Quantification of inhibitor activity of complement-dependent cytotoxicity using *iLite*® CD20 (+) Svar Luc 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-dependent cytotoxicity (CDC) is an important mechanism for clearance of antibody target cells. It is initiated when the complement factor C1q is bound to the Fc-domain of target-bound antibodies (1). This results in C1q complex that triggers the classical complement cascade and activates C3 and C5 convertase. This leads to formation of the membrane attack complex (MAC), which is destructing the cell membrane with pores resulting in lysis of the target cell (2,3).
CDC has an important role in the human immune response by eliminating pathogens, including bacteria, extracellular organisms, and tumor cells that would otherwise promote illness. Furthermore, CDC is a mechanism where therapeutic antibodies for cancer, targeting for example CD20 or CD38, may mediate their effect. Therefore, studying CDC activity can be a useful for investigating potential therapeutic antibodies that have anti-cancer effects (4,5).
We have developed reporter cells, named *iLite*® CD20 (+) Svar Luc Assay Ready Cells which offers a cell-based assay where potential inhibitors to the complement system can be investigated.

Principle of the assay

The *iLite*® CD20 (+) Svar Luc Assay Ready Cells are cells engineered for constitutive expression of Svar luciferase. The luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Svar luciferase signal is proportional to complement-dependent cytotoxicity in the sample, in this application note, triggered by the anti-CD20 antibody Rituximab. In the presence of inhibitory activity towards complement components, the induction of the complement-dependent cytotoxicity is diminished, resulting in a decreased release of Svar luciferase. Thus, the Svar luciferase signal is inversely proportional to the inhibitory activity towards complement components in a sample.

Specimen collection

The *iLite*® CD20 (+) Svar Luc Assay Ready Cells can be used for detection of inhibitory activity towards complement components in test samples containing human serum.

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Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® CD20 (+) Svar Luc Assay Ready Cells	Svar Life Science	BM5028
Diluent (RPMI 1640 containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin)	Gibco	61870 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Eculizumab (Anti-C5 antibody)	Evidentic	NA
Complement preserved human serum	NA	NA
Rituximab or analogues	Roche	NA
Svar luciferase substrate	Invivogen	Rep-qlc4lg, Quanti-luc 4 Lucia/Gaussia
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
Timer	NA	NA

Protocol

Preparation of C5 inhibitor Eculizumab calibrator

Anti-C5 antibody Eculizumab from Evidentic has successfully been used to neutralize CDC activity and inhibit the Svar luciferase release in *iLite*® CD20 (+) Svar Luc Assay Ready Cells (refer to the table and graph below).

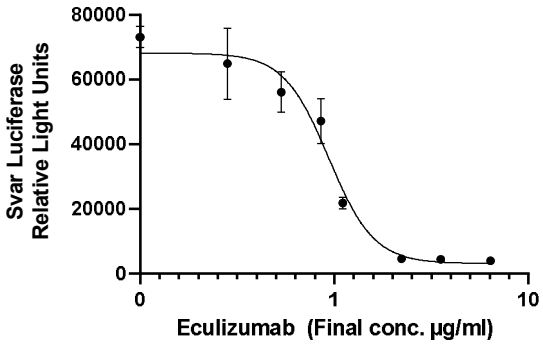

Figure 1. Example of C5-inhibitory curve.

Final 0.18 µg/mL Rituximab	Eculizumab (Anti-C5 Ab) Calibrator **solution conc.** (µg/ml)
A	70
B	39
C	24
D	12
E	9.4
F	5.9
G	3.1
H	0

Table 1. Suggested calibrator **solution concentrations** for Eculizumab.

Dilution of cells and pre-incubation with Rituximab

1. Design a plate layout. It is recommended to perform the test at least in duplicates.
2. Prepare 0.5 µg/ml Rituximab solution.
3. Thaw the vial *iLite*® CD20 (+) Svar Luc Assay Ready Cells in a 37°C water bath with gentle agitation for 1 minute. The cell suspension is mixed **very carefully** ten times with pipette to ensure a homogeneous distribution of cells.
4. Dilute 250 µL cell suspension with 2.75 mL diluent.
5. Add 20 µl of 0.5 µg/ml Rituximab solution to all wells (final concentration will be 0.18 µg/mL Rituximab).
6. Add 20 µL diluted cells to each wells. **Critical step: when the cell suspension is added into the rituximab solution in the wells, no mixing is needed. DO NOT USE plate-shaker.**
7. Place a lid on the plate and incubate for 30 minutes at 37 °C with 5% CO_2.

Preparation of Eculizumab calibrator

8. Perform a serial dilution of Eculizumab standard curve using 1:2 diluted serum solution as diluent.
9. Incubate the Eculizumab solutions for 30 minutes at 37°C and 5% CO_2.

Incubation of cell solutions with complement

10. Add 5 µl of the Eculizumab solutions to the wells containing cells and Rituximab (final concentration will be 1/11 of the solution concentrations in final 4.5% human serum). **Critical step: when the Eculizumab solution is added into the cell solution in the wells, no mixing is needed. DO NOT USE plate-shaker.**
11. Add 10 µl diluent to each well to a total volume of 55 µl. **Critical step: when the diluent is added into the cell solution in the wells, no mixing is needed. DO NOT USE plate-shaker.**
12. Place a lid on the plate and incubate for 5h at 37°C and 5% CO_2.

Adding substrate solutions

13. Equilibrate the plate and the substrate solution to room temperature.
14. Prepare the Svar luciferase substrate according to the manufacturer's instructions and add 75 µL per well. Mix by gentle pipetting and protect the plate from light. After 10 minutes incubation at room temperature read in a luminometer with read time 0.1 seconds. **Critical step: Mix gently to avoid damage to cells. DO NOT USE plate-shaker.**

Considerations should be taken into account

- **When handling of cells** – It is important to handle these cells with care since the constitutive expression of Svar luciferase causes increased assay background if handling induced cell rupture occurs. To avoid cell damage, pipette gently and sparsely, avoid creating bubbles, and do not use a plate shaker. Keep the cells on dry ice until antibody solutions have been plated.

- **When handling of serum samples** – To ensure preservation of complement activity in the serum, keep the serum samples on ice until use. Do not thaw serum using water warmer than 37°C and do not use repeatedly freeze-thawed serum.

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

Quantification of inhibitor activity of complement-dependent cytotoxicity using *iLite*® CD20 (+) Svar Luc Assay Ready Cells

1 Sample dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells at this stage**
- Prepare Rituximab spike sloution.
- Prepare serial dilution of Eculizumab in 1:2 diluted serum solution.
- Thaw the cell vial in a 37 °C water bath. Mix the cell suspension with a pipette to ensure a homogeneous cell solution. Dilute the cells.
- Add 20 µL Rituximab solution in duplicate to duplicate to assigned wells.
- Add 20 µL of diluted cells to assigned wells.

2 Incubation 30 min

- Incubate plate containing cells and Rituximab plus Eculizumab solutions at 37 °C with 5% CO_2 for 30 min.

3 Add ition of Eculizumab

- Add 5 µL of Eculizumab solutions to the wells with cells and Rituximab
- Add 10 µL diluent 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 Svar luciferase substrate and add 75 µL per well. Mix by gentle pippeting. Protect the plate from light.
- After 10 min incubation read in a luminometer with 0.1 second reading time.

Troubleshooting and FAQ

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

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