Detection of C5aR inhibitory activity using *iLite*® C5a Assay Ready Cells

For research and professional use only. Not for use in diagnostic procedures.

This application note contains a suggested protocol and performance data.
Each individual laboratory must set up their own method and perform relevant validations.

Background

Complement component 5a (C5a) is a 74 amino acid small protein fragment of complement protein 5 (C5). C5 is cleaved to C5a and C5b by C5 convertase enzymes as a result of complement activation. But C5 can also be cleaved by factors of the coagulation and fibrinolytic pathways. (1) C5a is a highly potent anaphylatoxin and chemoattracting peptide, with the ability to increase blood vessel permeability, stimulate cytokine release from myeloid cells, and expression of adhesion molecules on endothelial cells. (2)
Main pro-inflammatory effector functions are induced by C5a binding to the G protein-coupled seven transmembrane-containing receptor, C5aR1 (CD88). Rapidly after cleavage of C5 into C5a and C5b, C5a is metabolized by carboxypeptidases which removes the C-terminal arginine and forms C5a-desArg, a less potent ligand of C5aR1. C5a can also bind to a second receptor, C5aR2 (C5L2 or GPR77), however the biological effects associated with C5a binding C5aR2 are less well understood, both anti-inflammatory and pro-inflammatory effects have been described in literature. The C5a receptors are expressed in a wide range of cells and tissues, and the effect of C5a activation is dependent on the location of the C5a receptors. (3, 4)
While the complement system is an important part of the innate immune defense against pathogens, research has shown that excessive complement activation including C5a-C5aR interaction plays a central role in several autoimmune and neurodegenerative disorders as well as in acute and chronic inflammatory conditions. Given the strong evidence of a pathogenic role for C5a-C5aR in many conditions both ligand and receptor are promising therapeutic targets. Clinical studies of pharmacologic candidates inhibiting C5a and C5aR1 are ongoing. (2,3,5)

The *iLite*® platform offers a cell-based assay that enables the studies of C5a, receptor C5aR1 and their interaction.

Principle of the assay

The *iLite*® C5a Assay Ready Cells are engineered cells, optimized to express Firefly luciferase (FL) under the control of a C5a responsive promoter and exhibits a dose-dependent increase in firefly luciferase (FL) reporter gene activity following treatment of cells with increasing concentrations of human C5a. The cells also contain the Renilla luciferase (RL) internal standardization gene under the control of a constitutive promoter that renders assay results independent of cell number and provides a means for correcting for cytotoxic effects that may be encountered with some biological samples. Some increase in RL reporter-gene activity may be observed, however, at high non-physiologic concentrations of C5a and consequently normalization of C5a-induced FL activity relative to the expression of RL activity is not recommended for routine use. Normalization of C5a-induced FL activity relative to the expression of RL activity remains, however, a valuable means of correcting for sample cytotoxicity.
The luciferase signals can be measured in a luminometer following addition and incubation of luciferase substrate.

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The Firefly luciferase signal is proportional to the functional activity of C5a in the sample. In the presence of inhibitory activity against C5aR, the functional activity of the present C5a is reduced, resulting in a decreased stimulation of Firefly luciferase production. Thus, the Firefly luciferase signal is inversely proportional to the amount of inhibitory activity against C5aR in a sample (fig 1). The *iLite*® C5a Assay Ready Cells can therefore be utilized as an assay for detection of C5aR inhibitor activity in test samples, including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® C5a Assay Ready Cells	Svar Life Science	BM4075
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
C5aR antagonist W-54011	Tocris Bioscience	5455
C5a or analogues	R&D Systems	2037-C5
Firefly/Renilla luciferase substrate	Promega	E2920, Dual-Glo Luciferase Assay System
Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Svar Life Science for list of recommended suppliers	NA
Incubator, 37 °C with 5% CO_2	NA	NA
Water bath, 37 °C	NA	NA
Single-channel and multi-channel pipettes with polypropylene disposable tips	NA	NA
Polypropylene tubes or plate for dilution	NA	NA
Single-use polypropylene reservoir	NA	NA
Plate shaker	NA	NA
Timer	NA	NA

