Quantification of anti-HER2 ADCP activity using *iLite*® ADCP Effector 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

The immune system uses various mechanisms to kill specific pathogens, infected cells, and cancer cells. The Fab domains prevents the pathogen binding to the host but the majority of antibody effector function is delivered by the Fc-part, therefore Fc engineering strategies to increase the efficacy of anti-cancer antibodies is ongoing (1). The mechanisms of action by the Fc-part of the antibody are antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and programmed cell death (1).

ADCP is a mechanism whereby pathogenic cells are phagocytosed by various immune effector cells such as monocytes, macrophages, dendritic cells, and neutrophils (2). The antibody binds to a specific antigen on the pathogen/cancer cell, while the Fc part of the antibody binds and activates the Fc receptor on the effector cell resulting in activation of a pathway that triggers phagocytosis. The most dominant receptor in induction of ADCP is the low affinity Fc receptor FcγRIIa (CD32a).

Breast cancer is the most common cancer in women worldwide, and the second most common cancer overall. Survival rates have improved in the recent years, and stratification of patients into subgroups has vastly improved treatment options for many patients. As an example, patients with HER2 positive breast cancer generally have a poor prognosis, but treatment with Trastuzumab, a monoclonal antibody targeting the HER2 receptor, has shown to increase overall survival when given together with chemotherapy (3). During many years is has been postulated that Trastuzumabs' anti-tumor activity depends on multiple direct or indirect cytostatic and/or cytotoxic effects (3) and studies using new techniques has been able to show that the therapeutic effect by Trastuzumab is mediated by ADCC and ADCP (4).

The use of the *iLite*® ADCP Assay Ready Cells together with target cells of interest offers a technology platform to study the ADCP mechanism of a therapeutic antibody in a reliable and reproducible manner, facilitating the growing development of Trastuzumab biosimilars with ADCP activity (5).

Principle of the assay

The *iLite*® ADCP Assay Ready Cells are engineered cells that enable ADCP 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 FcγRIIa on the effector cells. 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 through the NFAT response element which triggers the expression of Firefly luciferase (FL) in the effector cells. In this application note, we describe the use of an effector cell line (*iLite*® ADCP Effector Assay Ready Cells) that over-express FcγRIIa and contain the FL reporter gene that responds to the principal transcription factors that mediate signaling from the FcγRIIa receptor, together with a positive target cell line which over-expresses the surface antigen HER2 (*iLite*® HER2 (+) Target Assay Ready Cells). *iLite*® ADCP Effector 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 HER2 expression (*iLite*® HER2 (-) 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 trastuzumab in the sample (Fig.1).

Specimen collection

Quantification of anti-HER2 ADCP activity using *iLite*® ADCP Effector Assay Ready Cells together with *iLite*® HER2 Target Assay Ready Cells can be performed in test samples including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® ADCP Effector Assay Ready Cells	Svar Life Science	BM5004
iLite® HER2 (+) Target Assay Ready Cells	Svar Life Science	BM5011
iLite® HER2 (-) Target Assay Ready Cells	Svar Life Science	BM5016
Diluent (RPMI 1640 + 9% heat inactivated FBS + 1% Penicillin Streptomycin)	Gibco	61870 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Trastuzumab 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% 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 (trastuzumab)

The ADCP effect of the trastuzumab antibody from Roche has successfully been measured in combination with a mix of ADCP Effector Assay Ready Cells and HER2 (+) Target Assay Ready Cells. As a negative control, a combination of ADCP Effector Assay Ready Cell and HER2 (-) 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 trastuzumab when making an 8-point calibration curve.

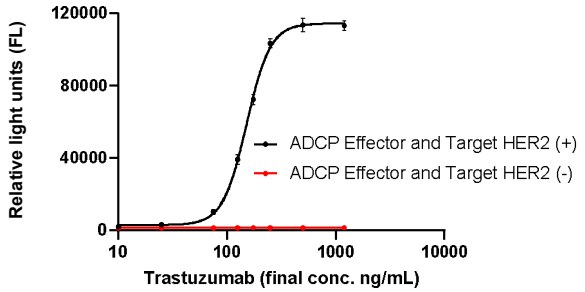

Figure 1. Example of Trastuzumab calibration curve using Firefly Luciferase substrate Dual-Glo® Luciferase Reagent. Values are shown as mean of triplicate ± SD and values on x-axis are given as **final concentration** in the wells before addition of Dual-Glo® Luciferase Reagent.

