Bioluminescent Assays for Studying Insulin Biology

Donna Leippe, Michael P. Valley, Gediminas Vidugiris, Natasha Karassina, James J. Cali and Jolanta Vidugiriene

Promega Corporation, 2800 Woods Hollow Rd, Madison, WI 53711

1. Introduction

The interplay between glucose, lipid, protein and amino acid metabolism is complex and must be finely regulated in response to changing environments. Hormones such as insulin and glucagon have a central role in communicating environmental conditions and coordinating metabolic pathways at the cellular, organ and whole organism level.

To facilitate studies of insulin, glucagon and islet function we have developed new bioluminescent homogeneous, in-solution immunoassays based on a luciferase complementation system. The system consists of two subunits that have weak binding affinity and must be brought into proximity to restore a functional luciferase and generate a light signal. The subunits were attached to two anti-insulin antibodies or two anti-glucagon antibodies to develop homogenous immunoassays with ranges of 10 pM to 8 nM for insulin and 0.5 pM to 1 nM for glucagon. The immunoassays have been used to measure GSIS in model systems including cell lines and human islets microtissues. The assay requires small volumes of sample and can be performed in 384-well plates to enable the rapid measurement of large numbers of samples, such as may occur during perifusion or screening experiments.

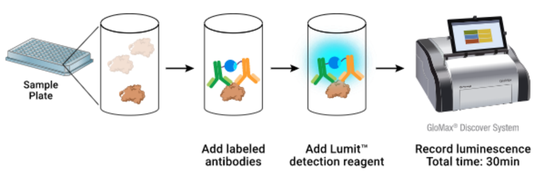
We have also used bioluminescent metabolite assays to study insulin action on specific cells types and processes. These metabolite assays are based on an enzyme-coupled, bioluminescent NADH detection technology that was developed for monitoring key metabolic pathways, such as glycolysis, glucose-uptake, lipolysis and lipogenesis. Hepatic gluconeogenesis was measured in a liver microtissue model system using a glucose assay to measure glucose production, which could be inhibited by insulin treatment. Effects of insulin on adipocytes were investigated using differentiated 3T3-L1 MBX adipocytes. In this cell system insulin was observed to increase glucose uptake and suppress lipolysis using glucose uptake and glycerol assays.

2. Lumit™ Immunoassays

- Lumit™ Immunoassays are bioluminescent immunoassays.
- They utilize the NanoBiT® technology, a structural complementation system based on a small and very bright NanoLuc luciferase.[1]
  - Large BiT: 18kDa domain with no luciferase activity (LgBiT)
  - Small BiT: a peptide (11 aa) which has low affinity for LgBiT (SmBiT)
  - The BiTs must be in proximity to generate an active luciferase
  - The BiTs can be conjugated to proteins, including antibodies

When labeled antibodies bind to their analyte, the BiTs are brought into proximity and form an active luciferase

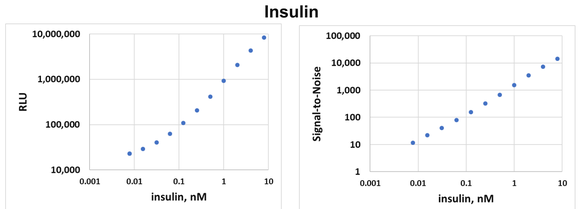
Lumit™ Immunoassays are in-solution, no-wash immnoassays



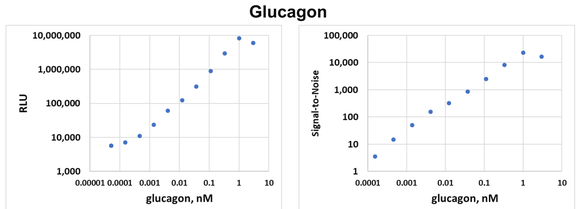
3. Lumit™ Insulin and Glucagon Immunoassays

Performance of the Immunoassays

Insulin



Glucagon



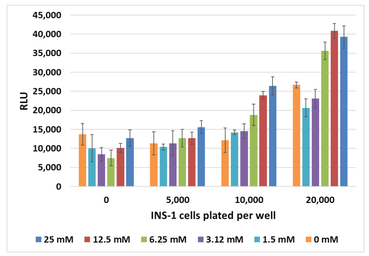
	Insulin	Glucagon
Linear Range	~10 pM to 8 nM	~1 pM to 1 -2 nM
Signal-to Background, maximum	~500	~500
L.O.D (at S/N = 3)	~ 10 pM (58 pg/ml)	~1 pM (3.4 pg/ml)

Insulin (human recombinant, Sigma #91077C) was serially diluted in GSIS buffer + 0.1% BSA. 50 µl was transferred to a 96-well plate and 50 µl of a mix of the two anti-insulin antibodies was added. After 1 hr at RT, 25 µl of NanoLuc Reagent was added and luminescence was recorded. An analogous experiment was performed using glucagon (synthetic, Sigma Cat,. #G2044) diluted in PBS + 0.1% BSA.

