

Quantification of anti-CD20 ADCC activity using *iLite*[®] ADCC 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

Antibody-dependent cell-mediated cytotoxicity (ADCC) is a mechanism whereby pathogenic cells are lysed by lymphocytes, most often Natural Killer (NK) cells. The mechanism involves binding of antibodies to surface antigens on the pathogen. Crosslinking of these antibodies to NK cells through the binding of the Fc-region to Fc receptor on the NK cells leads to activation of the NK cell and formation of an immune synapse with the pathogenic cell. The NK cell releases cytotoxic granules containing granzymes and perforin into the synapse, inducing apoptosis of the targeted cell (1).

The idea of employing ADCC to destroy dysfunctional cells by treating patients with antibodies has existed as long as the discovery of the ADCC mechanism. Rituximab, one of the first of such drugs approved, is a chimeric monoclonal antibody targeting CD20, a surface antigen primarily found on B-cells. The drug was first approved by the FDA in 1997 for treatment of chemotherapy resistant Non-Hodgkin B-cell lymphomas and today it is approved for several B-cell malignancies, inflammatory and autoimmune indications (2-4). ADCC has been shown as one of the mechanisms of therapeutic action of rituximab (5).

With the expiry of the patent for rituximab, several anti-CD20 biosimilars have entered the market and the clinic. By definition, a biosimilar shall demonstrate a similar nature compared to the reference product in terms of efficacy, safety, therapeutic mode of action, immunogenicity, pharmacokinetics and pharmacodynamics. In addition, other anti-CD20 monoclonal antibodies, with an epitope that either partially overlaps or an epitope that is distinct from that of rituximab have entered clinical use, such as ocrelizumab, a humanized (90-95%) antibody, and ofatumumab, a fully human monoclonal. Efforts are being made to enhance the ADCC activity of the antibodies by engineering the glycan patterns of the constant region, such the 3rd generation anti-CD20 monoclonal obinutuzumab (6).

iLite[®] ADCC Assay Ready Cells offers a technology platform to study the ADCC mechanism of a drug and to compare the ADCC mechanism of a biosimilar with the reference product.

Principle of the assay

The *iLite*[®] ADCC Assay Ready Cells are engineered cells that enable antibody-dependent cell-mediated cytotoxicity (ADCC) to be examined through the specific expression of Firefly luciferase. When the antibodies of interest bind to the antigens on the surface of the target cell, the target-bound antibodies will be presented to the Fc receptors (FcγRIIIa) on the effector cell. When the Fc-portion of the target-bound antibodies binds to the receptor, multiple cross-linking of the two cell types occurs. This will initiate a signaling cascade which triggers the expression of Firefly luciferase (FL) in the effector cell. In this application note, we describe the use of an effector cell line (*iLite*[®] ADCC Effector (V) Assay Ready Cells) that over-express FcγRIIIa and contain the FL reporter gene that responds to the principal transcription factors that mediate signaling from the FcγRIIIa receptor, together with a positive target cell line which over-expresses the surface antigen CD20 (*iLite*[®] CD20 (+) Target Assay Ready Cells).

iLite[®] ADCC Effector (V) Assay Ready Cells also contain the Renilla Luciferase (RL) reporter gene, under the control of a constitutive promoter, that allows drug-induced FL activity to be normalized with respect to the constitutive expression of RL.

This render assay results independent of variations in cell number, serum matrix effects, or lysis of the effector cells by the target cells. In addition, we also describe the use of a negative control in the form of a target cell line depleted of CD20 expression (*iLite*[®] CD20 (-) Target Assay Ready Cells). The Firefly luciferase signal can be measured in a luminometer following addition and incubation of luciferase substrate. The Firefly luciferase signal is proportional to the functional activity of Rituximab in the sample (Fig.1).

