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Quantification of anti-mVEGF 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, mainly 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 results in 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). ADCC is one mode of action for several antibodies targeting membrane bound antigens such as rituximab (target CD20) and trastuzumab (target HER2) while therapeutics directed to soluble targets mainly work by inhibiting the binding of the antigen to its receptor, such as infliximab (target TNF-alpha) and bevacizumab (target VEGF).

Vascular Endothelial Growth Factor (VEGF) is a signalling protein which is involved in both normal vascular growth and pathological angiogenesis (2). VEGF is secreted from cells and a large portion can be found bound to the extra cellular matrix (ECM) (3). Anti-VEGF treatment is widely used as cancer therapy, the drug inhibits angiogenesis and thereby limits the tumours growth and ability to metastasize (4). In 2004, Bevacizumab was the first anti-VEGF therapeutic antibody to be approved for treatment in metastatic colon cancer. Anti-VEGF therapeutics act as inhibitors of VEGF and its receptor interactions. Today, it is additionally approved for several other cancer forms (5). Bevacizumab biosimilar agents are also available and more are in clinical trials. Furthermore, anti-VEGF treatment (ranibizumab, aflibercept) is used as intravitreal injections to reduce the pathogenic neovascularization in wet age-related macular degeneration.

iLite[®] mVEGF Target Assay Ready Cells can be used for assessing the possibility of an anti-VEGF antibody drug inducing ADCC.

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. 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. After, the Fc-portion of the target-bound antibodies binds to the receptor, multiple cross-linking between 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 contains the FL reporter gene. The reporter gene responds to the principal transcription factors which mediate signaling from the FcγRIIIa receptor after cross-linking with a positive target cell line which over-expresses membrane bound VEGF (*iLite*[®] mVEGF (+) 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 without expression of membrane bound VEGF (*iLite*[®] mVEGF (-) 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 Bevacizumab 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> [®] mVEGF (+) Target Assay Ready Cells	Svar Life Science	BM5035
<i>iLite</i> [®] mVEGF (-) 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)
Bevacizumab or analogues	Roche	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% 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 (bavacizumab)

The ADCC effect of the bavacizumab antibody from Roche has successfully been measured in combination with a mix of ADCC Effector (V) Assay Ready Cells and mVEGF (+) Target Assay Ready Cells. As a negative control, a combination of ADCC Effector (V) Assay Ready Cell and mVEGF (-) 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 established. The table below shows recommended dilutions of bevacizumab when an 8-point calibration curve is prepared.

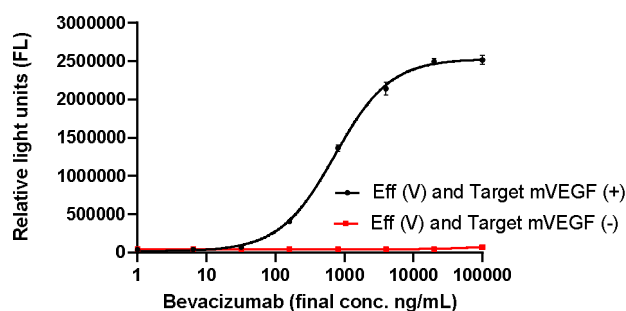


Figure 1. Example of bevacizumab calibration curve using Firefly Luciferase substrate Dual-Glo® Luciferase Reagent. Values are shown as mean of triplicate \pm SD and values on x-axis are given as **final concentration** in the wells before addition of Dual-Glo® Luciferase Reagent.

Calibrator	Bevacizumab
	Calibrator solution conc. (ng/mL)
1	200 000
2	40 000
3	8 000
4	1 600
5	320
6	64
7	13
8	0

