Quantification of anti-CD20 ADCC activity using *iLite*® ADCC Assay Ready Cells

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

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-portion to Fc receptors 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, leading to apoptosis of the targeted cell (1).

The idea of employing ADCC to destroy dysfunctional cells by treating patients with antibodies has existed since 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 approved by the FDA in 1997 for treatment of chemotherapy resistant Non-Hodgkin B-cell lymphomas, and has since also been approved in Europe for different inflammatory indications (2-4).

In addition, other anti-CD20 monoclonal antibodies have been developed, such as ocrelizumab, a humanized (90-95%) antibody, and ofatumumab, a fully human monoclonal. In addition, efforts are being made to enhance the ADCC activity of the antibodies by engineering the glycan patterns of the constant region, such as in obinutuzumab (5).

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*® ADCC Target CD20 (+) Assay Ready Cells). *iLite*® ADCC Effector (V) Assay Ready Cells also contain the Nano Luciferase (NL) 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 NL. 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*® Target CD20 (-) 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
iLite® ADCC Effector (V) Assay Ready Cells	Euro Diagnostica	BM4001
iLite® ADCC Target CD20 (+) Assay Ready Cells	Euro Diagnostica	BM4010
iLite® ADCC Target CD20 (-) Assay Ready Cells	Euro Diagnostica	BM4015

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/Nano luciferase substrate	Promega	N1650, Nano-Glo Dual-Luciferase Reporter Assay System
Plate; White walled micro well plate suitable for luminescence	PerkinElmer	6005680
Microplate Luminometer with appropriate reading software – no filter on luminometer	Contact Euro Diagnostica 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 ADCC Target CD20 (+) Assay Ready Cells. As a negative control, a combination of ADCC Effector (V) Assay Ready Cells and ADCC Target CD20 (-) 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.

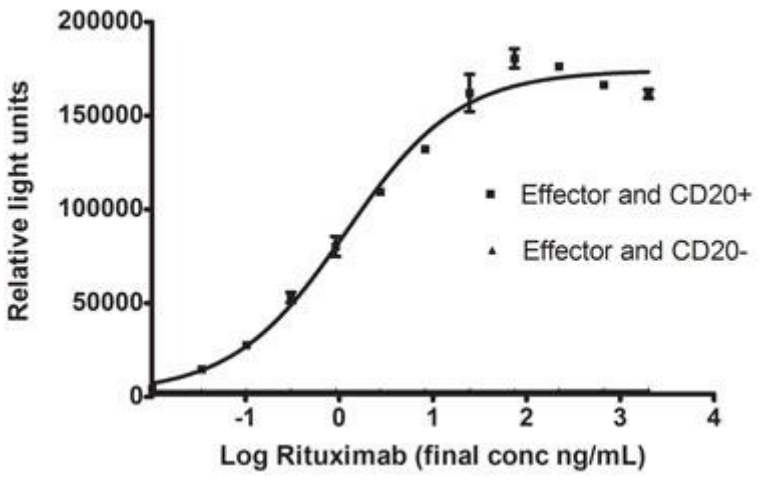

Figure 1. Example of Rituximab calibration curve using the Nano-Glo Dual-Luciferase Reporter Assay System. Values are shown as mean of triplicate ± SD.

Calibrator	Rituximab
	Calibrator conc. (ng/mL)
1	4 000
2	1 333
3	444
4	148
5	49
6	16
7	5.5
8	1.8
9	0.61
10	0.20
11	0.068
12	0

Table 1. Suggested solution calibrator concentrations for Rituximab

Protocol

Incubation

1. Design a plate layout.
2. Dilute calibrators, controls and samples to fall within the expected in assay values of 0-2000 ng/mL.
3. Add 40 µL calibrators, controls and samples in duplicate to assigned wells.
4. Thaw the vial of ADCC Effector (V) Assay Ready Cells and the vials of ADCC Target CD20 (+) Assay Ready Cells and ADCC Target CD20 (-) Assay Ready Cells in a 37°C water bath with gentle agitation.
5. Mix the cell suspensions very carefully several times with a pipette in order to ensure a uniform solution of cells.
6. Dilute 200 µL of the ADCC Effector (V) Assay Ready Cells and 200 µL the ADCC Target CD20 (+) Assay Ready Cells with 3.4 mL Diluent. The total volume of the diluted ADCC Effector (V) /Target CD20 (+) Assay Ready Cells mixture is 3.8 mL.
7. In a separate tube, dilute 62.5 µL of the ADCC Effector (V) Assay Ready Cells with 250 µL of the ADCC Target CD20 (-) Assay Ready Cells with 887.5 µL Diluent. The total volume of the diluted ADCC Effector (V) /Target CD20 (-) Assay Ready Cells mixture is 1.2 mL.
8. Add 40 µL of the diluted cells to each well to be tested.
9. Place the lid on the plate, mix and incubate for 4 hours at 37°C with 5% CO_2.

Adding substrate solutions

10. Equilibrate the plate and the substrate solutions to room temperature.
11. Prepare the **Firefly luciferase** substrate according to the suppliers instructions and add 80 µL per well. Mix and protect the plate from light. Read in a luminometer after the recommended incubation time at room temperature.
12. If appropriate, prepare the **NanoLuc luciferase** substrate according to the suppliers instructions and add 80 µL per well. Mix and protect the plate from light. Read in a luminometer after the recommended incubation time at room temperature.

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.

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. Biomonitor *iLite*® cell-based products are covered by patents which are the property of Euro Diagnostica 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

1 Sample dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute calibrators, controls and samples.
- Add 40 µL of calibrators, controls and diluted samples to pre-assigned wells.
- Thaw a vial of ADCC Effector (V) Assay Ready Cells, a vial of ADCC Target CD20 (+) Assay Ready Cells, and a vial of ADCC Target CD20 (-) Assay Ready Cells in a 37°C water bath. Mix the cell suspensions with a pipette in order to ensure a uniform solution.
- Prepare diluted cell mix of ADCC Effector (V)/Target CD20 (+) Assay Ready Cells, and ADCC Effector (V)/Target CD20 (-) Assay Ready Cells respectively.
- Add 40 µL diluted cell mixtures to each well.

2 Incubation 4 h

- Incubate at 37 °C with 5% CO_2 for 4 hours.

3 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. Read in a luminometer after incubation according to supplier's instructions.
- If appropriate, prepare the **NanoLuc luciferase** substrate according to the supplier's instructions and add 80 µL per well. Mix. Protect the plate from light. Read in a luminometer after incubation according to supplier's instructions.

Troubleshooting and FAQ

Please consult Euro Diagnostica's website www.eurodiagnostica.com

References

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5. Cang S, Mukhi N, Wang K, Liu D., *Novel CD20 monoclonal antibodies for lymphoma therapy.* J Hematol Oncol, 11;5:64 (2012)

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