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## **Complement Convertase Assay**

### **Kit Insert**

## **Summary**

Measurement of complement convertase is a sensitive and specific method to determine complement activation by biomaterials. During incubation with plasma, complement factors can bind to the surface of the material followed by the formation of a complement convertase complex. After washing, to remove unbound complement proteins, the biomaterials are incubated in a medium with a specific chromogenic substrate. Cleavage of the substrate is quantified by measuring the optical density at 405 nm. Positive and negative controls are included in the kit.

This method can be used to evaluate the biocompatibility of biomaterials and medical devices according to the international standard ISO 10993/Part 4.

## **Introduction**

Medical devices that come in contact with blood may activate the natural host defense mechanism of blood by a foreign-body reaction. One of the potential effects is activation of the complement system via the alternative pathway. In view of the wide-reaching biologic effects of the complement system, the consequences of uncontrolled complement activation could be devastating. Continued activation of the complement system attracts leukocytes that release lysosomal enzymes and oxygen radicals, which in turn, lead to necrosis of normal tissue.

Normally, tight controls are in effect which regulate the complement system to protect host tissue. The cascade is intrinsically moderated by the instability of the enzymes (convertases) formed. Once a component is activated, failure to rapidly combine with its substrate causes it to decay. However, during the use of medical devices these regulatory mechanisms often appear inadequate due to the foreign nature of the surface of these devices. Therefore, testing of complement convertase activity by materials used for the construction of medical devices is needed to ensure the use of materials with as low complement activation as possible.

This method was developed for in vitro testing of complement activation by materials, based on the formation of convertases on the material surface and chromogenic substrate conversion by the convertase (Complement Convertase Assay, or CCA). Since C5 convertase is biologically most relevant, a substrate with an amino-acid sequence similar to the C5a cleavage site is incorporated.

## Principle of the Test

A test material of interest (ideally the surface area should be known) is incubated with plasma. During incubation, complement factors can bind to the surface of the material followed by the formation of a complement convertase complex. After incubation, samples are washed and analyzed for complement convertase activity with a complement convertase-specific chromogenic substrate. The rate of color development depends on how much convertase has been generated on the surface of the biomaterial.

## Precautions

- The kit is intended for research use only.
- The kit should not be used beyond its expiration date.
- Do not combine reagents from CCA Kits with different lot nos.
- Plasma should always be treated as a potential biohazard during use and for disposal.
- Chemicals and reagents have to be treated as hazardous waste according to biohazard safety guidelines or regulations. For information on hazardous substances included in the kit please refer to the Material Safety Data Sheets, which are available upon request.
- Wear disposable (latex) gloves when handling specimens and reagents.
- Never pipette by mouth and avoid contact of skin and mucous membranes with reagents containing sodium azide and DMSO.
- Use disposable pipette tips throughout the procedure to avoid contamination of reagents.

## Contents of the Kit

• CCA Plasma	20 mL	2 bottles
• Wash Buffer 10x, Borate-NaCl solution, 0.01% sodium azide	32 mL	1 bottle
• Substrate, Convertase-specific chromogenic substrate in DMSO	580 $\mu$ L	2 vials
• Coloring Medium, Tris-Borate solution, 0.01% sodium azide	32 mL	1 bottle
• Reference 1, Low-density polyethylene (LDPE)	1 cm <sup>2</sup>	5 pcs
• Reference 2, Polydimethylsiloxane (PDMS)	1.4 cm <sup>2</sup>	5 pcs
• Reference 3, Medical steel (MS)	1.2 cm <sup>2</sup>	5 pcs

## **Additional Materials and Equipment**

The following materials and equipment are required but are not provided with the kit:

- (Calibrated) adjustable pipettes with disposable tips
- Incubator at 37 °C
- Timer
- 96-well plate
- Plate reader capable of measuring at 405 nm wavelength
- Tweezers
- Mixer
- Micro-centrifuge vials (0.5 mL and 1.5 mL)
- NaCl, 0.9%
- Optional, for cleaning of test samples: RBS (chlorinated trisodium phosphate, sodium metasilicate) or a comparable detergent.
- Sonicator
- Ultra pure water
- Ethanol (70%)

## **Test Procedure**

### Preparation of Materials for Testing

The CCA test can be used for coated or uncoated biomaterials. It is recommended that clean samples be tested. The following procedure is recommended to clean biomaterials:

1. Sonicate the biomaterial for 15 min in 2% RBS and wash three times with ultra pure water.
2. Sonicate the biomaterial for 5 min in 70% ethanol and wash three times with ultra pure water.
3. Dry the material in the air.