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

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 0-100 μ g/mL.
3. Add 40 μ L calibrators, controls and samples in duplicates to assigned wells.
4. Thaw a vial of ADCC Effector (V) Assay Ready Cells, and vials of mVEGF (+) Target Assay Ready Cells and mVEGF (-) Target Assay Ready Cells in a 37°C water bath with gentle agitation¹.
5. Mix the cell suspensions very carefully **at least 10 times with a pipette** to ensure a homogeneous distribution of cells.
6. Dilute 200 μ L of the ADCC Effector (V) Assay Ready Cells and 200 μ L the mVEGF (+) Target Assay Ready Cells with 3.44 mL Diluent. The total volume of the diluted ADCC Effector (V) /Target mVEGF (+) 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 mVEGF (-) Target Assay Ready Cells with 860 μ L Diluent. The total volume of the diluted ADCC Effector (V)/Target mVEGF (-) Assay Ready Cells mixture is 960 μ L.
8. Add 40 μ L of the mixed cell dilution to each test well.
9. Place the lid on the plate and mix on a plate shaker at a **minimum of 750 rpm** for 10 sec. Alternatively, mix the cell suspensions very carefully in the wells using a pipette. Insufficient mixing can cause reduced assay sensitivity.
10. Incubate for 6 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.

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.

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.

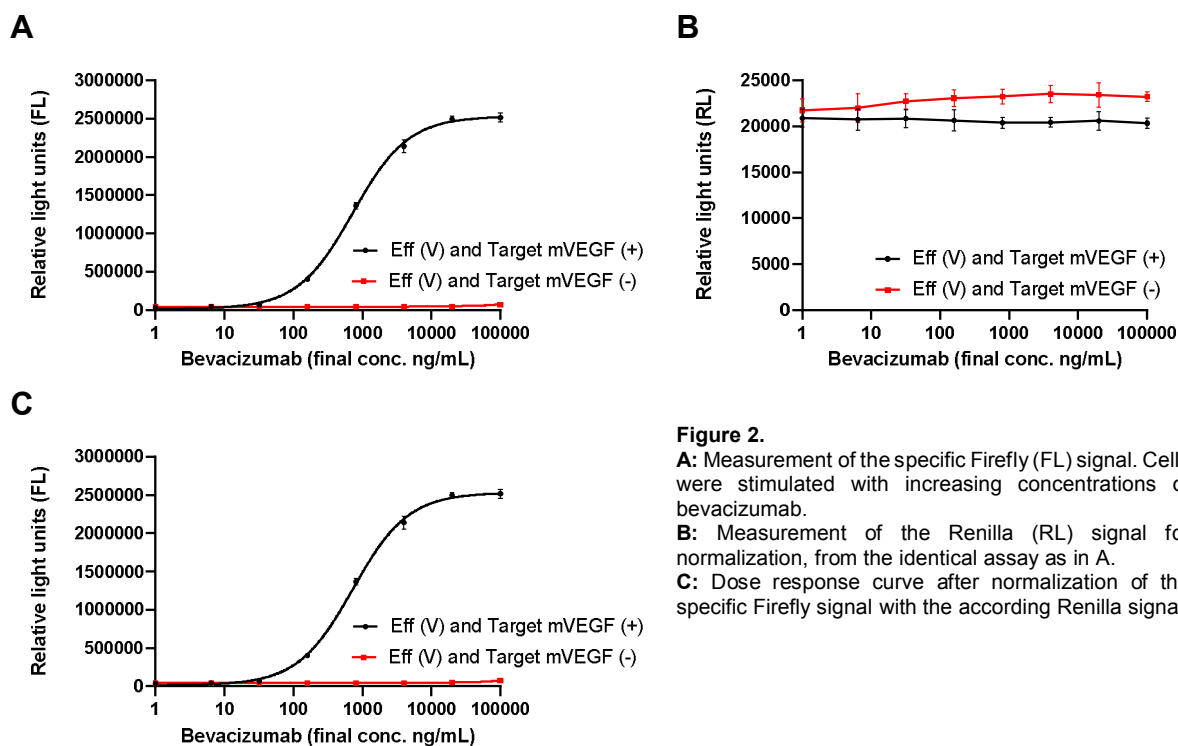


Figure 2.

