253	C4d (Neoantigen)	Binds C4 and C4d containing fragments	IgG1k
A254	Factor H #2	Does not inhibit the function	IgG1k
A255	Factor H #3	Does not inhibit the function	IgG1k

**Notes:**

All Complement Monoclonals are provided in 100 µl fill volumes at 1.0 mg/ml in Borate Buffered Saline. Suggested initial dilution for all antibodies in IHC applications is 1:1000; actual application and tissue type will determine proper dilution.

Tested Applications					Function Notes	Dilution ELISA
ELISA	RIA	IHC	W.Blot	FACS		
X	X	N/T	N/T	X	Probably does not inhibit function of C1q molecule	1:16000
X	X	N/A	X	N/A	C3a does not bind to cells so is not usually detected in FACS or IHC	
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	1:6200
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	1:10500
X	X	X	X	X	Only reactive to the activation product iC3b, not to C3b or other products	
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	1:5400
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	1:64000
X	N/T	N/T	N/T	N/T		
X	X	N/A	X	N/A		1:10000
X	X	X	N/T	X		
X	X	N/A	X	N/A		
X	N/T	X	X	X		
X	X	N/A	N/T	N/A		
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	
X	N/T	X	X	X		> 1:1000
X	X	X	X	N/T		1:34000
X	X	X	X	N/T		
X	X	X	N/T	N/T		
X	X	X	N/T	N/T		
X	X	X	X	X		
X	X	X	X	X		
X	X	X	X	X		
X	N/T	X				
X	N/T	X				
X	N/T	X				
X	X	X	X	X	Binds both the fragment and whole protein so is not suitable for all EIA applications	
X	X	X	X	N/T		> 1 µg/ml
X	X	X	N/T	N/T		> 1 µg/ml

For IHC the ABC method has proven to be the most reliable overall for these antibodies and analytes; frozen tissue is preferred. Antibodies indicated in **bold** are used in the EIA kits from QUIDEL. Suggested initial dilution for all antibodies in Westernblot 10 ug/ml. For FACS the recommended dilution is 10-40 ug/ml – 1:100 dilution – app 200 samples per vial.

## 10.2 Animal Species Crossreactivity Monoclonal Antibodies\*

### Species Crossreactivity Monoclonal Antibodies\*

ANTI-HUMAN ANTIBODY		CROSS-REACTING SPECIES														
		BABOON	CAT	CHICKEN	COW	DOG	GUINEA PIG	HAMSTER	HORSE	HUMAN	MONKEY	MOUSE	PIG	RABBIT	RAT	SHEEP
C1q	A201	-	-	-	-	-	-	-	-	+	slight	-	N/T	-	-	-
C3a	A203	+	-	-	-	-	-	-	-	+	+	-	+	-	-	-
iC3b (neo)	A209	N/T	-	-	-	-	-	-	-	+	N/T	-	N/T	-	-	-
C3c	A205	+	+	-	-	-	+	-	+	+	+	-	N/T	-	-	-
C3d	A207	-	-	-	-	-	-	-	-	+	-	-	slight	-	+	-
C4c	A211	+	-	-	-	-	-	-	-	+	+	-	N/T	-	-	-
C4d (neo)	A251	+	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	+	N/T	N/T	N/T	N/T	N/T
C4/C4d	A213	+	+	-	+	+	+	-	-	+	-	+	-	+	+	+
C5	A217	+	-	-	-	-	-	-	-	+	+	-	N/T	-	-	-
C6	A219	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T
C7	A221	-	+	-	+	+	-	+	+	+	N/T	-	+	+	+	-
C8	A249	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	N/T	N/T	N/T	N/T	N/T	N/T
C9	A223	+	-	+	+	-	+	+	+	+	+	-	N/T	-	-	-
SC5b-9 (TCC)(neo)	A239	+	-	-	-	-	-	-	-	+	+	-	N/T	-	-	-
Factor H (#1)	A229	+	-	-	-	-	-	-	+	+	+	-	N/T	-	-	-
Factor H (#2)	A254	+	-	-	-	-	-	-	-	+	+	-	N/T	-	-	-
Factor H (#3)	A255	+	-	-	-	-	-	-	-	+	+	-	N/T	-	-	-
Factor I (#1)	A247	-	-	-	-	-	-	-	-	+	N/T	-	N/T	-	-	-
S Protein	A237	-	-	-	-	-	-	-	-	+	N/T	-	N/T	-	-	-
Clusterin/SP40,40	A241	N/T	N/T	N/T	N/T	N/T	N/T	N/T	N/T	+	+	-	N/T	N/T	-	N/T

+ Crossreactivity

| Crossreactivity by Immunohistochemistry\*\*

N/T Not Tested

- No crossreactivity

\*Monoclonal antibodies were tested at QUIDEL for species crossreactivity in ELISA inhibition assays.

A more sensitive assay may detect cross reactivity where none has been indicated by ELISA inhibition.

\*\*Immunohistochemistry data are compiled from the observations of outside researchers; these experiments were not performed at QUIDEL and have not been confirmed.

## 10.3 Animal Species Crossreactivity Polyclonal Antibodies

### Species Crossreactivity Polyclonal Antisera

		CROSS-REACTING SPECIES														
		BABOON	CAT	CHICKEN	COW	DOG	GUINEA PIG	HAMSTER	HORSE	HUMAN	MOUSE	PIG	RABBIT	RAT	SHEEP	GOAT
Goat anti-human C1-INH	A300	+++	-	-	-	+	-	-	-	+++	-	++	+	-	-	-
Goat anti-human C1q	A301	+++	+++	-	-	+++	+++	+++	+	+++	+++	+++	+++	+++	-	-
Goat anti-human C1s	A302	+++	-	-	-	-	-	-	-	+++	-	-	-	-	-	-
Goat anti-human C3	A304	+++	-	-	-	-	+++	+++	+++	+++	+++	+++	+++	+++	-	-
Goat anti-human C4	A305	+++	+	-	-	+++	-	+++	+++	+++	+	+++	+++	+++	-	-
Goat anti-human C5	A306	+++	+	-	-	+++	+++	+	+++	+++	+	+++	+++	+++	-	-
Goat anti-human C6	A307	+++	+++	-	-	+++	-	-	+	+++	-	+++	+++	+++	-	-
Goat anti-human C7	A308	+++	+++	-	-	-	+++	+	+	+++	-	++	+++	-	-	-
Goat anti-human C8	A309	+++	+	-	-	+++	+++	+	+	+++	+	+++	+++	+	-	-
Goat anti-human C9	A310	+++	-	-	-	-	-	+	+++	+++	-	++	+	-	-	-
Goat anti-human Factor B	A311	+++	+++	-	-	-	+++	+++	+++	+++	+++	++	+++	+++	-	-
Goat anti-human Factor H	A312	+++	+++	-	-	+++	+	+++	+++	+++	+++	+++	+++	+++	-	-
Goat anti-human Factor I	A313	+++	-	-	-	-	-	-	-	+++	-	-	-	-	-	-
Chicken Anti-Human C3d (IgY)	A800	+++	-	-	+++	++	++	++	-	+++	+++	++	++	+++	++	++
Chicken Anti-Human C4b (IgY)	A801	+++	-	-	++	++	-	++	-	+++	++	++	++	++	+	++
Chicken Anti-Human SC5b-9 (IgY)	A802	+++	-	-	++	+++	-	++	-	+++	++	++	++	+++	+	++

- +++ Strong cross-reaction
- ++ Moderate cross-reaction
- + Weak cross-reaction
- No cross-reaction
- NT Not Tested

All antisera were tested for species crossreactivity by double immunodiffusion and/or one-dimensional immunoelectrophoresis.