Material and equipment needed

Material and equipment	Suggested supplier	Reference
<i>iLite</i> [®] ADCC Effector (V) Assay Ready Cells	Svar Life Science	BM5001
<i>iLite</i> [®] CD20 (+) Target Assay Ready Cells	Svar Life Science	BM5010
<i>iLite</i> [®] CD20 (-) Target Assay Ready Cells	Svar Life Science	BM5015
Diluent (RPMI 1640 + 9% heat inactivated FBS + 1% Penicillin Streptomycin)	Gibco	61870 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Rituximab or analogues	Roche	NA
Firefly/Renilla luciferase substrate	Promega	E2940, Dual-Glo [®] Luciferase Assay System
Plate; White walled micro well plate suitable for luminescence	Revvity	6055680
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

Preparation of calibrators (Rituximab)

The ADCC effect of the Rituximab antibody from Roche has successfully been measured in combination with a mix of ADCC Effector (V) Assay Ready Cells and CD20 (+) Target Assay Ready Cells. As a negative control, a combination of ADCC Effector (V) Assay Ready Cells and CD20 (-) Target Assay Ready Cells was used. In the present assay an Effector: Target ratio of 3:1 has been used. The optimal ratio is dependent on the antibody and target cells used and should be determined each time a new assay is set up. The table below shows recommended dilutions of Rituximab when making an 8-point calibration curve.

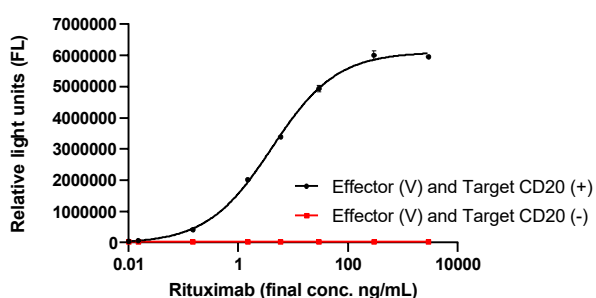


Figure 1. Example of Rituximab calibration curve using Firefly Luciferase substrate Dual-Glo® Luciferase Reagent. Values are shown as mean of triplicate \pm SD and values on x-axis are given as **final concentration** in the wells before addition of Dual-Glo® Luciferase Reagent.

Calibrator	Rituximab
	Calibrator solution conc. (ng/mL)
1	6 000
2	600
3	60
4	12
5	3.0
6	0.30
7	0.030
8	0

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

Protocol

Assay preparation and incubation

1. Design a plate layout.
2. Dilute calibrators, controls and samples to fall within the expected **in assay values (= final concentration)** of 0-3000 ng/mL.
3. Add 40 μ L calibrators, controls and samples in duplicate to assigned wells.
4. Thaw the vial of ADCC Effector (V) Assay Ready Cells and the vials of CD20 (+) Target Assay Ready Cells and CD20 (-) Target Assay Ready Cells in a 37°C water bath with gentle agitation.
5. Mix the cell suspensions very carefully **at least 10 times with a pipette** in order to ensure a homogeneous distribution of cells.
6. Dilute 200 μ L of the ADCC Effector (V) Assay Ready Cells and 200 μ L of the CD20 (+) Target Assay Ready Cells with 3.44 mL Diluent. The total volume of the diluted ADCC Effector (V) /Target CD20 (+) Assay Ready Cells mixture is 3.84 mL.
7. In a separate tube, dilute 50 μ L of the ADCC Effector (V) Assay Ready Cells with 50 μ L of the CD20 (-) Target Assay Ready Cells with 860 μ L Diluent. The total volume of the diluted ADCC Effector (V)/Target CD20 (-) Assay Ready Cells mixture is 960 μ L.
8. Add 40 μ L of the diluted cells to each well to be tested.
9. Place the lid on the plate and mix on a plate shaker at **minimum of 750 rpm** for 10 sec. Alternatively, mix the cell suspensions very carefully in the wells by pipette. Insufficient mixing can cause reduced assay sensitivity.
10. Incubate for 4 hours at 37°C with 5% CO₂.

Adding substrate solutions

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

Normalization

The reporter gene used for normalization, Renilla luciferase, is under the control of a tyrosine kinase promoter, and is thus constitutively expressed. Unspecific effects such as serum matrix effects or differences in cell number can be obviated by relating the specific Firefly signal with the Renilla normalization signal through simple division.