4. Insulin Secretion Measurements

Measurement of Glucose-Stimulated Insulin Secretion in Cell Models

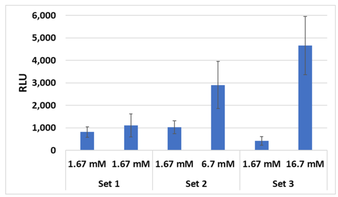
GSIS with INS-1 Cells



INS-1 rat insulinoma cells were plated at the indicated densities in 100 µl in the wells of a 96-well plate. After incubating for 3 days at 37°C, medium was exchanged with 100 µl GSIS Buffer containing glucose at the indicated concentration. The GSIS sample was collected after 1 hr at 37° and 10 µl was assayed in duplicate in 384-well plates.

GSIS with 3D Insight™ Human Islet Microtissues (InSphero AG)

Individual islet microtissues were provided as one each per well of a 96-well plate. Three sets of 6 microtissues were assayed for basal levels of insulin secretion by incubating with 50 µl 1.67 mM glucose. Each set was then incubated with 50 µl 1.67 mM, 6.7 mM or 16.7 mM glucose for 1 hr at 37°C. The GSIS sample was collected and 10 µl was used for the immunoassay. The 3D Insight™ Human Islet Microtissues were kindly provided by InSphero AG (Switzerland).

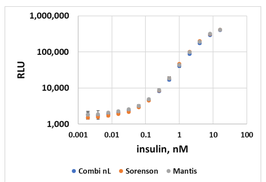


5. Compatibility with Miniaturization and Semi-Automated Dispensing

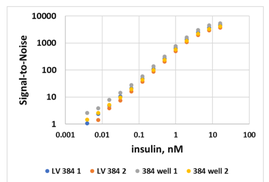
The immunoassays are amenable to the miniaturization and automation needed for rapid measurements of large numbers of samples

- The insulin immunoassay was performed in two types of 384-well plates: regular volume with 20 µl of sample and low volume (LV) with 6 µl of sample
- To accelerate the dispensing of the antibodies (20 or 6 µl) and NanoLuc Reagents (10 or 3 µl), 3 types of semi-automated reagent dispensers were used:
  - Multidrop™ Combi nL (ThermoFisher): nL to µL pressurized dispensing
  - BenchTop Pipettor, 96 channels (Sorenson): pipette tip dispensing
  - Mantis® Liquid Handler (Formulatrix®): positive displacement chips dispensing

Compatibility with 3 Dispensers (384-well RV plates)



Reproducibility between Plates (Combi nL Dispenser)

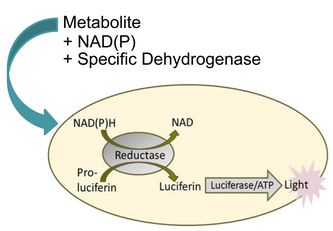


Dilutions of insulin were prepared in PBS + 0.1% BSA and transferred using a Tecan Evo MCA 96-tip robotic arm into multiple 384-well regular and low volume plates. Each insulin concentration was assayed in 16 wells per plate. The average RLU was used to calculate Signal-to-Background, Signal-to-Noise and Z' values. The Z' factor is a parameter used to express the quality of an assay for HTS, with a value of >0.5 indicating an acceptable assay.[2] Error bars are +/- 1 s.d. unless otherwise noted.

