Determination of neutralizing antibodies against VEGF inhibitors using *iLite*® VEGF 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

Vascular Endothelial Growth Factor (VEGF) is a signalling protein which is involved in both normal vascular growth and pathological angiogenesis. Without angiogenesis, growth of solid tumours would be limited by oxygen and nutrient supply. Tumours which express VEGF can overcome this limitation and are thus able to grow and metastasize. For this reason, different anti-cancer therapies targeting VEGF have emerged, e.g. a humanized anti-VEGF antibody bevacizumab (Avastin™, Genentech) is currently widely used as a first-line therapy for colorectal cancer (1,2). Prolonged therapies with VEGF inhibitors may lead to development of neutralizing antibodies (NAbs), which may counteract the VEGF antagonist activity of the inhibitors. The *iLite*® VEGF Assay Ready Cells can be used for measurements of VEGF inhibitor activity and presence of neutralizing antibodies to VEGF inhibitors.

Principle of the assay

The *iLite*® VEGF Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of a VEGF responsive promoter. Binding of VEGF to the VEGF receptor 2 (VEGFR2) results in activation of the VEGF regulated Firefly luciferase reporter gene construct. 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 VEGF in the sample. In the presence of inhibitory activity against VEGF, the amount of free VEGF is reduced, resulting in a decreased stimulation of Firefly luciferase production. In the absence of VEGF inhibitor activity and suspected NAb presence in test samples, a known amount of drug is added to quench the Firefly signal and the presence of NAbs is measured as a restored signal. The *iLite*® VEGF Assay Ready Cells can therefore be utilized as an assay for determination of neutralizing antibodies against VEGF inhibitors in test samples, including human serum.

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Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® VEGF Assay Ready Cells	Svar Life Science	BM4021
Diluent (DMEM containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Anti-bevacizumab antibody	Abnova	MAB11128
Bevacizumab or analogues	NA	NA
VEGF or analogues	Gibco	PHC9394
Firefly substrate	Promega	E2620, Bright-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

Protocol

Preparation of neutralizing antibodies against VEGF inhibitor

An anti-bevacizumab antibody from Abnova has successfully been used to neutralize bevacizumab (VEGF inhibitor) and restore the VEGF regulated Firefly luciferase expression in *iLite*® VEGF Assay Ready Cells (refer to the table and graph below).

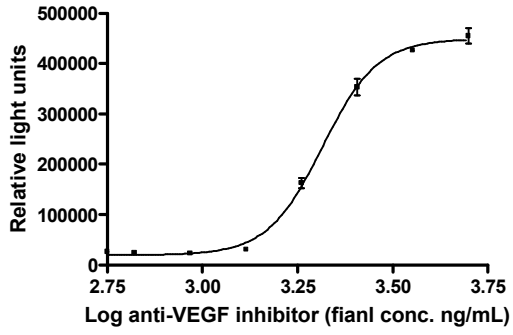

Figure 1. Example of anti-VEGF inhibitory curve

Final 25 ng/mL VEGF and 1000 ng/mL Bevacizumab	Anti-bevacizumab Suggested **solution** concentrations, **µg/mL**
A	40
B	29
C	20
D	15
E	10
F	7.4
G	5.3
H	0

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

Assay performance and incubation

1. Design a plate layout. It is recommended to perform the test at least in duplicate.
2. Perform a serial dilution of the reference anti-bevacizumab antibody. Ensure matrix consistency between reference antibody solutions, control solutions, and sample solutions.
3. Add 20 µL of the reference anti-bevacizumab antibody dilutions, controls and samples to assigned wells (final concentration will be one-eighth of solution concentration).
4. Add 20 µL of 8.0 µg/mL bevacizumab to all wells (final concentration will be 1000 ng/mL bevacizumab).
5. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
6. Add 40 µL of 100 ng/mL VEGF to all wells (final concentration will be 25 ng/mL VEGF).
7. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
8. Transfer references, controls and samples to new wells, adding 40 µL per well.
9. Thaw the vial of *iLite*® VEGF Assay Ready Cells in a 37°C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with pipette in order to ensure a homogeneous distribution of cells.
10. Dilute 250 µL cells with 5.75 mL Diluent.
11. Add 40 µL diluted cells to each well.
12. Place the lid on the plate, mix and incubate for 18 hours at 37 °C with 5% CO_2.

Adding substrate solutions

13. Equilibrate the plate and the substrate solutions to room temperature.
14. Prepare the **Firefly luciferase** substrate according to the suppliers' instructions and add 80 µL per well. Mix and protect the plate from light. After 2 minutes incubation at room temperature read in a luminometer.

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 supplier's/manufacturer's 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

Determination of neutralizing antibodies against VEGF inhibitors using *iLite*® VEGF Assay Ready Cells

1 Plate 1
- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Serial dilute reference anti-bevacizumab antibody
- Add 20 µL of ref. anti-bevacizumab antibody solutions, controls and samples to pre-assigned wells
- Add 20 µL of bevacizumab to each well

2 Incubation 1 30 min
- Incubate at 37 °C with 5% CO_2 for 30 minutes

3 Incubation 2 30 min
- Add 40 µl of VEGF to all wells
- Incubate at 37°C with 5% CO_2 for 30 minutes

4 Plate 2 Add cells
- Transfer references, controls and samples to new wells
- Thaw the vial of cells in a 37°C water bath. Mix cell suspension with a pipette in order to ensure a homogeneous distribution of cells. Dilute the cells
- Add 40 µL diluted cells to each well

5 Incubation 3 18 h
- Incubate at 37°C with 5% CO2 for 18 hours

6 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 2 min incubation read in a luminometer

Troubleshooting and FAQ

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

References

1. **Wang Y, Fei D, Vanderlaan M, Song A.** *Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro.* Angiogenesis 7:335-345 (2004).
2. **Risau W.** *Mechanisms of angiogenesis.* Nature 386: 671-674 (1997).