Calibrator	Trastuzumab Calibrator **solution conc.** (ng/mL)
1	2 400
2	1 000
3	500
4	350
5	250
6	150
7	50
8	0

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

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 0-1200 ng/mL.
3. Add 40 µL calibrators, controls, and samples in duplicate to assigned wells.
4. Thaw the vial of ADCP Effector Assay Ready Cells and the vials of HER2 (+) Target Assay Ready Cells and HER2 (-) 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 µL of the ADCP Effector Assay Ready Cells and 50 µL the HER2 (+) Target Assay Ready Cells with 3.59 mL Diluent. The total volume of the diluted ADCP Effector /Target HER2 (+) Assay Ready Cells mixture is 3.84 mL.
7. In a separate tube, dilute 40 µL of the ADCP Effector Assay Ready Cells with 10 µL of the HER2 (-) Target Assay Ready Cells with 718 µL Diluent. The total volume of the diluted ADCP Effector /Target HER2 (-) Assay Ready Cells mixture is 768 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.

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.

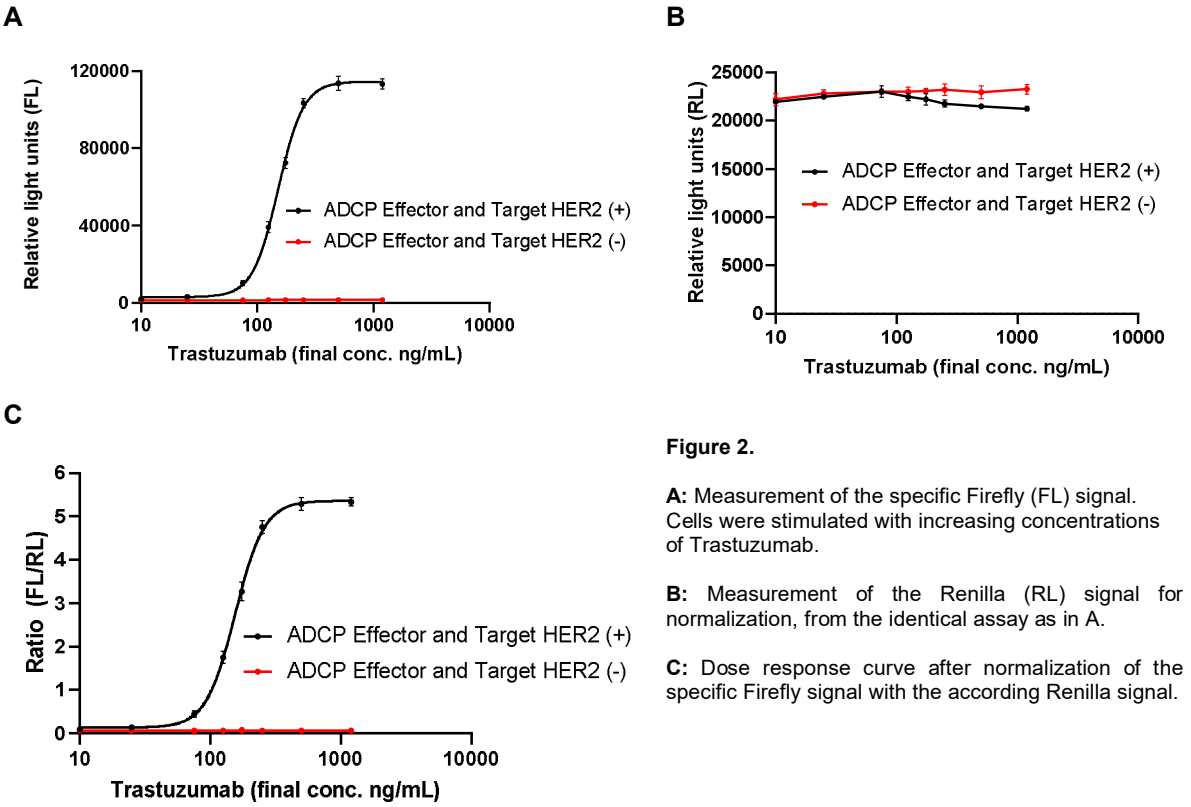

Figure 2.

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

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.

QUICK GUIDE

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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 ADCP Effector Assay Ready Cells, a vial of HER2 (+) Target Assay Ready Cells, and a vial of HER2 (-) 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 ADCP Effector/Target HER2 (+) Assay Ready Cells, and ADCP Effector/Target HER2 (-) Assay Ready Cells respectively.
- Add 40 µL diluted cell mixtures to each well and mix.

2 Incubation 6 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.

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.

Troubleshooting and FAQ

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

References

1. Liu R, Oldham RJ, Teal E, Beers SA, and Cragg MS. *Fc-Engineering for Modulated Effector Functions—Improving Antibodies for Cancer Treatment, Antibodies.* 9(4):64 (2020)
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3. Valabrega G, Montemurro F, Aglietta M. Trastuzumab: mechanism of action, resistance and future perspectives in HER2-overexpressing breast cancer. Ann Oncol.18(6):977–84 (2007)
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5. Thill M. Biosimilar Trastuzumab in Clinical Trials: Differences or Not? Breat Care (Basel) 14(1):17-22 (2019)