### **Notes:**

- Certain materials might be affected by RBS, sonication and/or ethanol, therefore this cleaning procedure is a recommendation only; each user should determine their own optimal procedure.
- Cleaned materials should always be handled with tweezers.
- The reference materials provided in the kit are clean and ready for use.

### Reagent Preparation

Determine the volumes of reagents and number of multi-well strips required.

Prepare reagents as follows:

- **CCA Plasma:** Defrost a vial with CCA plasma at 37 °C and store at room temperature until use. Thawed plasma must be used within 4 hours and cannot be refrozen. Do not unnecessarily expose the plasma to 37 °C at this point.
- **Wash buffer:** Dilute the concentrated Wash buffer 10x with ultra pure water. Diluted wash buffer can be stored for 48 h at 2-8 °C. After thawing Wash buffer 10x can be refrozen for later use.
- **Substrate solution:** Add 14 mL of Coloring medium to a vial of substrate. Prepare freshly prior to use; this reagent cannot be stored beyond 1 day. Store at room temperature until use.

## **Assay Procedure**

### **Notes in Advance:**

- Never use pipettes or vials of glass since glass is a material that may substantially activate the plasma.
- Select 2 or 3 types of reference materials to which the activities of the test materials can be compared.
- The reference materials provided in the kit have been cleaned by the protocol described above and are ready for use.
- The volumes given in this procedure are based on pieces of material of 1.0 x 0.5 cm (two-sided surface area of approximately 1 cm<sup>2</sup>). Test materials should be prepared in pieces of approximately the same size.

1. Place the selected reference and (cleaned) test materials in 1.5 mL micro-centrifuge vials using tweezers. Fix the materials vertically between the walls of the vials to prevent floating.
2. Place Reference 1 in an identical vial for the blank.
3. Add 650 µL CCA plasma to each vial with test or reference material. Ensure that the biomaterial is completely immersed in the plasma.
4. Add 650 µL 0.9% NaCl to the blank(s).
5. Incubate the vial(s) for 15 min at room temperature.
6. During this incubation, prepare an identical number of 1.5 mL micro-centrifuge vials, each containing 1.5 mL wash buffer.
7. Add, after the 15 min incubation, 1 mL wash buffer to the vials with specimens. Remove the specimens with a pair of tweezers, briefly blot dry with a piece of tissue or filter paper and transfer to vials with 1.5 mL of wash buffer. Do not mix or vortex.
8. Remove the specimens as previously and transfer to 0.5 mL vials. Add 0.5 mL substrate solution to each vial.
9. Incubate up to 24 h at room temperature. The rate of color development depends on how much convertase has been

generated on the surface of the biomaterial and is linear up to OD405 nm = 0.75.

10. Transfer 225  $\mu\text{L}$  of each tube to a 96-well plate. As a template, Table 1 can be used.

11. Read the optical density within 30 min at 405 nm (OD405).

## Calculations

1. Correct the determined OD405 values for the mean blank result (BLK).
2. Calculate the CCA result of the test and reference materials in OD405/24 h/cm<sup>2</sup>.

## Assay Criteria

The result of the blank (BLK) should be OD405  $\leq$  0.075.

All measured OD405 values should be  $\leq$  0.75.

See the 'Certificate of Analysis' for recommended target ranges for the reference materials.

Table 1. Suggested 96-well template for the CCA assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK	BLK	SPL 5	SPL 5	SPL 13	SPL 13						
B	CTRL 1	CTRL 1	SPL 6	SPL 6	SPL 14	SPL 14						
C	CTRL 2	CTRL 2	SPL 7	SPL 7	SPL 15	SPL 15						
D	CTRL 3	CTRL 3	SPL 8	SPL 8	SPL 16	SPL 16						
E	SPL 1	SPL 1	SPL 9	SPL 9	SPL 17	SPL 17						
F	SPL 2	SPL 2	SPL 10	SPL 10	SPL 18	SPL 18						
G	SPL 3	SPL 3	SPL 11	SPL 11	SPL 19	SPL 19						
H	SPL 4	SPL 4	SPL 12	SPL 12	SPL 20	SPL 20						

## Characteristics

CCA results are classified as follows:

INACTIVE:	OD405/24 h/cm <sup>2</sup>	$\leq$ 0.06
LOW:	0.06 < OD405/24 h/cm <sup>2</sup>	$\leq$ 0.20
MEDIUM:	0.20 < OD405/24 h/cm <sup>2</sup>	$\leq$ 0.60
HIGH:	OD405/24 h/cm <sup>2</sup>	> 0.40