Quantification of anti-EGFR 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-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).

Deficient signaling of the EGFR and other receptor tyrosine kinases in humans is associated with diseases such as Alzheimer's, while over-expression is associated with the development of a wide variety of tumors. Interruption of EGFR signaling, either by blocking EGFR binding sites on the extracellular domain of the receptor or by inhibiting intracellular tyrosine kinase activity, can prevent the growth of EGFR-expressing tumors and improve the patient's condition. Therefore, many novel therapeutic approaches are aimed at the EGFR, for example the monoclonal antibodies cetuximab and panitumumab. Since the activity of these therapeutic antibodies is mediated in part by antibody-dependent cell-mediated cytotoxicity (ADCC) there is a need to assess the ADCC activity of these therapeutic antibodies, which can be performed with *iLite*® ADCC Assay Ready Cells.

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 EGFR (*iLite*® EGFR (+) 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 renders 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 EGFR expression (*iLite*® EGFR (-) 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 cetuximab in the sample (Fig 1).

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Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® ADCC Effector (V) Assay Ready Cells	Svar Life Science	BM5001
iLite® EGFR (+) Target Assay Ready Cells	Svar Life Science	BM5035
iLite® EGFR (-) Target Assay Ready Cells	Svar Life Science	BM5036
Diluent (RPMI 1640 + 9% heat inactivated FBS + 1% Penicillin Streptomycin)	Gibco	61870 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Cetuximab or analogues	Lilly	NA
Firefly/Renilla luciferase substrate	Promega	E2940, 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% CO2	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 (cetuximab)

The ADCC effect of the cetuximab antibody from Roche has successfully been measured in combination with a mix of ADCC Effector (V) Assay Ready Cells and EGFR (+) Target Assay Ready Cells. As a negative control, a combination of ADCC Effector (V) Assay Ready Cell and EGFR (-) Target Assay Ready Cells was used. In the present assay an Effector: Target ratio of 4:1 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 cetuximab when making an 8-point calibration curve.

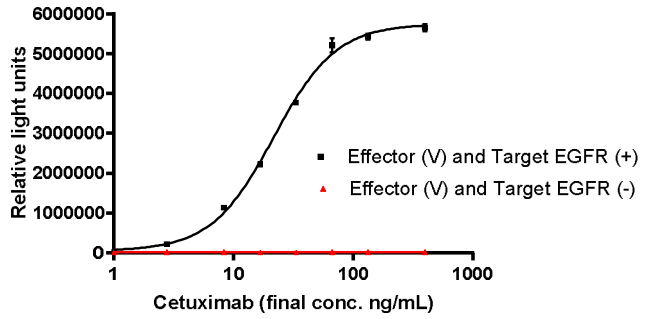

Figure 1. Example of cetuximab calibration curve using Firefly Luciferase substrate Dual-Glo® Luciferase Reagent. Values are shown as mean of triplicate ± SD and values on x-axis are given as **final concentration** in the wells before addition of Dual-Glo® Luciferase Reagent.

Calibrator	Cetuximab
	Calibrator **solution conc.** (ng/mL)
1	800
2	267
3	133
4	67
5	33
6	17
7	5.7
8	0

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

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 0-400 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 EGFR (+) Target Assay Ready Cells and EGFR (-) Target Assay Ready Cells in a 37°C water bath with gentle agitation[1].
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 µL of the ADCC Effector (V) Assay Ready Cells and 200 µL the EGFR (+) Target Assay Ready Cells with 3.44 mL Diluent. The total volume of the diluted ADCC Effector (V) /Target EGFR (+) Assay Ready Cells mixture is 3.84 mL.
7. In a separate tube, dilute 50 µL of the ADCC Effector (V) Assay Ready Cells with 50 µL of the EGFR (-) Target Assay Ready Cells with 860 µL Diluent. The total volume of the diluted ADCC Effector (V)/Target EGFR (-) Assay Ready Cells mixture is 960 µL.
8. Add 40 µL 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% CO_2.

