Evaluation and validation of the HTRF insulin assay as a replacement for a commercially available ELISA

Jeffrey Hixon
Elixir Pharmaceuticals
Cambridge, MA USA

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Elixir Pharmaceuticals is a pharmaceutical company focused on the discovery, development and commercialization of novel pharmaceuticals for the treatment of metabolic diseases and obesity.
Type II Diabetes

- >180M people worldwide have diabetes
- Type II accounts for 80-95% of all cases
- Frequently undiagnosed
- Sixth leading cause of death by disease
  - Leading cause of kidney disease
  - Leading cause of non-traumatic limb amputations
  - Leading cause blindness among young adults
Type II Diabetes

- Complicated metabolic disorder

- Characterized by:
  - Loss of sensitivity to insulin
  - Decrease in the body’s ability to produce insulin
  - Overproduction of glucose by the liver
  - Uncontrolled diabetes leads to abnormally high blood sugar levels
    - A condition known as hyperglycemia

- Six classes of drugs approved for treatment
  - Still unmet medical need for diabetes / weight control drugs
Insulin

- Produced in the beta islet cells of the pancreas
- Stimulate uptake of glucose from the blood
- Critical in the control of glucose homeostasis
- Insulin deficiency is the hallmark of type I diabetes
- Hyperinsulinemia and insulin resistance characterize type II diabetes

- Diabetes research often involves rat and mouse models for *in vivo* studies
  - Diet induced obesity (DIO) model: animals fed high fat diet to induce an imbalance in blood glucose and insulin levels
  - Glucose tolerance test (GTT): where a bolus of glucose is administered and plasma insulin and glucose levels are measured over time

Key to diabetes research is the ability to measure insulin effectively and accurately
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- Traditional assays for insulin
  - Radio-immunoassay (RIA)
  - Enzyme linked immunosorbant assay (ELISA)

- Cisbio’s HTRF insulin assay
**Insulin ELISA**

- 96 well antibody coated plate
- Dispense 95μl sample diluent
- Pipette 5μl plasma sample or insulin standard
- Incubate 2 hours at 4C
- Wash 5 times with wash buffer
- Dispense 100μl anti-insulin conjugate
- Incubate 30 min at room temperature
- Wash 5 times with wash buffer
- Dispense 100μl enzyme substrate solution
- Incubate 40 min at room temperature
- Stop reaction by adding stop solution
- Measure A₄₅₀ and subtract A₆₃₀ values
- Calculate insulin concentrations using the standard curve
Cisbio’s HTRF insulin assay

- Dispense 5μl sample or insulin standard
- Dispense 2.5μl each of anti-insulin Ab-cyptate and anti-insulin Ab-XL665
- Incubate 2 hours at room temperature
- Read on an HTRF compatible reader
- Calculate insulin concentration using the standard curve
## Assay comparison

<table>
<thead>
<tr>
<th></th>
<th><strong>ELISA</strong></th>
<th><strong>HTRF</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample volume required</strong></td>
<td>5μl</td>
<td>5μl</td>
</tr>
<tr>
<td><strong>Standard curve range</strong></td>
<td>0 - 6.4ng/ml</td>
<td>0 - 10ng/ml</td>
</tr>
<tr>
<td><strong>Plate format</strong></td>
<td>96 well</td>
<td>384 well</td>
</tr>
<tr>
<td><strong>Miniaturizable</strong></td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td><strong>Total # steps to perform assay</strong></td>
<td>13</td>
<td>5</td>
</tr>
<tr>
<td><strong>Total time to perform assay</strong></td>
<td>4+hrs</td>
<td>~2hrs</td>
</tr>
<tr>
<td><strong>Wash steps</strong></td>
<td>10</td>
<td>zero</td>
</tr>
<tr>
<td><strong>Cost per well (US$)</strong></td>
<td>$3.46</td>
<td>$0.13</td>
</tr>
<tr>
<td><strong>Specificity</strong></td>
<td>r, m</td>
<td>r, m, p, h</td>
</tr>
</tbody>
</table>
Comparison of ELISA and HTRF standard curves

**ELISA insulin standard curve**

- **OD (450nm-630nm)** vs. **Insulin (ng/ml)**
  - Values range from 0.00 to 1.50 for OD, and from 0 to 7 for Insulin.

**HTRF insulin standard curve**

- **Ratio** vs. **Insulin (ng/ml)**
  - Values range from 0 to 1500 for Ratio, and from 0 to 10 for Insulin.