# 11 Appendix

## 11.1 Assay protocols (examples)

11.1.1 Testing of Complement Activation Using the Example of Liposome Preparations

11.1.2 Quantifying Complement Activation in a Non-human Specimen (CxH50)

## 11.2 Technical Data sheet and certificate of analysis (examples)

Normal human complement serum (A113)

HAGG Activator (A114)

Activator Cobra Venom Factor (A600)

Specimen stabilizer (A9576)

C3 Depleted Serum (A508)

Normal human complement Standards (A100)

## 11.1.1 Protocol:

### Testing of Complement Activation Using the Example of Liposome Preparations

Determination of complement activation product C3a

Reagents and Materials	Storage	Contents	Cat.-No.
<b>Quidel Complement ELISA</b>	2 – 8 °C	96 wells	Axxx
<b>Quidel Normal Human Serum Complement</b> Lot 906680, e.g. C3a 800 ng/ml (see Certificate of Analysis)	-70 °C	5 ml 2.5 ml	A113 A112
<b>EDTA, heparin and re-calcified plasma are not suitable</b>			
<b>Cobra Venom Factor/Activation of the alternative pathway</b> Lot 905605, Titer: 686 U/ml (see Certificate of Analysis) 8-20 U/ml is adequate to convert nearly all C3 to C3 fragments when incubated with neat human serum for 30 - 90 minutes at 37 °C	-70 °C	1 ml	A600
<b>Complement Activator Quidel – classical pathway</b> HAGG, 10 ul for 1 ml = complete activation after 30 minutes at 37 °C	-70 °C	0.2 ml	A114
<b>Specimen Stabilizer/Quidel</b> Dilute samples 1:1	2 – 20 °C	25 ml	A9576
Complement inhibitor: <b>EDTA</b> EDTA 10 mMol final concentration – ice-cold			

#### Note:

Prepare appropriate bowl with ice Eppendorf PPE tubes  
Mix the samples gently, but do not vortex

#### Handling of Reagents – Storage

Cobra Venom	Thaw quickly at 37 °C and immediately transfer to ice Store unused material in aliquots at -70 °C Calculation of required quantity: e. g. titer: 685 units/1000 ul 20 units in 1000 ul of serum are required for complete activation; equal to 29 ul $1000/685 \times 20 = 29$ ul
HAGG	Immediately store unused material at -70 °C 10 ul for 1 ml of serum
Specimen Stabilizer	Dissolve in a water bath at 37 °C and mix. Allow it to cool down to room temperature.
Normal Serum = NHS	Thaw quickly at 37 °C and immediately transfer to ice Freeze serum that is not needed aliquoted into PPE tubes at -70 °C Prepare 5 aliquots of 100 ul (50 ul) each in Eppendorf tubes on ice Immediately add 100 ul (50 ul) Specimen Stabilizer to 1 aliquot
NHS/Buffer	Corresponding to the percentage of buffer in the samples; e. g. 80:20 Prepare 5 aliquots of 100 ul (50 ul) each in Eppendorf tubes on ice Immediately add 100 ul (50 ul) Specimen Stabilizer to 1 aliquot
NHS – activated	Add 5 ul Activator or 15ul CVF to 500 ul NHS and immediately transfer 5 aliquots of 100 ul to ice
NHS – inhibited	500 ul plus EDTA – transfer 5 aliquots of 100 ul each to ice

Lipos/NPs

Prepare 5 aliquots in different dilutions  
Immediately transfer aliquots of 100 µl (50 µl) to ice

**When working with complement activation assays it should always be kept in mind that contamination with bacteria must be avoided; i.e. pipette tips and reaction vessels have to be ultra-pure (sterile) to avoid unwanted activation of lipopolysaccharides.**

#### Test Procedure

1. ELISA equipment
2. Plate reader, washer, multichannel pipettes, PP tubes, sterile pipette tips  
Appropriate pipettes and PP vessels to prepare dilutions
3. Prepare ice bath
4. Prepare incubation at 37 °C under rotation

#### 5. Provide Materials / Controls / Reagents

- a) Human Normal Serum
- a) Activator: HAGG or Cobra venom or Zymosan
- b) Inhibitor - EDTA
- d) Liposomes/Nanoparticles/Therapeutics preparations
- e) All buffers
- f) Specimen Stabilizer

#### 6. Prepare samples and controls for incubation – must be performed on ice

- a) Control 1: Human Normal Serum without additives – concentration listed in Certificate of Analysis
- b) Human Normal Serum plus activator – increase to 10-fold concentration may occur
- c) Human Normal Serum plus EDTA – inhibition control
- d) Prepare all buffers to be used in the same ratio as in the preparations with Human Normal Serum
- e) Prepare liposome/nanoparticle preparations in appropriate ratio with Human Normal Serum (20:80)

e. g. prepare 500 µl, prepare 5 aliquots of 100 µl each on ice

#### 7. Incubation of all controls and samples

- a) Control 1: Human Normal Serum without additives – concentration listed in Certificate of Analysis

	Pipet	Incubation intervals at 37 °C on rotator				on ice
		15 min	30 min	60 min	120 min	120 min
NHS	5 x 100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
NHS plus activator	5 x 100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
NHS plus inhibitor	5 x 100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Buffer	5 x 100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Sample	5 x 100 µl	100 µl	100 µl	100 µl	100 µl	100 µl

#### 8. Add 100 µl Specimen Stabilizer (1+1) to aliquots after incubation intervals and hold on ice

9. Dilute samples with **Sample Diluent** according to test procedure and expected response -

**Example C3a:** all samples are **already** diluted **1+1 with Stabilizer** and are held on ICE

- a) Human **Normal Serum** without additives – concentration e. g. C3a 800 ng/ml
- b) Human Normal Serum plus **activator** – positive control of the method
- c) Human Normal Serum plus **EDTA – inhibition** control = see Control a
- d) Prepare all **buffers** to be used in the same ratio as in the preparations **with Human Normal Serum**
- e) Prepare **liposome/nanoparticle** preparations in an appropriate ratio with Human Normal Serum (e. g. 20:80)

and test different concentrations of the therapeutic

	Dilution with Sample Diluent				on ice
	15 min	30 min	60 min	120 min	120 min
NHS	1:500	1:500	1:500	1:500	1:500
NHS plus activator	1:1000	1:1000	1:5000	1:5000	1:5000
NHS plus inhibitor	1:500	1:500	1:500	1:500	1:500
Buffer	1:500	1:500	1:500	1:500	1:500
Sample Lipos/NPs	1:500	1:1000	1:1000	1:1000	1:1000

Final dilution – due to predilution 1+1 with Sample Stabilizer					
NHS	1:1000	1:1000	1:1000	1:1000	1:1000
NHS plus activator	1:1000	1:2000	1:10000	1:10000	1:10000
NHS plus inhibitor	1:1000	1:1000	1:1000	1:1000	1:1000
Buffer	1:1000	1:1000	1:1000	1:1000	1:1000
Sample Lipos/NPs	1:1000	1:2000	1:2000	1:2000	1:2000

The following dilutions cover a range

1:500 up to 2500 ng (2.5 ng \*2\*500 = 2.500 ng)  
 1:1000 up to 5000 ng (2.5 ng \*2\*1000 = 5.000 ng)  
 1:5000 up to 25000 ng (2.5 ng \*2\*5000 = 25.000 ng)

**Note:**

It is recommended to perform, for example, preliminary tests with 2 samples using different concentrations and incubation times.

Test activation, inhibition and buffers in a separate run.