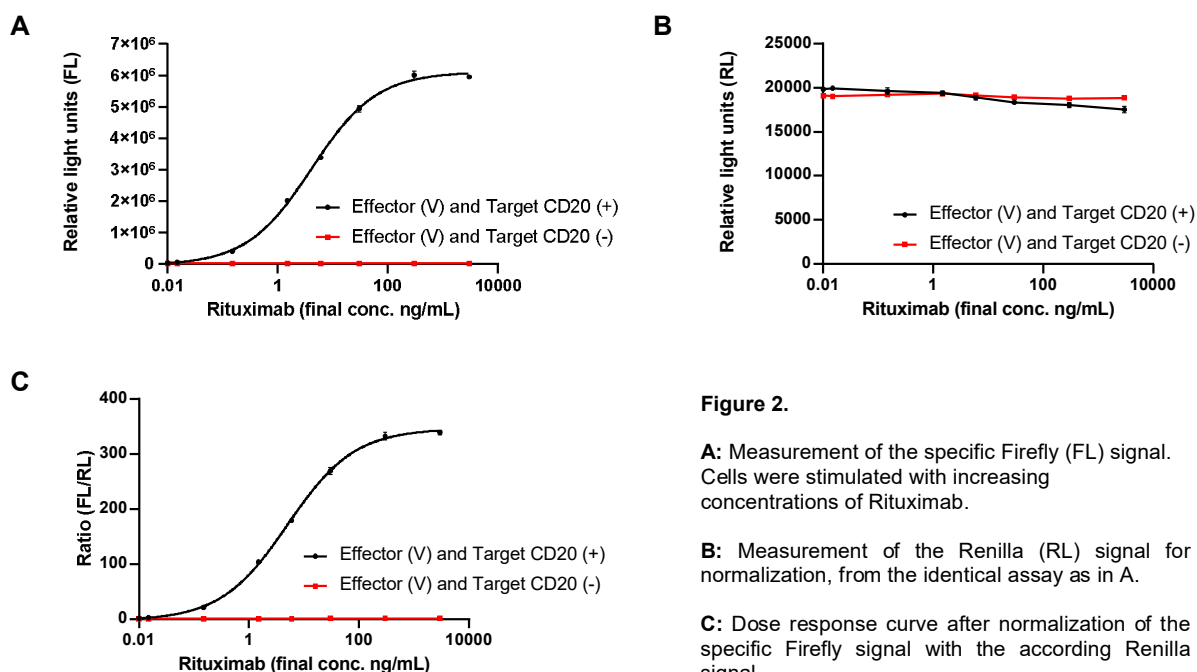


Figure 2.

A: Measurement of the specific Firefly (FL) signal. Cells were stimulated with increasing concentrations of Rituximab.

B: Measurement of the Renilla (RL) signal for normalization, from the identical assay as in A.

C: Dose response curve after normalization of the specific Firefly signal with the according Renilla signal.