Both curves show a linear relationship between the measured values and the insulin concentrations.
Miniaturization assessment

Insulin standard curves
Scaling assay volume

Ratio

Insulin (ng/ml)

- 20ul
- 15ul
- 10ul
- 5ul
- 3ul
- 2ul
Rodent and Human insulin standard curves

Rat/mouse insulin standard curve

- Ratio vs. [Insulin] ng/mL

Human insulin standard curve

- Ratio vs. [Insulin] ng/mL

- 20uL assay
- 15uL assay
- 10uL assay
Time course in variable volume assay

**Rat / mouse insulin HTRF assay**
10 ng/ml insulin

**Human insulin HTRF Assay**
10 ng/ml insulin
Replicate standard curves

Rat / mouse insulin standard curves

<table>
<thead>
<tr>
<th>Insulin standard concentration (ng/ml)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td>0.15625</td>
<td>3.6</td>
</tr>
<tr>
<td>0.3125</td>
<td>4.7</td>
</tr>
<tr>
<td>0.625</td>
<td>4.4</td>
</tr>
<tr>
<td>1.25</td>
<td>4.7</td>
</tr>
<tr>
<td>2.5</td>
<td>2.8</td>
</tr>
<tr>
<td>5</td>
<td>1.7</td>
</tr>
<tr>
<td>10</td>
<td>1.8</td>
</tr>
</tbody>
</table>
Variability testing on unknown plasma sample

- Unknown Plasma sample

CV = 3.3%
Insulin measurement in pancreatic islets

Glucose Dose Response of Insulin Secretion in Pancreatic Islets isolated from mice (n=3)

![Graph showing glucose dose response of insulin secretion](#)
Ghrelin is a key metabolic regulator

- Peptide hormone
- Secreted from stomach
- Interacts with receptors in the brain and periphery
- Controls and integrates a variety of metabolic functions
- Part of an intricate neuroendocrine system
Ghrelin signaling as a validated target in metabolic disease

- Ghrelin KO and ghrelin receptor KO mice resist diet-induced obesity (DIO)
- KO mice resist decline in metabolic parameters when placed on a high fat diet
- Block of ghrelin inhibits body wt gain, food intake and fat mass content in rodents
- Vaccination against ghrelin causes lack of weight gain and increased relative fat free mass in rodents
- A small molecule ghrelin antagonist inhibits body weight gain and insulin levels in DIO mice
Favorable metabolic profile in ghrelin receptor KO mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>GhrR +/-</th>
<th>GhrR +/-</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>46.2 ± .8</td>
<td>38.9 ± 1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>118.6 ± 4.7</td>
<td>98.6 ± 4.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>.97 ± .10</td>
<td>.53 ± .05</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>7.38 ± .92</td>
<td>3.34 ± .43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CLAMP (GI R, mg/Kg/min)</td>
<td>26.7 ± 2.4</td>
<td>37.3 ± 3.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>% HbA1c</td>
<td>4.20 ± .10</td>
<td>3.93 ± .08</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>TG (mg/dl)</td>
<td>101.1 ± 3.7</td>
<td>103.2 ± 5.0</td>
<td>n.s.</td>
</tr>
<tr>
<td>TC/HDL-C</td>
<td>1.22 ± .03</td>
<td>1.14 ± .02</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Improved insulin sensitivity

From: Longo et al [2008] Regul Pept. 150:55
Dramatic improvement in insulin sensitivity in both male and female DIO GhrR KO mice

**Male DIO GhrR KO vs WT mice**

**GTT-glucose (± SEM)**

![Graph showing blood glucose levels for male DIO GhrR KO vs WT mice](image1)

**GTT-insulin (± SEM)**

![Graph showing insulin levels for male DIO GhrR KO vs WT mice](image2)

**Female DIO GhrR KO vs WT mice**

**GTT-glucose (± SEM)**

![Graph showing blood glucose levels for female DIO GhrR KO vs WT mice](image3)

**GTT-insulin (± SEM)**

![Graph showing insulin levels for female DIO GhrR KO vs WT mice](image4)
GhrR antagonism recapitulates the insulin sensitivity of HFD fed GhrR KO mice

**DAY 28**

**GTT-glucose**

![Graph showing glucose levels](image)

**GTT-insulin**

![Graph showing insulin levels](image)

**DAY 56**

**GTT-glucose**

![Graph showing glucose levels](image)

**GTT-insulin**

![Graph showing insulin levels](image)

- Decreased plasma glucose excursion
- Dramatically decreased insulin requirement
Conclusion

The HTRF insulin assay allows:

- Seamless migration from ELISA to HTRF format
- Extremely cost effective assay compared to ELISA
  - ~4% of the cost
- Comparable sample requirements
- Time savings over the ELISA
  - >2 hours savings
- Assay volumes scalable to screening levels
- Easy measurement of insulin levels across multiple systems
  - *In vivo*: rodent and human plasma or serum samples
  - *In vitro*: pancreatic β cells
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