Even if optimal dilution and optimal incubation intervals with maximum activation are found, further runs with preparations should include aliquots with 0 and maximum values as controls.

**Evaluation:**

1. ELISA kit controls within the range?
2. Normal Human Serum in accordance with Certificate of Analysis in 0 value sample/increase at 37 °C to 2-3-fold
3. Activated Normal Serum: may increase up to the 10-fold of the baseline value
4. Inhibited material: no increase even during incubation
5. Normal Serum with buffer: pay attention to the ratio (80:20), thus, the target value is not 800 but 640 ng/ml – if buffer is neither activated nor inhibited
6. How does serum containing Lipos/NPs behave in comparison to Normal Serum?

	Evaluation	Example C3a ng/ml
Serum with activator	Shows maximum possible activation	18.235
Serum with inhibitor	Confirms complement inhibition, despite incubation at 37 °C values remain at the level of 0 value (ice)	< 1000
Normal Serum	0 min 60 min 120 min	0.75 1.848 1.98
Lipo/Nano samples 20 % sample/80 % serum	Activation is evaluated in relation to Normal Serum 0 min (iced sample) 30 min 60 min 120 min 240 min	0.985 2.392 2.716 4.963 4.946
	Preparation 2 0 min (iced sample) 60 min 120 min 180 min 240 min	1.142 2.175 2.356 3.013 3.432
Buffer / Serum 20 % Buffer / 80 % Serum	No activation and no inhibition was observed	< 1000

#### Examples for appropriate dilution steps

##### 1:500

1. Step: 240 ul Diluent plus 10 ul Sample – 1:25
2. Step: 380 ul Diluent plus 20 ul Dilution I – 1:20

##### 1:1000

1. Step: 245 ul Diluent plus 5 ul Sample – 1:50
2. Step: 380 ul Diluent plus 20 ul Dilution I – 1:20

##### 1:5000

1. Step: 495 ul Diluent plus 5 ul Sample – 1:100
2. Step 490 ul Diluent plus 10 ul Dilution 1 – 1:50

The dilutions have been selected such that only minimum amounts of diluent are used.  
The final quantity is sufficient to dispense 2x 100 ul into the ELISA plate with the multichannel pipette.

## 11.1.2 Protocol:

### Quantifying Complement Activation in a Non-human Specimen (CxH50)

Operation Performed by: ..... Date: .....

Reviewed by:..... Date: .....

#### I. Purpose

To define the procedure and reagents used in the CxH50 assay for serum.

	Lot Number	Expiration Date
<b>Gelatin Veronal Buffer (GVB++)</b> 5X Veronal Buffer with Ca/Mg		
Gelatin		
Deionized water		
Sheep Erythrocytes in Alsevers Solution		
Hemolysin (Rabbit Anti-Sheep Erythrocyte)		
Bulk, EDTA 0.2M		
Gelatin Veronal Buffer with Ca/Mg (GVB++)		
Hemolysin-Treated Sheep Erythrocytes (EAs)		
On-Test Normal Guinea Pig Serum		
On-Test Complement Activated Guinea Pig Serum		
C3 depleted human serum (Quidel P/N A508)		
Normal human complement standard (Quidel P/N A100)		
Control (as appropriate)		

#### Materials/Reagents/Equipment required

Thermometer  
 Balance  
 Water Bath, 37°C  
 Heat Block/Stir Plate  
 Stir Bar  
 Assorted Glassware  
 Pipettes  
 Parafilm  
 50 mL Conical Tubes (polypropylene)  
 Refrigerated Table-Top Centrifuge (e.g., Dupont/Sorval RT6000)  
 Spectrophotometer (capable of 540 nm wavelength)  
 13 x 100 mm disposable glass test tubes  
 12 x 75 mm disposable glass test tubes  
 Ice bucket with ice

## II. References

- [1] Mayer, M.M. (1961) Complement and complement fixation.  
**In Experimental Immunochemistry,**  
edited by E.A. Kabat and M.M. Mayer. 2nd Ed. Charles C. Thomas, Springfield, IL, pp 133-240.
- [2] Nilsson, U.R. and B. Nilsson.  
**Simplified Assays of Hemolytic Activity of the Classical and Alternative Complement Pathways.**  
Journal of Immunological Methods, 72 (1984), pp 49-59.
- [3] Ruddy, S. (1985) Complement.  
**In Laboratory Diagnostic Procedures in the Rheumatic Diseases,**  
Grune & Stratton, pp 137-161.

## III. Procedure

1. **Gelatin Veronal Buffer (GVB++)** (Makes 1 Liter):
  - a. Add 2.0 grams of gelatin to 200 mL of D.I. water in a heat-resistant glass container. Heat to boiling to dissolve with constant stirring.
  - b. Measure out 200 mL of 5X VB++ in a clean graduated cylinder. Add an additional 500 mL of D.I. water to the cylinder and mix well.
  - c. Add 100 mL of the dissolved gelatin and bring to a final volume of one liter with D.I. water. Cover with Parafilm® and mix well by repeated inversion of the container.
  - d. Filter the Gelatin Veronal Buffer (GVB++) through a 0.45 µm filter and store in a sterile container at 4°C. Label container with date of preparation, description, your initials, and the expiration date. (Note: The GVB++ is good for 3 months when stored at 2-8°C and protected from contamination.)

2. **Sensitized Sheep Erythrocytes (EAs)** (Makes about 25 mL of EA stock solution for adjustment; can be repeated and pooled for larger volumes):

Wash SRBCs as follows:

- a. Place 5 mL of SRBCs in a 50 mL conical tube and bring to 50 mL with cold GVB++. Keep the GVB++ on ice throughout the procedure. Invert the tube several times to suspend the cells and then spin 900 to 1500 Xs g (2000 rpm in QCT#348 centrifuge) at 2 – 8 °C for 10 minutes. Decant supernatant and discard while saving the pellet of cells. The pellet should be about 1 mL in volume. Re-suspend the cell pellet into 50 mL of fresh GVB++ by gently inverting the tube and spinning again as described above. Continue to wash the cells in this manner until the supernatant is clear and colorless.
- b. Re-suspend the pellet into 20 mL of GVB++. In a separate container combine 27 mL of GVB++, 3.0 mL of 0.2M EDTA solution, and the optimal dilution of Hemolysin for the assay (usually 1:200 to 1:1000) as determined for each lot of Hemolysin (see Section 5 for titration of Hemolysin). Add this second mixture to the suspended SRBCs in GVB++ and mix by gentle inversion of the conical tube. (NOTE: The dilution of Hemolysin is based upon the total 50 mL mixture volume of GVB++, EDTA, and suspended EAs.)
- c. Incubate the SRBCs, Hemolysin, EDTA mixture at 37 °C for 30 minutes with frequent, gentle mixing (e.g., inversion) every 5 minutes.
- d. Spin the cell suspension as described above, and wash at least two times with GVB++ or until the supernatant is clear and colorless. Re-suspend the cells in 20 to 25 mL of GVB++ and store at 4 °C. Label this suspension with, "EA Stock Solution," the date of preparation, the expiration date, and your initials. The EA Stock Solution is used to make the daily working solution of EAs used in the CxH50 Assay. The EA Stock Solution is good for 10 days when stored at 2 – 8 °C and protected from contamination.

