Poster no 34, 12th Annual Essential Protein Engineering Summit (PEGS Boston), April 2016

Establishment of a Novel *iLite*™ Reporter Gene Assay for the Quantification of the Activity and Neutralizing Antibody response to Insulin Products

Christophe Lallemand, Maud Simansour, Feifei Li ang, Rosa Ferrando-Miguel, and Michael G. Tovey
Biomonitor SAS, Villejuif Bio Park, 1 Mail du Professeur Georges Mathé, 94800 Villejuif France

Introduction

We have shown previously that reporter gene assays based on the establishment of a cell line transfected with a luciferase reporter-gene placed under the control of a drug responsive chimeric promoter, provide highly sensitive and reproducible assays (*iLite*™) for quantifying the activity of recombinant biopharmaceuticals, (1-3). The aim of this work was to develop an *iLite* assay responsive to insulin. Insulin exerts its activity by binding to a high affinity heterodimeric receptor (CD220) that possesses intrinsic tyrosine kinase activity (4). Binding of insulin to the insulin receptor alpha (Ira) chain results in receptor dimerization, receptor auto-phosphorylation, and signaling via the IR beta (IRb) chain resulting in phosphorylation of the insulin receptor substrate-1 (IRS-1) and recruitment of several kinases including phosphatidylinositol 3-kinase (PI3K), protein kinase B (PKB), and glycogen synthase kinase-3 (GSK-3). IR also activates directly the signal transducer and activator of transcription-5 (STAT5) that interacts directly via its SH2 domain with Tyr 960 of the IRb chain (5).

Numerous methods have been employed to assess IR activity in cell-free systems in vitro including radiologic, fluorescence, or colorimetric assays employing isolated IR kinase activity and various exogenous substrates (6). Regulatory authorities, however, recommend the use of cell-based assays that reflect the mode of action of a drug for the detection and quantification of neutralizing anti-drug antibodies (7,8).

Method - Cloning Strategy

To develop an insulin responsive cell line the avian B-cell line DT-40 (ATCC CRL2111) was transfected with the gene encoding the human insulin receptor together with a 5 x tandem repeat of a non-canonical STAT5 recognition sequence and a human STAT5 expression vector. The human insulin receptor associated kinase can activate the STAT5 pathway directly independently of PI3K, AKT or JAK kinases. DT-40 cells were also co-transfected with the insulin receptor and the Renilla Luciferase (RL) normalization gene under the control of a constitutive promoter (Figure 1).

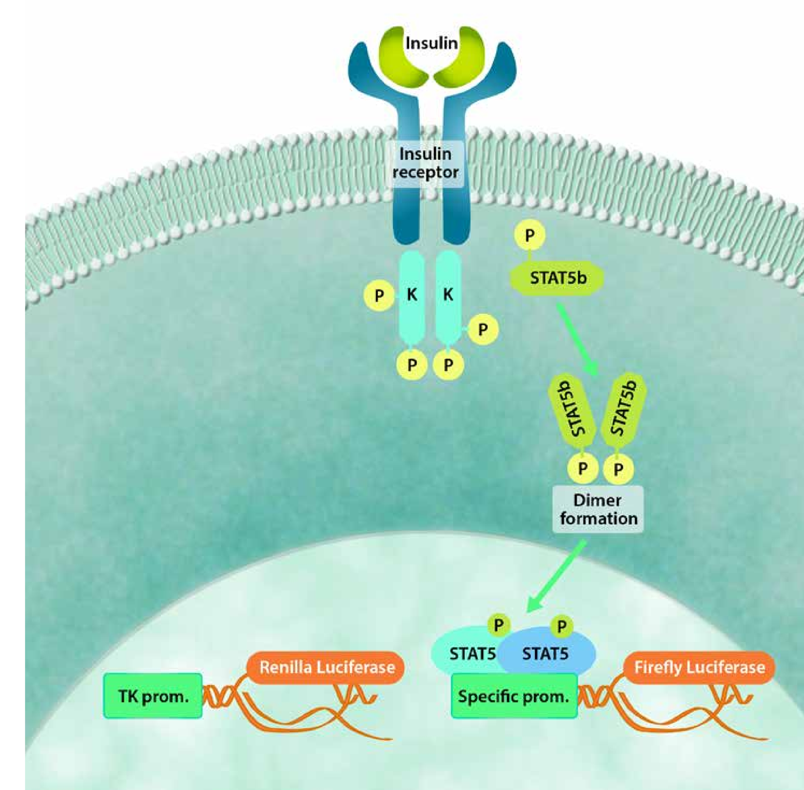


Figure 1. Schematic illustration of the signalling pathway of the insulin responsive cell line.

Results

The resulting cells, insulin-responsive *iLite* reporter gene cells, provide the basis for the establishment of a rapid, sensitive, precise, and highly specific assay for the quantification of insulin activity that can be completed within 5 hours, figure 2.

