

Determination of neutralizing antibodies against VEGF inhibitors using *iLite*[™] VEGF 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

Vascular Endothelial Growth Factor (VEGF) is a signaling protein which is involved in both normal vascular growth and pathological angiogenesis. Without angiogenesis, growth of solid tumors would be limited by oxygen and nutrient supply. Tumors 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 towards 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. The Firefly luciferase signal is thus inversely proportional to the amount of inhibitory activity against VEGF in a sample. 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 a highly sensitive assay for determination of neutralizing antibodies against VEGF inhibitors in test samples, including human serum.



Material and equipment needed

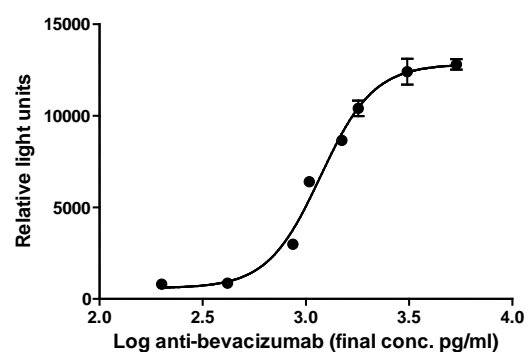
Material and equipment	Suggested supplier	Reference
<i>iLite</i> [™] VEGF Assay Ready Cells	Euro Diagnostica	BM4020
Diluent (DMEM + 9% heat inactivated FBS + 1% Penicillin Streptomycin)	Gibco	31966-021 (DMEM) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Anti-bevacizumab antibody	Abnova	MAB11128
Bevacizumab or analogues	NA	NA
VEGF or analogues	Gibco	PHC9394
Firefly/Renilla luciferase substrate	Promega	E2920, 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 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

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).

Final 25 ng/mL VEGF and 1000 ng/mL Bevacizumab	Anti-bevacizumab ab
	Suggested solution concentrations, pg/mL
A	42 800
B	24 800
C	14 300
D	12 000
E	8 300
F	6 900
G	3 300
H	0



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.
4. Add 20 μL of 8 $\mu\text{g}/\text{mL}$ bevacizumab to all wells.
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.
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 a pipette in order to ensure a uniform solution 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

1. Equilibrate the plate and the substrate solutions to room temperature.
2. Prepare the **Firefly luciferase** substrate according to the supplier's instructions and add 80 μL per well. Mix and protect the plate from light. Read in a luminometer after 10 minutes incubation at room temperature.
3. If appropriate, prepare the **Renilla luciferase** substrate according to the supplier's instructions and add 80 μL per well. Mix and protect the plate from light. Read in a luminometer after 20 minutes incubation 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/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.

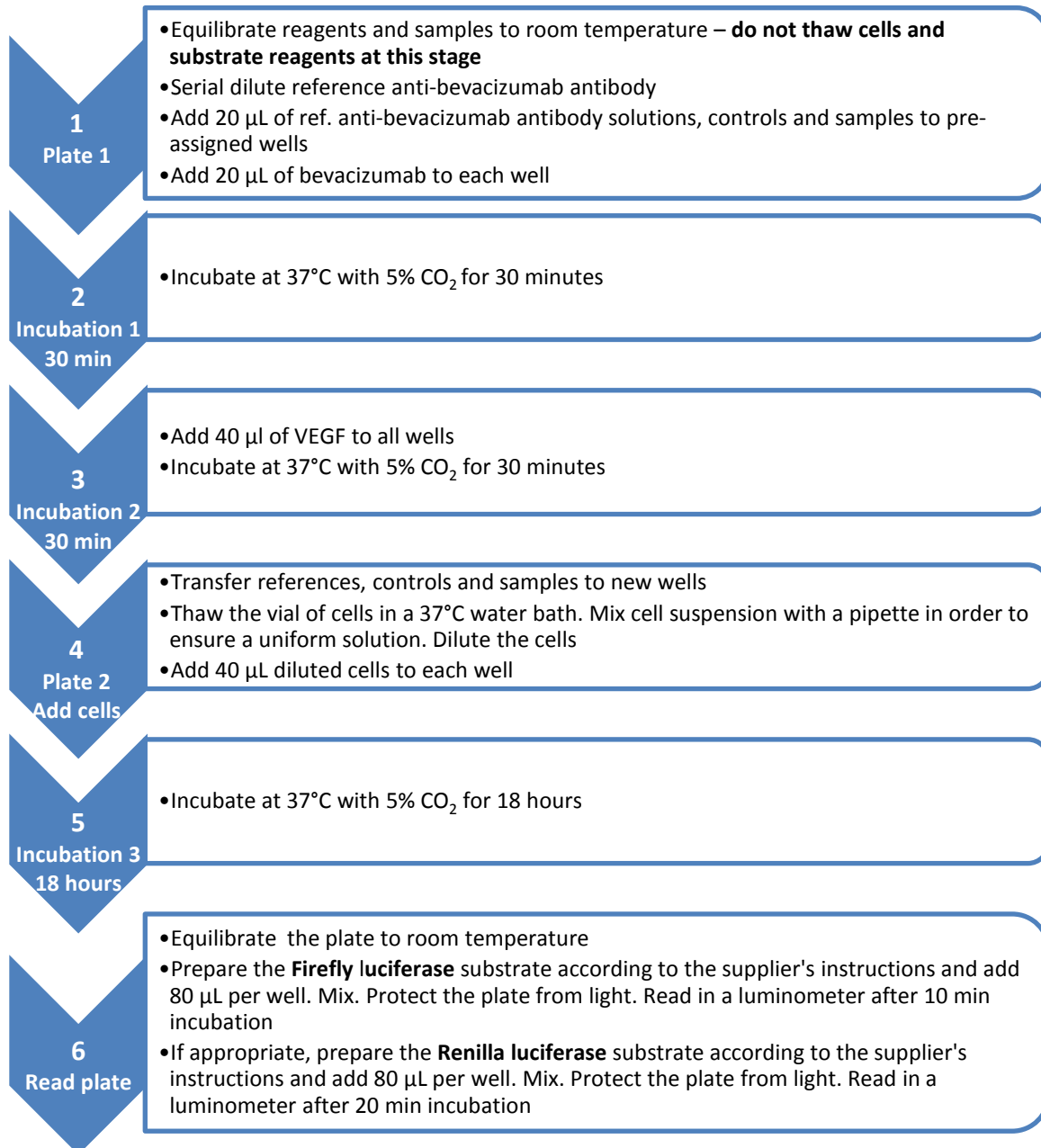
- Residues of chemicals and preparations are generally considered as biohazardous waste, and should be inactivated prior to disposal by autoclaving or using bleach. All such materials should be disposed of in accordance with established safety procedures.

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 – Determination of neutralizing antibodies against VEGF inhibitors using *iLite*[™] VEGF Assay Ready Cells



Troubleshooting and FAQ

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