[1] The color of the cell suspension might vary from salmon pink to a more yellowish red. The difference in color is due to small variations in pH of the solution and do not affect function of the cells.

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 µL 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 µL 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.

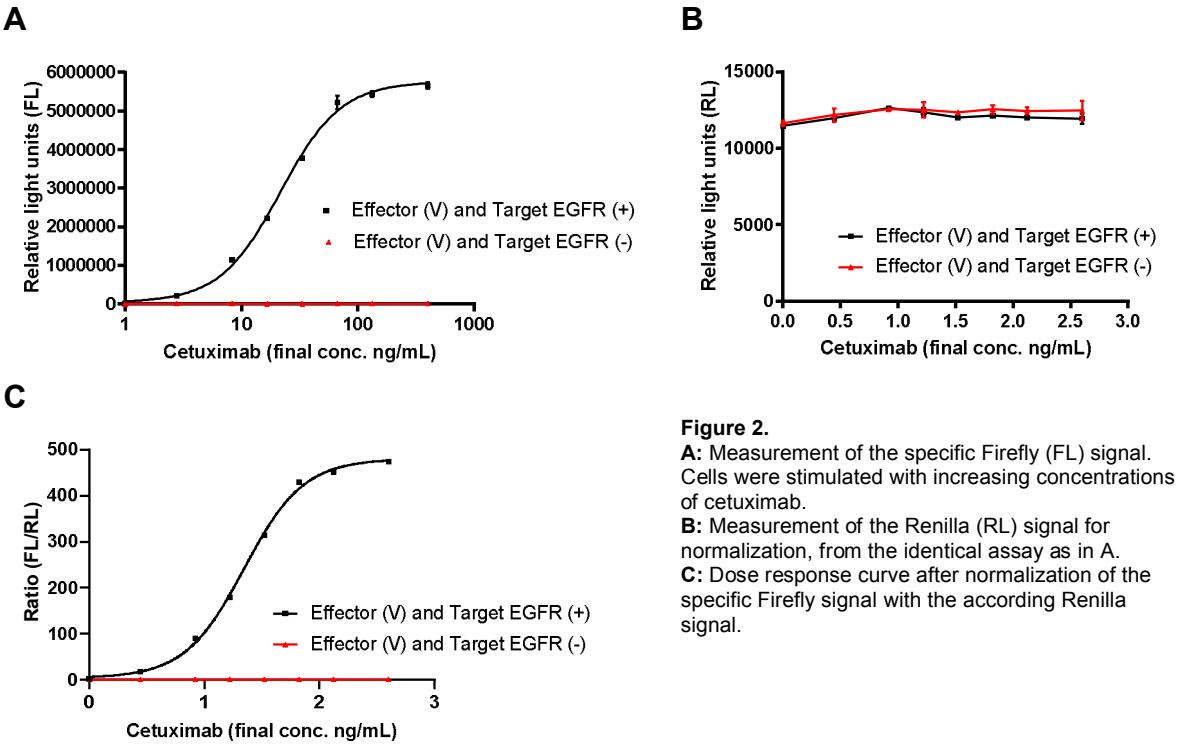

Figure 2.
A: Measurement of the specific Firefly (FL) signal. Cells were stimulated with increasing concentrations of cetuximab.
B: Measurement of the Renilla (RL) signal for normalization, from the identical assay as in A.
C: Dose response curve after normalization of the specific Firefly signal with 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-EGFR 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 EGFR (+) Target Assay Ready Cells, and a vial of EGFR (-) Target Assay Ready Cells in a 37°C water bath. Mix the cell suspensions with a pipette in order to ensure a homogeneous distribution of cells.
- Prepare diluted cell mix of ADCC Effector (V)/Target EGFR (+) Assay Ready Cells, and ADCC Effector (V)/Target EGFR (-) Assay Ready Cells respectively.
- Add 40 µL diluted cell mixtures to each well and mix.

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. 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

1. **Weiner GJ.** *Building better monoclonal antibody-based therapeutics.* Nat Rev Cancer 15(6): 361-70 (2015).