- e. Adjust a portion of the cells to a final concentration of  $6.5 \times 10^7$  cells per mL to make a **daily** working solution of EAs as described below. The working solution is best made in the morning and can be used throughout the day. Make a freshly washed and adjusted working EA solution for each day of testing.
- f. Make sure the cells are washed at least twice or until the supernatant is clear and colorless using GVB++ as the washing solution. Using 12 x 75 glass tubes, mix 1.3 mL of water plus 0.2 mL of cell suspension to test the concentration of the EAs. Read the O.D. at **540 nm** using water as a blank. If the mixture is  $1.2 \leq OD \leq 1.6$ , the cells are at  $6.5 \times 10^7$  cells per mL. Dilute or spin down and re-suspend in an appropriate volume should any adjustments become necessary. Only a portion of the EA Stock Solution need be adjusted wto produce the working solution for any particular day.

### 3. Optimization of CxH50 Assay

**Keep all testing materials on ice. Thaw all materials quickly in a 37° C water bath then place immediately on ice.**

- a. Prepare a 1:10 – 1:1 X  $10^6$  dilutions of Normal Guinea Pig Serum (NGPS) in GVB++ these will be the initial test samples. The amount of NGPS can be altered if necessary.
- b. To the 13 x 100 glass tubes, add 345  $\mu$ L of cold GVB++ and 10  $\mu$ L of the C3 depleted sample, 120  $\mu$ L of Ea and 25  $\mu$ L of diluted Normal Guinea PigSerum. Prepare individual tubes for entire dilution series. Vortex samples thoroughly, but gently.

Sample	$\mu$ L of cold GVB ++	$\mu$ L of the C3 depleted serum	$\mu$ L of Ea	$\mu$ L of sample
Test Sample (sample: NGPS)	345	10	120	25
Reference (sample: NHS: A100)	345	10	120	25
Positive sample: sterile H20)	0	0	120	380
Background (sample: NGPS)	355	0	120	25
Negative (no sample)	380	0	120	25

- c. Prepare controls and samples in triplicate– Positive control (D.I. water), Background Control (NGPS, GVB++), Negative Control (GVB++), and Reference Control (GVB++ + NHS). Add the sample to the tubes last.
- d. Place the tubes in a 37°C water bath for 30 minutes, vortexing very gently every 5 minutes. Handle tubes carefully so no water gets inside as this will cause lysis.
- e. Centrifuge the tubes at 900 to 1500 Xg (2000 rpm) for 10 minutes at 2 – 8 °C.
- f. Read supernatants at 540nm using GVB++ as the blank.
- g. Attach printout, and calculate data as described in step 4.

#### 4. Calculations

- a. Calculate the percent lysis (**Y**) in decimal form as follows:

$$Y = \frac{\text{O.D. 540 nm Experimental}}{\text{Avg. O.D. 540 nm Positive Control}}$$

- b. Next, determine the value of  $Y/1-Y$  for each experimental value.

**NOTE: Since the CH50 assay is used to determine the 50 % point of lysis as compared to the Positive Control, the point at which  $Y/1-Y = 1.0$ , i.e., the 50 % lysis point, must be calculated.**

Example: With an experimental O.D. 540nm = .554, and a Positive Control O.D. 540 nm = 1.483, the percent lysis is calculated as follows:

$$Y = 0.554 / 1.483 = \mathbf{0.374} \text{ and } Y/1-Y = 0.374 / (1-0.374) = \mathbf{0.597}$$

- c. Determine the volume of serum that corresponds to the 50% point of lysis using either of the following methods:
- Using log-log graph paper, plot the volume ( $\mu\text{L}$ ) of serum used on the Y-axis vs. the  $Y/1-Y$  value on the X-axis per assay tube for each serum tested. Draw a best fit line through the points. Graphically determine the volume ( $\mu\text{L}$ ) of sample needed to give a  $Y/1-Y$  value of 1.0 (i.e., the 50% lysis point).

or

- Using a calculator that is capable of linear regression analysis, enter the values as described above for the assay tubes that bracket the  $Y/1-Y = 1.0$  level of lysis. Determine the volume ( $\mu\text{L}$ ) of serum that would generate the 50 % point of lysis using point-to-point regression.

- To calculate the CH50 units/ml present in the original undiluted serum, use the following formula:

$$\text{CH50 units/ml} = \frac{1000 \mu\text{L/mL}}{\mu\text{L of serum giving } Y/1-Y \text{ of } 1.0}$$

#### 5. Hemolysin Titration for the Production of EAs

A determination of the optimal concentration of hemolysin used for the production of EAs must also be performed using the methods described previously. This procedure must be done for every new lot of hemolysin purchased or when the activity of any hemolysin preparation is in question.

The titration assay will include various concentrations/volumes of hemolysin (e.g., final dilutions of 100-, 200-, 500-, 1000-, and 2000-fold) used to produce small lots of EAs. These EAs are then examined against a dilution of serum known to generate a high level (80 – 100%) of lysis of EAs as compared to the positive control (EAs plus water). In this manner, every lot of hemolysin will be examined for optimal performance. The concentration of hemolysin chosen to produce future preparation of EAs should be the concentration that results in maximal lysis using a serum of known CH50 activity.

#### IV. Additional Information

- If the data points do not fall on a straight line, possible problems include pipetting errors, reaction mixtures were not vortexed completely or often enough during the assay, EAs not functioning properly (too old), or contaminated GVB++.
- Human plasma can also be examined using these methods since the original dilution of 80- to 100-fold will prevent clot formation during the assay.
- If the series of tubes for a serum does not afford the presence of a 50 % lysis point somewhere in the series, adjust the original serum dilution and repeat the assay using a less concentrated or more concentrated serum dilution as appropriate.

## V. Determination of optimal Dilution for later Testing

1. C3H50 is determined by the dilution yielding a 50% lysis of Ea.
2. The dilution of the normal guinea pig serum at which 50% lysis occurs can then be used as the optimal dilution for all future experiments.
3. Normal human serum can be used as an experimental control for the process.
4. The positive control sample (DI water only) is used as the 100 % lysis point.
5. Normal guinea pig serum is a background value.
6. Once the optimal (50 % lysis) dilution is determined in an unactivated normal control all future assays can be use this dilution as 100 %.

## VI. Determination of C3H50

1. To determine C3H50 in test sera samples are run versus a normal unactivated control (100 %) at the optimal dilution determined in section V.
2. Dilute all experimental samples with cold GVB++ to the optimal dilution. For assay controls NGPS (100 % control) and NHS (assay control), DI water (100 % lysis control). Perform the hemolytic assay.
  - a. Prepare a optimal dilution of Normal Guinea Pig Serum (NGPS) and test specimens in GVB++ these will be samples.
  - b. To the 13 x 100 glass tubes, add 345  $\mu$ L of cold GVB++ and 10  $\mu$ L of the C3 depleted sample, 120  $\mu$ L of Ea and 25  $\mu$ L of optimally diluted Normal Guinea Pig Serum. Prepare individual tubes for all test specimens. Vortex samples thoroughly, but gently.

Sample	$\mu$ L of cold GVB ++	$\mu$ L of the C3 depleted serum	$\mu$ L of Ea	$\mu$ L of sample
100% Normal (sample: NGPS@ optimal dilution)	345	10	120	25
Reference (sample: NHS: A100)	345	10	120	25
Positive (sample: sterile H2O)	0	0	120	380
On Test (sample: test GP serum)	345	10	120	25
Negative (no sample)	380	0	120	0

- c. Prepare controls and samples in triplicate– Positive control (D.I. water), Background Control (NGPS, GVB++), Negative Control (GVB++), and Reference Control (GVB++ + NHS). Add the sample to the tubes last.
- d. Place the tubes in a 37 °C water bath for 30 minutes, vortexing very gently every 5 minutes. Handle tubes carefully so no water gets inside as this will cause lysis.
- e. Centrifuge the tubes at 900 to 1500 X g (2000 rpm) for 10 minutes at 2 – 8 °C.
- f. Read supernatants at 540 mn using GVB++ as the blank.
- g. Read supernatants at 540 mn using GVB++ as the blank.
- h. Use the A540 of NGPS as 100 % control. From the A540 of the test samples calculate % of 100% control and plot accordingly.