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

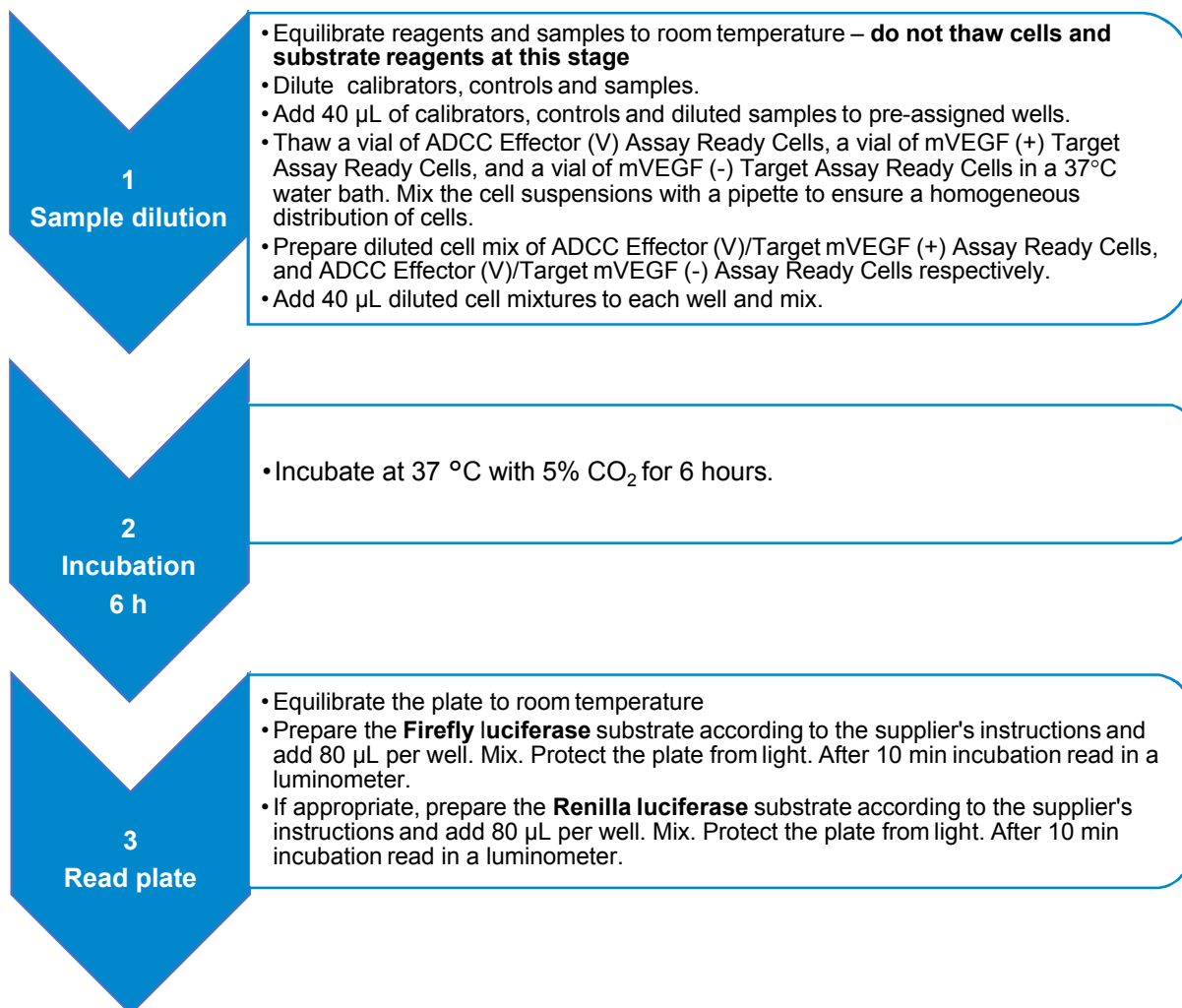
- This application note is intended for professional laboratory research use only. The data and results originating from following the Application Note should not be used either in diagnostic procedures or in human therapeutic applications.
- Use and handle the material and instruments referenced according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

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Troubleshooting and FAQ

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

References

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2. Risau, W. *Mechanisms of angiogenesis*. Nature 386: 671 – 674 (1997).
3. Guzmán-Hernández M et al. Secretion of VEGF-165 has unique characteristics, including shedding from the plasma membrane. Mol Biol Cell. 2014 Apr;25(7):1061-72.
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