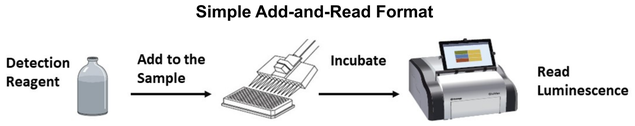
6. Bioluminescent Metabolite Assays

- The Metabolite Assays are based on a bioluminescent NAD(P)H detection technology and coupled-enzyme reactions
- They can be used for the rapid detection of multiple metabolites
- Sample types include cell culture media, cell lysates and tissue homogenates

Metabolites
Lactate
Glucose
Glutamine
Glutamate
Glycerol
Triglycerides
Cholesterol
Cholesterol Esters
Glucose Uptake
BCAA
Glycogen



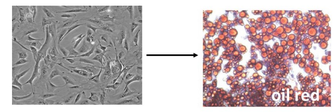
Simple Add-and-Read Format



7. Insulin Action on Lipid Metabolism

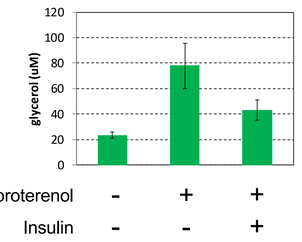
- Cellular glucose and lipid metabolism can be altered in response to insulin
- These changes can be detected using metabolite assays in several cellular model systems including adipocytes, myotubes and hepatocytes

3T3-L1 MBX Fibroblasts → Adipocytes



Adipocytes generated by differentiating 3T3-L1 MBX fibroblasts serve as a useful model for studying the effects of insulin on glucose uptake and lipolysis.

Insulin Inhibition of Lipolysis Measured by Glycerol Secretion

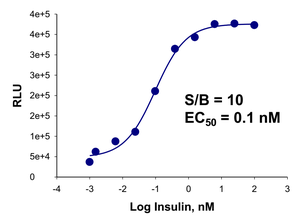


Adipocytes were treated for 90 min with combinations of isoproterenol (25 nM) and insulin (150 nM). Lipolysis was assayed by measuring glycerol release into the medium. Isoproterenol-stimulated lipolysis was inhibited 2-fold by insulin.

8. Insulin Action on Glucose Metabolism

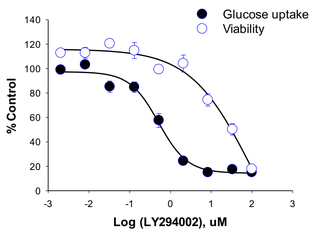
Insulin Stimulation of Glucose Uptake in Adipocytes

Increased Glucose Uptake



Treatment of adipocytes with insulin increased glucose uptake in a dose dependent manner. This is due to the insulin-induced translocation of GLUT4 to the plasma membrane. The bioluminescent assay measures the uptake of the commonly used glucose analog 2DG.[4]

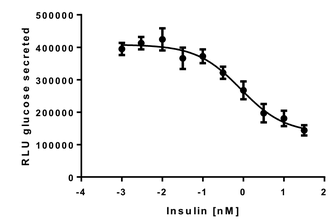
Inhibition of Glucose Uptake



Insulin stimulated glucose uptake could be inhibited by the PI3K inhibitor LY294002. Cells were treated with inhibitor for 30 min and then 100 nM insulin for 1 hr. Viability and 2DG uptake were then measured. IC_{50}'s: 0.5 µM for glucose uptake, 88 µM for viability.

Inhibition of Gluconeogenesis Measured by Glucose Secretion In a Liver Cell Model

Hepatocyte Spheroids formed from 2000 cells (iCell® Hepatocytes 2.0; FUJIFILM Cellular Dynamics) were incubated with 10 mM lactate, 2 µM forskolin, and 10 nM insulin for 6 hrs. Glucose secreted into the medium was measured using a bioluminescent glucose detection assay.



9. Summary

Bioluminescent Assay can facilitate the study of insulin and glucagon secretion and action

- Bioluminescent immunoassays were developed to measure insulin and glucagon with picomolar sensitivity and large linear range
- The Insulin Immunoassay was used to measure secreted insulin in functional GSIS tests of cells and islet microtissues
- The assays are compatible with low volumes and semi-automated dispensing to accelerate the measurement of large numbers of samples

- Bioluminescent metabolite assays have been developed for several key metabolites that can serve as markers for important cellular metabolic pathways
- The assays were used to study the action of insulin in model systems to measure cellular responses such as increases in glucose uptake, decreases in glucose production and decreases in lipid catabolism

References:
1. Dixon, A.S., *et al.* ACS Chem. Biol. 11:400-408 (2016)
2. Zhang, J.H., *et al.* J. Biomol Screen, 4:67-73 (1999)
3. Leippe, D.M., *et al.* SLAS Discov. 22(4):366-377 (2017)
4. Valley, M.P., *et al.* Anal. Biochem. 505:43-50 (2016)

Corresponding author: donna.leippe@promega.com