## 11.2 Technical Data sheet and certificate of analysis (examples)

### Complement

### Technical Data Sheet

#### Normal Human Serum Complement

**For Research Use Only. Not for use in Diagnostic Procedures.**

##### Background

Quidel human serum complement standard is a uniform pool of human serum complement that has been characterized for levels of complement activation fragments (complement split products) as well as CH50. Because the anticoagulants used in the preparation of plasma (EDTA, ACD and even heparin) can interfere with complement activation in vitro, serum has long been used as a human complement source for experimentation. Many commercially available sera, however, are not processed appropriately to conserve complement activity. Lyophilization and plasma recalcification can raise background levels of complement split products (SC5b-9 or TCC, iC3b, Bb, C4d and particularly the anaphylatoxins C3a, C5a and C4a) and decrease CH50. Sera, however, must be processed quickly and correctly to prevent complement turnover. For this reason, sera obtained from blood banks and other large pools may not be suitable for many experimental procedures. Conversely, in house serum draws are complicated by several factors including the necessity of maintaining standardized pools of complement for ongoing experimentation and the confidentiality requirements relating to infectious disease testing. Quidel Normal Human Serum Complement is designed to address these specific issues.

##### Storage and Handling

This product should be stored at or below -70 °C. When needed, it should be thawed rapidly at 37 °C and immediately placed on ice until use. Any remaining sera should be aliquotted into polypropylene tubes in a convenient volume and re-frozen at -70 °C or below. Avoid repeated freeze thaw. **Storage at temperatures warmer than -70 °C is not recommended.**

##### Applications

Normal human serum complement is suitable for in vitro experiments relating to complement activation. It has been widely used in biomaterials research, pharmaceutical development and cytotoxicity assays. It is ideal for experiments and assays for which a high level of in vitro complement activity is necessary or a low level of complement activation fragments is required.

##### Specifications

Catalog Number: A113  
Volume/Vial: 5.0 ml  
Storage: ≤ -70°C  
Form: Frozen Liquid

##### References

- [1] Idusogie, E. E., Presta, L., et al.  
**Mapping of the C1q binding site on Rituxan, a chimeric antibody with a human IgG1**  
FC. J. Immunol 164:4178-4184 (2000).
- [2] Hinton, PR., Xiong, J., et al.  
**An Engineered Human IgG1 Antibody with Longer Serum Half-Life.**  
J Immunol 176:346-356 (2006).
- [3] Dall'Acqua, W.F., Cook, K.E., et al.  
**Modulation of the Effector Functions of a Human IgG1 through Engineering of Its Hinge Region.**  
J Immunol 177:1129-1138 (2006).

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## Guinea Pig Serum Complement

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### Background

Quidel guinea pig serum complement is a uniform pool of guinea pig serum that has been characterized for both classical and alternative pathway activity. Guinea pig serum has been in use from the beginning of the twentieth century to the present for the study of complement activity and its relationship to immunity [1-4]. The discovery of guinea pigs deficient in specific complement fragments has furthered this research. Guinea pig serum is recommended by both the U.S. and European Pharmacopoeia for testing the anticomplementary activity of immunoglobulin.

### Storage and Handling

This product should be stored at or below  $-70^{\circ}\text{C}$ . When needed, it should be thawed rapidly at  $37^{\circ}\text{C}$  and immediately placed on ice until use. Any remaining sera should be aliquotted into polypropylene tubes in a convenient volume and re-frozen at  $-70^{\circ}\text{C}$  or below. Avoid repeated freeze thaw. **Storage at temperatures warmer than  $-70^{\circ}\text{C}$  is not recommended.**

### Applications

Guinea pig serum complement is suitable for in vitro experiments relating to complement activation. It has been widely used in biomaterials research and pharmaceutical development. It is ideal for experiments and assays for which a high level of in vitro complement activity is necessary or a low level of complement activation fragments is required.

### Specifications

Catalog Number:	A119
Volume/Vial:	1.0 ml
Concentration:	>40 mg protein/ml
Activity:	>500 CP50 Units/ml >50 AP50 Units/ml
Storage:	$\leq -70^{\circ}\text{C}$
Form:	Frozen Liquid
Origin:	Manufactured in the USA

### References

- [1] Moore, H.D.  
**Complementary and opsonic functions in their relation to immunity: A study of the serum of guinea-pigs naturally deficient in complement.**  
J. Immunol 4:425-441 (1919).
- [2] Hyde R.R. and Parsons E.I.  
**Quantitative interdependence of sensitizer and complement in hemolysis.**  
Am. J. Epidemiol. 7(1):11-21 (1927).
- [3] Muller F. and Segerling M.  
**A factor in guinea-pig serum with accelerating effect on immune immobilization of Treponema pallidum (IAF): Isolation, purification and differentiation from the known haemolytic complement components and from lysozyme.**  
Immunology 27:33-41 (1974).
- [4] Wooley, R.E. et al.  
**Comparison of chicken plasma and guinea pig serum in a quantitative microtiter method of determining microbial complement resistance.**  
Avian Diseases 35(4):897-900 (1991).

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## Complement Activator (HAGG) Heat Aggregated Gamma Globulin

**For Research Use Only. Not for use in Diagnostic Procedures.**

### Background

The classical pathway of complement can be triggered by antibody bound to a foreign particle (immune complex, cell or other material). Particles of this type interact directly with C1q. C1q can then activate the C1r(2)C1s(2) sub-units of the C1 complex. The activated C1s cleaves C4 to C4b near the amino terminus of the gamma chain releasing C4a in the process. Activation of the Classical Pathway is under stringent control in vivo. C4b is rapidly degraded to iC4b and then to C4c and C4d. Because of the short life of the C4b molecule, much of the C4d is free and circulates in serum. As such C4d is an excellent marker for classical complement activation in vivo or in vitro and is therefore the basis of the MicroVue™ C4d EIA Kit (Item A008).

Heat Aggregated Gamma Globulin acts as a potent classical complement pathway activator, functioning similarly to naturally occurring immune complexes. When activated by a potent activator, such as Quidel's Complement Activator, nearly all the C4 in a given complement source may be converted into cleavage products.

The activity of the resulting complement activator is assessed using the MicroVue C4d EIA kit. 10 µl of Activator is incubated with a reference serum (Normal Human Serum Complement, Cat. A113) at 37 °C for 90 minutes. At fixed time points, samples of the reference serum are taken. Ice cold 20 mM EDTA is added to each sample to block further complement activation; the samples are stored on ice until assay. Upon completion of the activation phase, the resulting samples are assayed in the MicroVue C4d kit. The results (µg C4d/mL) are graphed vs. time for each sample. Full activation is shown by a rapid increase in C4d concentration followed by a plateau. Quidel's complement activator will fully activate the classical complement pathway of the reference sera within 30 minutes at 37 °C.