Precautions

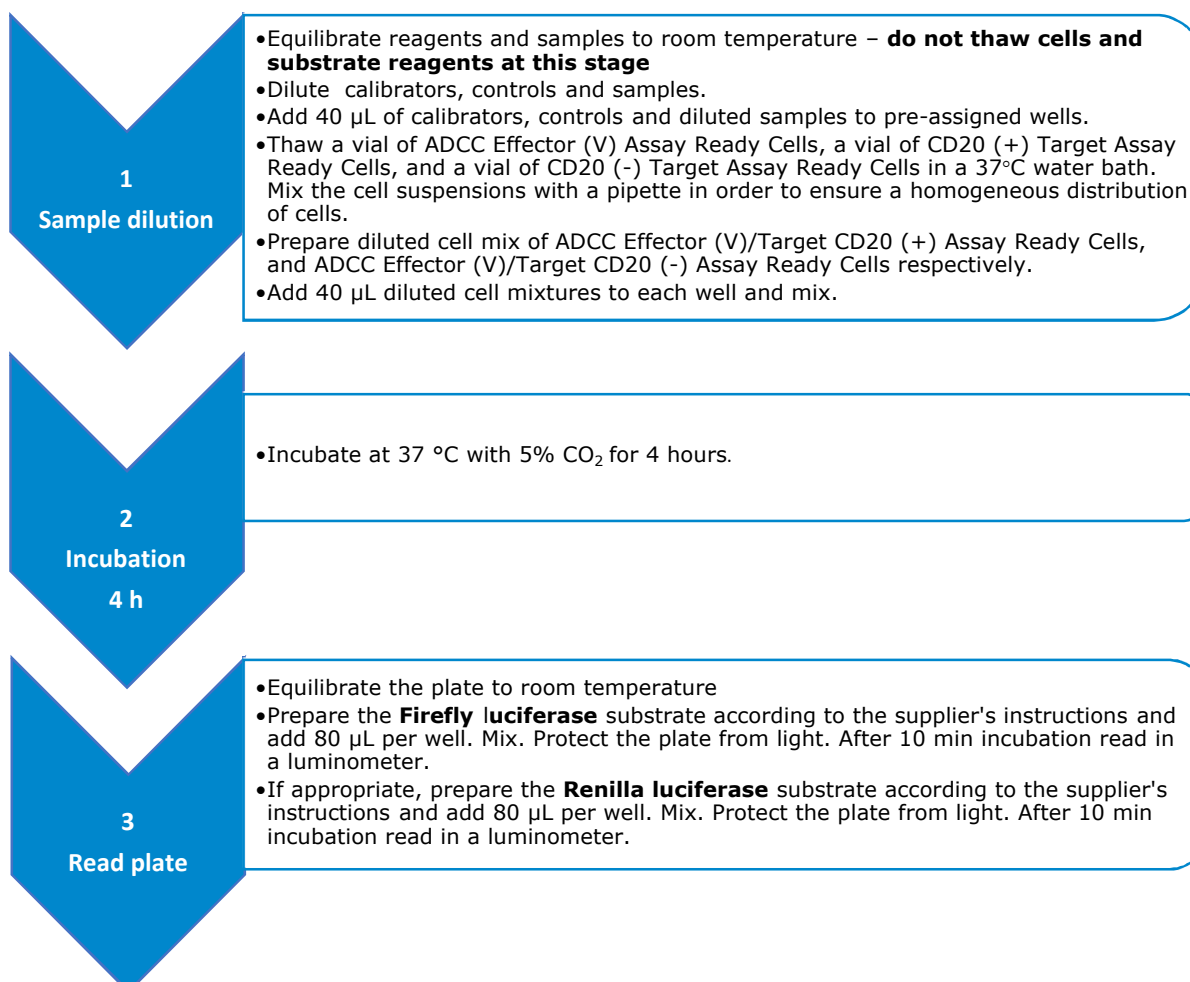
- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according to the suppliers'/manufacturers' 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.

Proprietary 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 anti-CD20 ADCC activity using *iLite*[®] ADCC Assay Ready Cells



Troubleshooting and FAQ

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

References

1. Weiner GJ. *Building better monoclonal antibody-based therapeutics*. Nat Rev Cancer 15(6): 361-70 (2015).
2. Maloney DG et al. *IDEC-C2B8 (Rituximab) anti-CD20 monoclonal antibody therapy in patients with relapsed low-grade non-Hodgkin's lymphoma*. Blood, 15; 90(6):2188-95 (1997)
3. <https://www.cancer.gov/about-cancer/treatment/drugs/fda-rituximab> (2016-10-24)
4. EPAR summary for the public, EMA, EMA/424820/2016, (2016)
5. Weiner GJ. *Rituximab: mechanism of action*. Semin Hematol. 2010 Apr;47(2):115-23.
6. Cang S et al. *Novel CD20 monoclonal antibodies for lymphoma therapy*. J Hematol Oncol, 11;5:64 (2012)