Determination of anti-insulin neutralizing antibodies using *iLite*® Insulin 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

Insulin is a peptide hormone produced by the beta cells of the pancreas. Its main function is to enhance the uptake of glucose by target cells and thereby regulate the metabolism of carbohydrates, fats and proteins. Release of insulin into the circulation is dependent on the blood glucose levels.

The effect of insulin can be interrupted either by direct destruction of the beta cells (Type I diabetes) or decreasing the ability of the target cells to take up glucose (Type II diabetes). In both forms of diabetes, administration of exogenous insulin is an essential part of the treatment. However, therapies with protein-based drugs such as insulin may lead to development of neutralizing antibodies counteracting the therapeutic effect. The immunogenicity of therapeutic insulin and development of neutralizing antibodies is affected by drug related factors like formulation, structure and purity but also by patient related factors like administration route, age and genetic susceptibility. (1,2)

Principle of the assay

The *iLite*® Insulin Assay Ready Cells are engineered cells optimized to express Firefly luciferase under the control of an insulin responsive promoter. Insulin exerts its activity by binding to a high affinity heterodimeric receptor, CD220, which possesses intrinsic tyrosine kinase activity. Binding of insulin to the insulin receptor alpha chain results in receptor dimerization, receptor auto-phosphorylation, and signalling via the IR beta chain which activates the insulin 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 insulin in the sample. In the presence of neutralizing antibodies against insulin, the amount of free insulin is reduced, resulting in a decreased stimulation of Firefly luciferase production. Hence, the Firefly luciferase signal is inversely proportional to the number of neutralizing antibodies in a sample.

The *iLite*® Insulin Assay Ready Cells can be utilized as a highly sensitive assay for determination of anti-insulin neutralizing antibodies in test samples including human serum.

Material and equipment needed

Material and equipment	Suggested supplier	Reference
iLite® Insulin Assay Ready Cells	Svar Life Science	BM3060
Diluent (RPMI containing 9% heat inactivated FBS + 1% Penicillin-Streptomycin).	Gibco	61870-044 (RPMI) 26140-079 (FBS) 15140-122 (Penicillin-Streptomycin)
Anti-Insulin antibody	Abcam	Ab7842 (de-salted)
Insulin or analogues	Life Technologies	12585-014

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 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 Insulin inhibitor

Insulin inhibitor from Abcam has been used to effectively neutralize insulin and inhibit the insulin regulated Firefly luciferase expression in *iLite*® Insulin Assay Ready Cells (refer to the table and graph below).

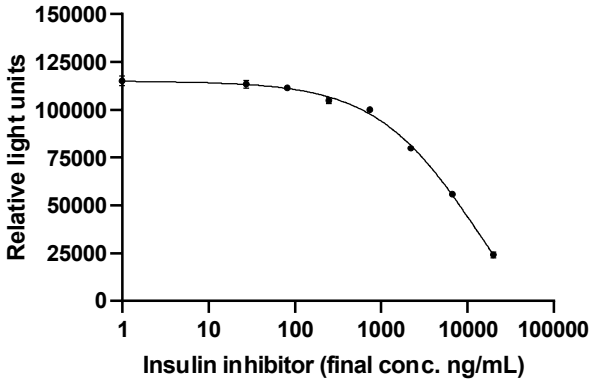

Figure 1. Example of Insulin inhibitory curve

Final concentration 250 ng/mL Insulin	Anti-Insulin antibody: Suggested calibrator solution concentrations, ng/mL
A	80 000
B	26 667
C	8 889
D	2 963
E	988
F	329
G	110
H	0

Table 1. Suggested calibrator **solution concentrations** for anti-Insulin Ab7842

Assay preparation and incubation

1. Design a plate layout. It is recommended to perform the tests at least in duplicates.
2. Prepare a serial dilution of the reference Insulin inhibitor. Ensure matrix consistency between reference antibody, controls, and sample solutions.
3. Add 20 µL of the reference Insulin inhibitor dilutions, controls and samples to assigned wells (final concentration will be a quarter of the solution concentration).
4. Add 20 µL of 1000 ng/ml Insulin to all wells (final concentration will be 250 ng/mL Insulin).
5. Place the lid on the plate, mix and incubate the plate for 30 minutes at 37 °C with 5% CO_2.
6. Thaw a vial of *iLite* Insulin Assay Ready Cells in a 37°C water bath with gentle agitation. The cell suspension is mixed very carefully ten times with a pipette to ensure a homogeneous distribution of cells.
7. Dilute 250 µL cell solution with 5.75 mL Diluent
8. Add 40 µL diluted cells to each well.

9. Place the lid on the plate, mix and incubate for 5 hours at 37 °C with 5% CO_2.

Adding substrate solutions

10. Equilibrate the plate and the substrate solutions to room temperature.
11. Prepare the **Firefly luciferase** substrate according to 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 the plate in a luminometer.
12. 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. After 10 minutes incubation at room temperature read the plate 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 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 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 Insulin neutralizing antibodies using *iLite*® Insulin Assay Ready Cells

1 Sample dilution

- Equilibrate reagents and samples to room temperature – **do not thaw cells and substrate reagents at this stage**
- Dilute reference Insulin inhibitor, controls and samples
- Add 20 µL of ref. Insulin inhibitor solutions, controls and diluted samples to pre-assigned wells
- Add 20 µL of Insulin

2 Incubation 30 min

- Incubate at 37 °C with 5% CO2 for 30 minutes.

3 Add cells

- Thaw the vial of cells in a 37°C water bath. Mix cell suspension with a pipette in order to ensure a homogeneous cell solution. Dilute the cells.
- Add 40 µL diluted cells to each well

4 Incubation 5h

- Incubate at 37°C with 5% CO2 for 5 hours

5 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
- 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. Fineberg SE, Krasner AS et al. *Immunological responses to exogenous insulin.* Endocr. Rev. 2007 Oct;28(6):625-52.
2. Hu X, Chen F. *Exogenous insulin antibody syndrome (EIAS): a clinical syndrome associated with insulin antibodies induced by exogenous insulin in diabetic patients.* Endocr Connect. 2018 Jan;7(1):R47-R55.