### Specifications

Catalog Number:	A114
Protein Concentration:	~ 50 mg/ml
Volume/Vial:	0.2 ml/vial
Storage:	≤ -70°C
Buffer:	Phosphate Buffered Saline (pH 7.3, 20 mM CaCl and MgCl)



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## Cobra Venom Factor

**For Research Use Only. Not for use in Diagnostic Procedures.**

### Background

Cobra Venom Factor (CVF), sometimes referred to as C3b(Cobra), is the non-toxic, complement activating component of cobra venom.<sup>1-3</sup> Like naturally occurring C3b, CVF forms a complex with complement components Factor B and Factor D. This CVFBbD convertase is capable of activating C3 in a wide variety of species via the alternative complement pathway. Unlike the naturally occurring convertase (C3bBbD), the C3b(Cobra)BbD convertase is Factor H resistant and is therefore not inactivated by Factor I or CR1. Given appropriate incubation time, CVF will convert nearly 100 % of the C3 to C3 end products. Unlike CVF purified from the *Naja naja* species, CVF from *Naja naja kaouthia* activates the terminal pathway directly by forming a C5 convertase.<sup>4,5</sup> This depletes C5 in a manner analogous to that described above for C3. Levels of iC3b, C3a, SC5b-9, C5a and the Factor B cleavage product Bb are all extremely high in CVF treated sera.

### Storage and Handling

Purified CVF may be stored at -70°C until the expiration date listed on the vial and the accompanying Certificate of Analysis. CVF should be thawed rapidly at 37°C and immediately placed on ice until use.

### Applications

**Note:** When using any CVF *in vivo* or *in vitro*, it is important to monitor units of activity rather than µg/ml as activity/µg can vary slightly between preparations and suppliers. **In general, one unit of CVF is equal to 2 – 6 µg of CVF.**

Quidel's CVF has been used in a variety of *in vitro* and *in vivo* models to deplete complement. For *in vitro* experiments, 8 – 20 units/ml of serum is adequate to convert nearly all the available C3 to C3 fragments when incubated with neat human serum for 60 – 90 minutes at 37° (data on file at Quidel). This will also convert nearly all the available C5 to C5a and SC5b-9.

Quidel's CVF has also been used successfully in a variety of animal models,<sup>6-8</sup> including mice, rats, guinea pigs, various primates, dogs, pigs and sheep to deplete complement *in vivo*. This application has not been tested or verified at Quidel. For a list of studies, please refer to Quidel's expanded bibliographic references for this product, available upon request from Quidel Technical Service.

### Specifications

Catalog Number: A600  
 Concentration: 1.0 – 1.2 mg/ml  
 Purity: ≥ 95 % by SDS PAGE  
 Volume/Vial: 1.0 ml  
 Activity/Vial: ≥ 350 units  
 Storage: ≤ -70 °C  
 Buffer: Phosphate Buffered Saline (pH 7.2 ± 0.2)

### References

- [1] Fritzing, D.C., Bredehorst, R., Vogel, C-W  
**Molecular cloning and derived primary structure of cobra venom factor.**  
 PNAS 91:26, 12775-12779 (1994).
- [2] O'Keefe, M.C., Caporale, L.H., Vogel, C-W.  
**Comparison of the Alpha Chain Fragments of C30 and C3c and CVF implications for C3 convertase formation.**  
 Complement 4:3-4 (1987).
- [3] Gowda D.C., et al.  
**Modulation of the Effector Functions of a Human IgG1 through Engineering of Its Hinge Region.**  
 J Immunol 177:1129-1138 (2006).
- [4] Van Den Berg, C.W., et al.  
**In vivo anti complementary activities of cobra venom factors from *Naja naja* and *Naja haje*.**  
 J Immunol Meth 12:6,287-294 (1991).
- [5] Bauman, N.  
**Lack of complement C5 convertase generating activity in *Naja haje* cobra venom factor.**  
 J Immunol 120:5, 1763-1764 (1978).
- [6] Till, G.O., et al.  
**Activation of C5 by CVF is required in neutrophil-mediated lung injury in the rat.**  
 Am J Pathol 129:144-53 (1987).
- [7] Rajasinghe, H., et al.  
**Key role of the alternative pathway in hyperacute rejection of rat hearts transplanted into fetal sheep.**  
 Transplantation 62:3, 407-426, 1996.
- [8] Koymada, N., Bach, F.  
**Transient complement inhibition plus T-Cell immunosuppression induces long term graft survival of mouse to rat cardiac xenografts.**  
 Transplantation 66:9, 1210-1215 (1998).



## Specimen Stabilizing Solution

**For Research Use Only. Not for use in Diagnostic Procedures.**

### Background

The Quidel Specimen Stabilizing Solution, when used in accordance with the procedure described below, will retard the generation of complement activation fragments and complexes in human serum and plasma during processing and storage. This product extends the stability of the specimens significantly reducing the likelihood of in-vitro generated elevation of complement fragments. The Quidel Specimen Stabilizing Solution should be used for stabilizing serum and plasma specimens that cannot be tested or stored at or below -70 °C, within four hours after phlebotomy. The Quidel Specimen Stabilizing Solution is intended for use with all assays designed to assess complement activation fragments, specifically the MicroVue™ iC3b, C3a, C4d, Bb Plus and SC5b-9 Plus EIA kits.

### Preparation

Warm the bottle containing the Specimen Stabilizing Solution in a 37 °C water bath to dissolve the contents. Mix thoroughly until the solution is clear. Allow the contents to return to room temperature prior to use.

### Procedure

- Dispense each specimen to be stabilized into convenient, single-use volumes (e.g., 50 µL). To ensure the lowest level of complement activation; this should be performed on ice.
- To each aliquot add an equal volume of stabilizing solution.
- Mix thoroughly.

### Specimen Storage

Stabilized specimens may be stored for up to six days at 4 °C prior to testing or at -20 °C or below for one month. Storage of unstabilized specimens at -20 °C or warmer will result in complement activation.

### Use of Stabilized Specimens

Stabilized specimens should be tested as soon after thawing as possible. As with any complement specimen, thaw at 37 °C. Remove to ice as soon as the specimen has thawed. Do not leave specimens at 37 °C. Since the specimen is already diluted 1:2 in specimen stabilizer, the required assay dilution should be reduced two-fold (e.g., if the required dilution is 1:50, make a 1:25 of the stabilized specimen). Normal ranges should be determined for stabilized specimens independently. In some assays, the concentration of the analyte will differ slightly between stabilized and unstabilized specimens.

### Specifications

Catalog Number: A9576

Volume/Vial: 25 mL

Storage: 2 – 20 °C



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## Normal Human Complement Standard

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### Background

Quidel's human complement standard is a uniform pool of human serum complement that has been characterized for levels and activities of a variety of complement proteins. Unlike lyophilized complement sources, Quidel complement standard maintains high levels of complement hemolytic activity and antigenic integrity. For this reason it is an ideal external standard for a variety of laboratory functional and antigenic assays for specific complement proteins (e.g., CH50, APH50, RID, C3 H50, etc.)

Sera obtained from blood banks and other large pools may not be suitable for many procedures due to high levels of background complement activation. Conversely, in house serum draws are complicated by several factors including the necessity of maintaining standardized pools of complement for ongoing experimentation and the confidentiality requirements with regard to infectious disease testing.

### Characterization and Testing/Analysis and Testing

Quidel's Human Complement Standard has been tested for antigenic levels of **C1q, C2, C3, C4, C5, C6, C7, C8, C9, Factor B, Factor H and Factor P**, by Radial Immuno-Diffusion (**RID**). Functional levels of the complement proteins **C1q, C2, C3, C4, C5, C6, C7, C8, C9 and Factor B and Factor D** have been determined by standard hemolytic assay. Classical pathway hemolytic activity (**CH50**) and alternative pathway hemolytic activity (**AH50**) have also been measured by standard techniques. Levels of the complement fragment **iC3b**, a measure of C3 cleavage, were assessed using the MicroVue™ iC3b EIA kit.