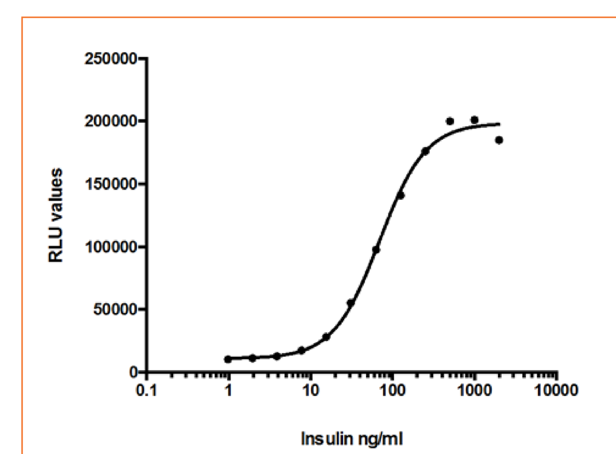
The assay was also precise with both intra and inter assay coefficients of variation (% CV) of 10 % or less. Neither bovine fetal serum (FBS) nor a pool of normal human serum had any significant effect on insulin-induced FL activity at a final concentration of 1:10.

Insulin had no significant effect on the level of expression of the Renilla Luciferase (RL) normalization gene under the control of a constitutive promoter demonstrating that expression of (RL) provides a means for correcting for non-specific cyto-toxic effects or serum matrix effects and allows cytokine levels to be quantified even in sera with a relative high level of cytotoxicity.

The assay is highly specific for human insulin and human insulin like growth factor-1 (IGF-1) had no significant effect on FL expression (Figure 3). Although human insulin like growth factor-2 (IGF-2) was able to activate FL activity at markedly higher concentrations than human insulin both IGF-1 and IGF-2 can easily be removed from samples by simple molecular filtration.

Over-expression of the human insulin receptor on the surface of DT-40 cells (Figure 4a), enabled the phenomenon of negative insulin receptor cooperativity, previously predicted from theoretical studies (9), to be demonstrated for the first time (Figure 4b). This phenomenon is specific to the insulin receptor and was not observed following transfection of the insulin-responsive cells with the human G-CSF and IL-23 receptors and treatment of DT-40 cells with either human G-CSF or IL-23 (Figure 5).

Insulin-responsive *iLite* reporter gene cells also provide the basis for the establishment of a sensitive and precise assay for the quantification of neutralizing anti-insulin antibodies (Figure 6).



Time of incubation	5 h
EC50	70 ng/ml
LLOQ	16 ng/ml
Dynamic range	20-fold

Figure 2. Dose-response curve illustrating the sensitivity o fthe insulin-responsive *iLite* reporter gene cells.

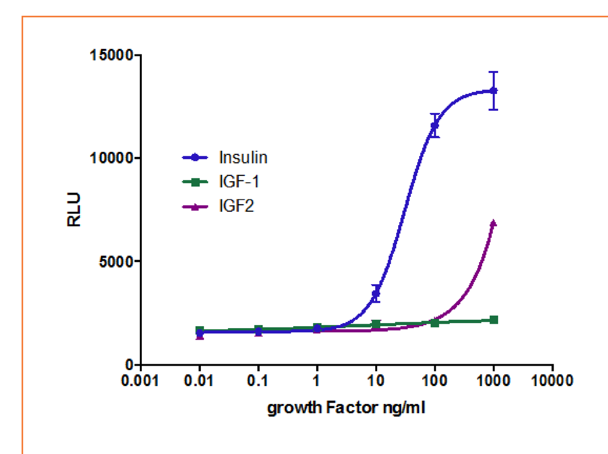


Figure 3. Specificity of the *iLite* Insulin Assay Ready Cells vs IGF-1 and IGF-2. Both IGF-1 and IGF-2 can be readily removed from samples by simple molecular filtration.

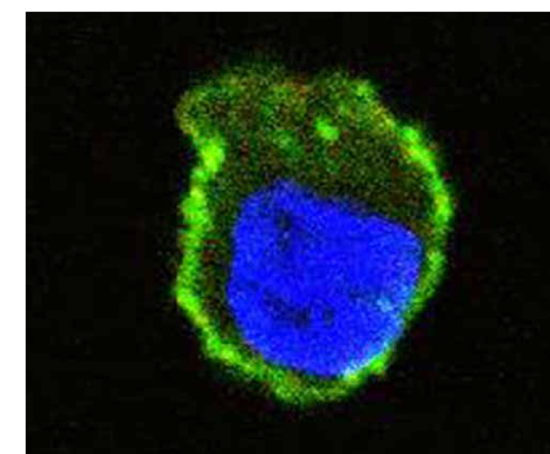


Figure 4a. Visualization of the phenomenon of negative insulin receptor cooperativity.