Protocol

Preparation of C5a receptor inhibitor

The C5aR antagonist W-54011 from Tocris Bioscience has successfully been used to neutralize the functional effect of C5a induced Firefly luciferase expression in *iLite*® C5a Assay Ready Cells (refer to the table and graph at page 3)

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Perform a serial dilution of the reference C5aR inhibitor. Ensure matrix consistency between reference solutions, control solutions, and sample solutions.
3. Add **20 µL** of the reference C5aR inhibitor dilutions, controls, and samples to assigned wells (final concentration will be a quarter of solution concentration).
4. Thaw the vial of *iLite*® C5a Assay Ready Cells in a 37 °C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with pipette to ensure a homogeneous distribution of cells.
5. Dilute 250 µL cell suspension with 5.75 mL Diluent.
6. Add **40 µL** diluted cells to each well.
7. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
8. Add **20 µL** of 16 ng/ml C5a to all wells (final concentration will be 4 ng/mL C5a).

9. Place the lid on the plate, mix and incubate for 5 hours at 37 °C with 5% CO_2.

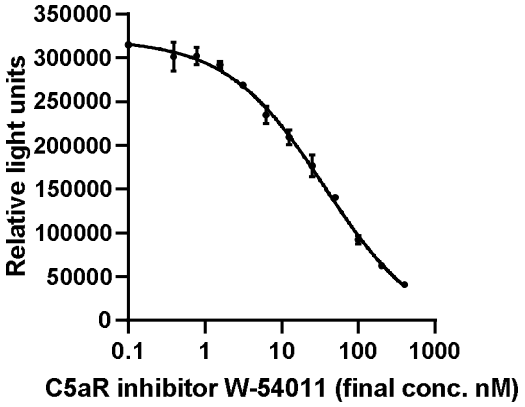

Figure 1. Example of C5aR inhibitory curve

Final 4 ng/mL C5a	W-54011 Suggested calibrator solution concentrations, nM
1	1600
2	800
3	400
4	200
5	100
6	50
7	25
8	13
9	6.3
10	3.1
11	1.6
12	0

Table 1. Suggested calibrator solution concentrations for C5aR inhibitor

Adding substrate solutions

10. Equilibrate the plate and the substrate solution to room temperature.
11. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.
12. If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer.

Precautions

- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the supplier's/manufacturer's instructions or product specifications accompanying the individual material and instruments.
- Dispose of all sample specimens, infected or potentially infected material in accordance with good microbiological practice. All such materials should be handled and disposed as though potentially infectious.
- Residues of chemicals and preparations are generally considered as biohazardous waste and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

Propriety Information

In accepting delivery of *iLite*® Assay Ready Cells the recipient agrees not to sub-culture these cells, attempt to sub-culture them or to give them to a third-party recipient, and only to use them directly in assays. *iLite*® cell-based products are covered by patents which are the property of Svar Life Science AB and any attempt to reproduce the delivered *iLite*® Assay Ready Cells is an infringement of these patents.

QUICK GUIDE

Detection of C5aR inhibitory activity using *iLite*® C5a Assay Ready Cells

1 Sample dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute reference C5aR inhibitor, controls and samples.
- Add **20 µL** of ref. C5a inhibitor solutions, controls and diluted samples to pre-assigned wells.
- Thaw the cell vial in a 37 °C water bath. Mix cell suspension with a pipette to ensure a uniform cell suspension. Dilute the cells
- Add **40 µL** diluted cells to each well.

2 Incubation 30 min

- Incubate at 37 °C with 5% CO_2 for 30 min.

3 Add C5a

- Add **20 µL** C5a to each well.

4 Incubation 5 h

- Incubate at 37 °C with 5% CO_2 for 5 hours.

5 Read plate

- Equilibrate the plate to room temperature
- Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.
- If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. After 10 min incubation read in a luminometer.

Troubleshooting and FAQ

Please consult the Svar Life Science website www.svarlifescience.com

References

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