### Storage and Handling

This product should be stored at or below -70 °C. **Storage at temperatures warmer than -70 °C is not recommended.** When needed, it should be thawed rapidly at 37 °C and immediately placed on ice until use. Refer to the Certificate of Analysis for this product for specific handling instructions. Any remaining sera should be aliquotted into polypropylene tubes and refrozen at -70 °C or below. Avoid repeated freeze thaw.

### Applications

This product is intended for use in a research setting as a standard material for complement assays. Protocols for the RID, hemolytic and other assays used in the characterization of this product are available from Quidel Technical Service. **This product should not be used in experiments where measurement of *in vitro* complement activation (e.g. complement biocompatibility) is required. Quidel provides Item A113 expressly for use in these applications.**

### Specifications

Catalog Number: A100  
Volume/Vial: 1.0 ml  
Storage: ≤ -70°C  
Form: Frozen Liquid



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Fax: (408) 616-4310

**CERTIFICATE OF ANALYSIS**

NORMAL HUMAN SERUM COMPLEMENT

Item #: A113                      Lot Number:                     906680                                          Exp Date:                     2018-03                    

<u>Characteristic</u>	<u>RESULT</u>
Volume	5 mL/vial
Test Results:	
iC3b	<u>21.82</u> µg/mL
Bb	<u>2.465</u> µg/mL
C4d	<u>8.142</u> µg/mL
SC5b-9	<u>708.4</u> ng/mL
C3a	<u>798.7</u> ng/mL
C5a	<u>14.99</u> ng/mL
CH50	<u>121.3</u> U Eq/mL

**INFECTIVITY**

All raw materials derived from human origin used in the manufacture of this product have been tested for and found non-reactive for the following:

- Hepatitis B Surface Antigen by a test approved for such use by the FDA.
- Antibody to Human Immunodeficiency Virus by a test approved for such use by the FDA (HIV 1 and HIV 2).
- Hepatitis C Virus by a test approved for such use by the FDA.

Be advised that all blood products should be treated as potentially infectious. Use Universal Precautions. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents.

This is a research product intended for use in Quidel's Complement Fragment Immunoassays. It is not for human or drug use.

**HANDLING INSTRUCTIONS:**

This product should be thawed rapidly with frequent mixing in a water bath at 37°C. Thawing at room temperature or 4°C may result in complement activation, decreased CH50, and elevated levels of complement fragments.

Transfer thawed material immediately to ice to prevent complement activation prior to use. During the thawing procedure, do not leave product at 37°C for longer than is required for thawing, as complement activation may occur. Do not leave product on ice for more than 4 hours, as complement activation may occur.

Repeated freezing and thawing is not recommended. If this product is to be re-frozen for future analysis, Quidel suggests freezing aliquots in polypropylene tubes to prevent complement activation resulting from multiple freeze/thaw cycles.



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CERTIFICATE OF ANALYSIS

COMPLEMENT ACTIVATOR

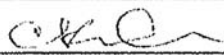
Item #: A114                      Lot Number: 1M0373-5                      Exp Date: 2013-03

<u>TEST</u>	<u>SPECIFICATION</u>	<u>RESULT</u>
Buffer	Phosphate Buffered Saline, pH 7.3 ± 0.3, ≤ 0.2% Sodium Azide, Magnesium Chloride, Calcium Chloride	As Specified
Complement Activation	10 µL of activator will fully activate 1 mL of A113 NHS when incubated at 37°C for 30 minutes according to the C4d EIA (P/N A008) Package Insert.	<b>PASS</b> / FAIL

STORE AT ≤ -70°C  
AVOID REPEATED FREEZE/THAW

FOR RESEARCH USE ONLY

NOT INTENDED FOR INCORPORATION INTO A FINISHED IVD PRODUCT.  
NOT FOR USE AS A COMPARATOR TO A CE MARKED IVD PRODUCT AND/OR TO DETERMINE THE  
SUITABILITY OF PRODUCT PRIOR TO CE MARKING.

Reviewed By: <u></u>	Date: <u>9/5/13</u>
---	---------------------

Example  
only

**CERTIFICATE OF ANALYSIS**

**COBRA VENOM FACTOR**

Item #: A600                      Lot Number:           **905605**                                Exp Date:           2012-04          

<u>TEST</u>	<u>SPECIFICATION</u>	<u>RESULT</u>
Buffer	PBS, pH 7.2 ± 0.2	<b>As Specified</b>
Protein Concentration	1.0 – 1.2 mg/mL	<u>          1.0          </u> mg/mL
Functional Titer*	≥ 350 units /mL	<u>          685          </u> units /mL
Purity	≥ 95% by SDS-PAGE	<b>PASS</b> / FAIL
Phospholipase Activity	None	<b>PASS</b> / FAIL

\* One unit of Cobra Venom Factor is described as the amount of Cobra Venom Factor required to inhibit 50% lysis of 5 x 10<sup>7</sup> EA when first incubated with 500 µL of a 1:40 dilution of normal human serum for 30 minutes at 37°C in a final volume of 1400 µL Gelatin Veronal Buffered Saline (GVB<sup>++</sup>).

**STORE AT ≤ -70°C  
AVOID REPEATED FREEZE / THAW**

**FOR RESEARCH USE ONLY**

**NOT INTENDED FOR INCORPORATION INTO A FINISHED IVD PRODUCT.  
NOT FOR USE AS A COMPARATOR TO A CE-MARKED IVD PRODUCT,  
AND/OR TO DETERMINE THE SUITABILITY OF PRODUCT PRIOR TO CE MARKING**

Reviewed By: <u>          <i>[Signature]</i>          </u>	Date: <u>          5/26/09          </u>
--	--

Example  
only



**CERTIFICATE OF COMPLIANCE**

QUIDEL certifies that the product listed below has been manufactured according to applicable internal standard operating procedures, current U.S. Good Manufacturing Practices (GMPs), FDA Quality System Regulation (QSR) and ISO 9001/ISO 13485.

PRODUCT: Specimen Stabilizing Solution

PART No.: A9576

LOT: 902673

EXPIRATION DATE: 2007-10

This product has been tested against and has passed all established specifications and procedures.

Mary Reinhardt  
Quality Assurance

12-30-05  
Date



Example only

10165 McKellar Court  
San Diego, CA 92121  
Phone: (408) 616-4301 or  
(800) 524-6318 (US only)  
Fax: (408) 616-4310

**CERTIFICATE OF ANALYSIS**

**C3-DEPLETED HUMAN SERA**

Item #: A508      Lot Number: 904980      Exp Date: 2013-05

<u>TEST</u>	<u>SPECIFICATION</u>	<u>RESULT</u>
<b>CLASSICAL PATHWAY ACTIVITY*</b>		
Recommended volume of C3-Dpl serum per assay	≤ 30 µL	<u>6</u> µL
Input of highly purified C3 required to yield one C3H50 unit at the recommended input of C3-Dpl serum	≤ 500 ng	<u>184</u> ng
Input of C3 in NHS Complement Standard required to yield one C3H50 unit at the recommended input of C3-Dpl	≤ 500 ng	<u>160</u> ng
Background O.D. reading at 412 nm of EA blank reaction mixture at the recommended input of C3-Dpl serum	≤ 0.30	<u>0.235</u>
<b>CH50 classical pathway functional activity upon C3 reconstitution**</b>		
CH50 units/mL C3-Dpl serum reconstituted with C3 (1.2 mg C3/mL C3-Dpl)	≥ 60 units/mL	<u>143</u> U/mL
CH50 units/mL NHS Complement Standard	≥ 80 units/mL	<u>167</u> U/mL
Functional Activity Ratio	$\frac{\text{CH50/mL C3-Dpl + C3}}{\text{CH50/mL NHS Std}} \geq 0.60$	<u>0.86</u>