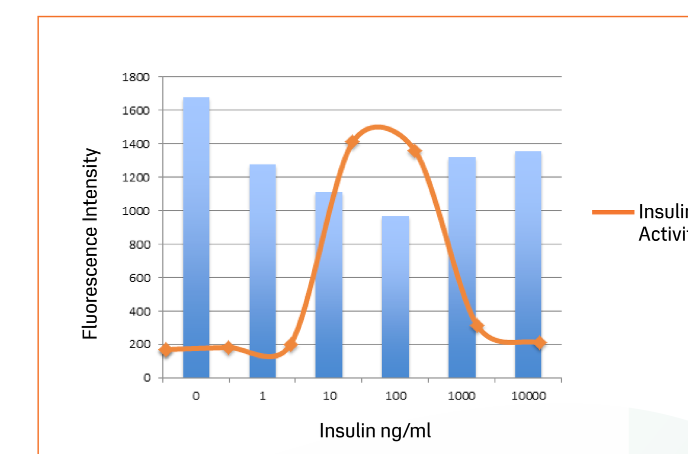
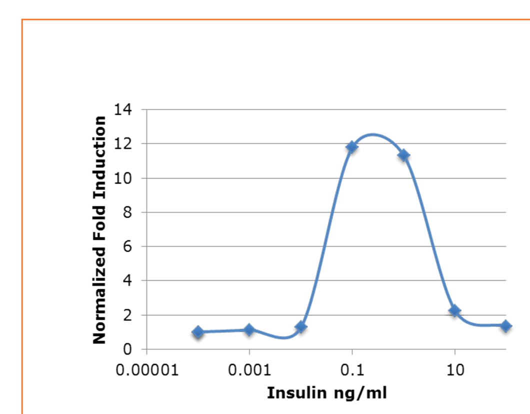
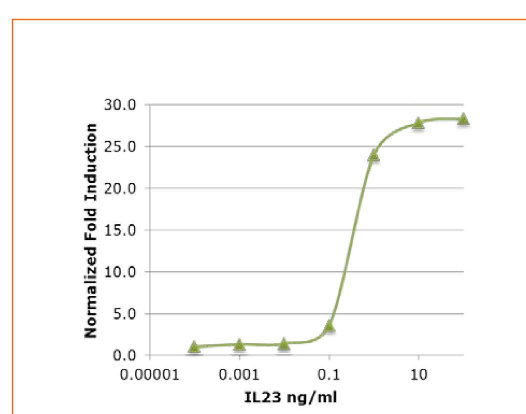


Figure 4b. Correlation between the Expression of Free Insulin Receptors & Insulin-Induced FL Activity. Pre-incubation of cells for 20' min at 37°C in the presence of increasing concentrations of insulin, followed of the detection of free insulin receptors on the cell surface using an anti-CD220 MAb.





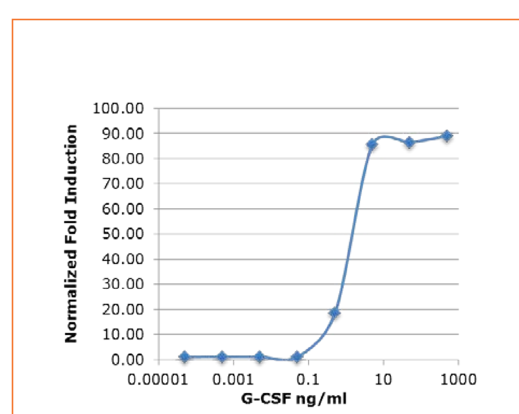


Figure 5. Negative receptor cooperativity could only be demonstrated for the insulin receptor and not for IL-23 or human G-CSF receptors. 5-hour assay using OPH17 cells.

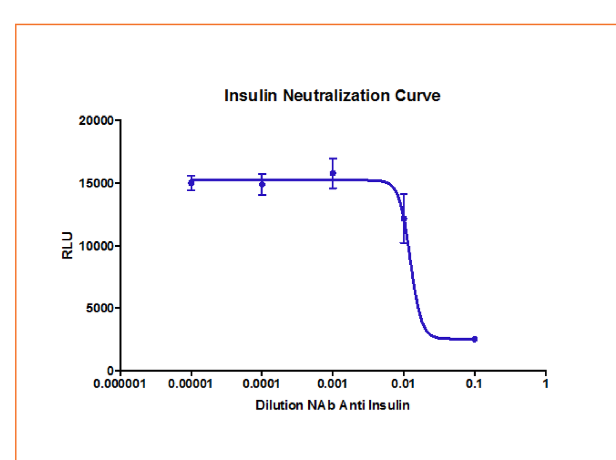


Figure 6. The *iLite* Insulin cell line can also be used for quantification of neutralizing insulin-antobodies.

References

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Conclusion

Assay-ready frozen *iLite* reporter gene cells described herein eliminate the principal inconveniences of cell-based assays in general and provide the basis for the establishment of rapid, precise and highly specific assays for both the quantification of human insulin activity and neutralizing anti-insulin antibodies even in the presence of human serum.

A vial with frozen cells, *iLite* Insulin Assay Ready Cells, can simply be thawed and an assay can be carried out immediately without the need for cell culture.

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