**CERTIFICATE OF ANALYSIS (Cont'd)**

**C3-DEPLETED HUMAN SERA**

Item #: A508

Lot Number: 904980

Exp Date: 2013-05

**ALTERNATIVE PATHWAY (AP) ACTIVITY\*\*\***

APH50 units/mL C3-Dpl reconstituted with C3 (1.3 mg C3/mL C3-Dpl)	≥ 50 APH50 units/mL	<u>200</u>	U/mL
Input of C3-Dpl plus C3 required to yield one APH50	≤ 20 µL	<u>5.0</u>	µL
APH50 units/mL NHS Complement Standard	≥ 50 APH50 units/mL	<u>158</u>	U/mL
Input of NHS Complement Standard required to yield one APH50	≤ 20 µL	<u>6.3</u>	µL
AP Functional Activity Ratio	$\frac{\text{APH50/mL C3-Dpl+C3}}{\text{APH50/mL NHS Std}} \geq 0.60$	<u>&gt; 1.0</u>	

**INFECTIVITY DATA**

All raw materials derived from human origin used in the manufacture of this product have been tested for and found non-reactive for the following:

- Hepatitis B Surface Antigen by a test approved for such use by the FDA.
- Antibody to Human Immunodeficiency Virus by a test approved for such use by the FDA (HIV 1 and HIV 2).
- Hepatitis C Virus by a test approved for such use by the FDA.

Be advised that all blood products should be treated as potentially infectious. Use Universal Precautions. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents.

\* One C3H50 unit measured by classical pathway activation is defined as the amount of C3 required to yield 50% lysis of  $3 \times 10^7$  EA when incubated in the presence of the recommended volume of C3-Dpl serum for 30 minutes at 37°C in a total reaction volume of 500 µL GVB\*\*.

\*\* One CH50 unit of whole classical pathway complement functional activity is defined as the input of C3-Dpl, reconstituted with C3, or NHS Complement Standard yielding 50% lysis of  $1 \times 10^8$  EA when incubated at 37°C for 60 minutes with frequent mixing in a total reaction volume of 1.5 mL GVB\*\*.

\*\*\* One unit of whole alternative pathway activity (APH50) is defined as the input of C3-Dpl reconstituted with purified C3 or NHS Complement Standard yielding 50% lysis of  $1.5 \times 10^7$  rabbit erythrocytes (Er) when incubated for 30 minutes at 37°C in a total reaction volume of 75 µL GVB\* containing a final Mg-EGTA concentration of 13.3 mM.

**STORE AT ≤ -70°C  
AVOID REPEATED THAW/FREEZE**

**FOR RESEARCH USE ONLY**

**NOT INTENDED FOR INCORPORATION INTO A FINISHED IVD PRODUCT.  
NOT FOR USE AS A COMPARATOR TO A CE MARKED IVD PRODUCT AND/OR TO DETERMINE THE  
SUITABILITY OF PRODUCT PRIOR TO CE MARKING.**

Reviewed By: <u>Jessica Morales</u>	Date: <u>6/23/08</u>
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only

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**CERTIFICATE OF ANALYSIS**

**NORMAL HUMAN SERUM COMPLEMENT STANDARD**

Item #: A100                      Lot Number:           **903791**                                Exp Date:           2015-01          

<u>TEST</u>	<u>ACCEPTABLE RANGE</u>	<u>TEST RESULT</u>
<b>Component Antigen Concentration</b>	<b>(µg/mL)</b>	
C1q	51 – 125	<u>84.6</u>
C2	22 – 45	<u>24.8</u>
C3	800 - 1650	<u>1050</u>
C4	130 – 450	<u>210</u>
C5	38 – 113	<u>81.5</u>
C6	28 – 72	<u>61.1</u>
C7	24 – 90	<u>69.6</u>
C8	43 – 106	<u>55.1</u>
C9	33 – 95	<u>77.9</u>
Factor B	147 – 350	<u>293.6</u>
Factor H	160 – 412	<u>295.1</u>
Factor P	13 - 68	<u>40.8</u>
iC3b Fragment	0 – 100	<u>29.3</u>
<b>Component Functional Activity</b>	<b>(Units/mL)</b>	
CH50 (Total classical pathway activity by hemolytic assay)	> 75	<u>249</u>
APH50 (Alternative pathway activity by hemolytic assay)	> 80	<u>105</u>
C1qH50	>2000	<u>2256</u>
C2H50	> 5000	<u>30,450</u>
C3H50	> 2500	<u>31,067</u>
C4H50	> 280,000	<u>1,390,901</u>
C5H50	> 7000	<u>22,660</u>
C6H50	> 9000	<u>15,274</u>
C7H50	> 7000	<u>21,045</u>

Component Functional Activity	(Units/mL)	
C8H50	> 9000	32,617
C9H50	> 9000	19,241
Factor BH50	> 180	230
Factor DH50	> 100	174

**INFECTIVITY DATA**

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- Antibody to Human Immunodeficiency Virus by a test approved for such use by the FDA (HIV 1 and HIV 2).
- Hepatitis C Virus by a test approved for such use by the FDA.

Be advised that all blood products should be treated as potentially infectious. Use Universal Precautions. No known test method can offer complete assurance that products derived from human blood will not transmit infectious agents.

**Recommended rapid thawing procedure:**


A slow thaw cycle has been shown to activate complement in serum. For this reason we recommend a rapid thawing procedure. First, remove the product from -70°C storage and place in a 37°C water bath. Remove the vial frequently from the water bath and vortex until approximately 90% of the ice in the liquid is melted. Next, immediately transfer the vial to crushed ice, and continue vortexing until all the frozen material is thawed. The product should be kept on crushed ice until used.

**STORE AT ≤ -70°C**

**AVOID REPEATED FREEZE/THAW**

**FOR RESEARCH USE ONLY**

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NOT FOR USE AS A COMPARATOR TO A CE MARKED IVD PRODUCT AND/OR TO DETERMINE THE  
SUITABILITY OF PRODUCT PRIOR TO CE MARKING.**

Reviewed By: 	Date: 5/29/07
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[www.quidel.com](http://www.quidel.com)

**TECOmedical AG**  
Headquarters **TECOmedical Group**

Gewerbestrasse 10  
4450 Sissach  
Switzerland

phone +41 (0) 61 985 81 00  
fax +41 (0) 61 985 81 09

mail [info@tecomedical.com](mailto:info@tecomedical.com)  
web [www.tecomedical.com](http://www.tecomedical.com)



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• EN ISO 9001  
• EN ISO 13485

### Germany

**TECOmedical GmbH**

Wasserbreite  
32257 Bünde  
Germany

phone +49 (0) 52 23 985 99 99  
fax +49 (0) 52 23 985 99 98

mail [info@tecomedical.com](mailto:info@tecomedical.com)  
web [www.tecomedical.com](http://www.tecomedical.com)

### France

**TECOmedical SARL**

20 rue du Bois Chaland  
91090 Lisses  
France

phone 0800 100 437  
fax 0800 100 480

mail [chdu@tecomedical.com](mailto:chdu@tecomedical.com)  
web [www.tecomedical.com](http://www.tecomedical.com)

### Benelux

**TECOmedical NL**

't Hazeveld 34  
3862 XB Nijkerk  
The Netherlands

phone +31 (0) 33 4951 473  
fax +31 (0) 33 4951 635

mail [sbk@tecomedical.com](mailto:sbk@tecomedical.com)  
web [www.tecomedical.com](http://www.tecomedical.com)