Introduction

The metabolism involved in the regulation of food intake and processing is one of the most fundamental in all multi-cellular life forms. In mammals, the main source of energy obtained from food is glucose. As such, its abundance and distribution need careful regulation.

The primary mediators of glucose metabolism are hormones known as insulin and glucagon. These act as complementary modulators to regulate the amount of glucose in blood and tissues. High levels of insulin are generated when glucose is abundant. This hormone promotes cell incorporation of glucose as energy, and removes excess glucose by stimulating fatty acid synthesis or stimulating excretion. In contrast, high levels of glucagon are present when glucose is scarce. This hormone activates the breakdown of fatty acids and the retention of glucose. Both hormones also regulate each other to maintain homeostasis.

The relative level of the two hormones is a critical indicator of metabolism in humans. Normally, levels of one hormone should be elevated and the other hormone lowered. Simultaneous high levels generally indicate hyperglycemia or Type 2 diabetes, while simultaneous low levels indicate Type 1 diabetes or starvation.
Both regulation and the design of synthetic analogs of these hormones are of prime interest for the pharmaceutical industry. This necessitates the ability to test levels of these hormones in test subjects and in biochemical assays. Because the ratio of insulin vs. glucagon is critical, a test that can manage both targets in a single assay with ease-of-use and high sensitivity is needed.

AlphaPlex™ offers a complete assay for all-in-one-well measurement of both insulin and glucagon in biological samples (Figure 1). The insulin assay generates a specific signal at 545 nm (from Terbium-based beads), while the glucagon assay generates signal at 615 nm (from Europium-based beads). The assay is simple (only two to three steps), requires very low amounts of sample, and demonstrates good sensitivity.

Materials and Methods

Required material:
- AlphaLISA® glucagon detection kit (PerkinElmer #AL312)
- AlphaPlex insulin detection kit (PerkinElmer #AP204TB)
- 384-well Optiplate™ assay plates (PerkinElmer #6007290)
- AlphaScreen® mirror block (PerkinElmer #2101-4010) (for sequential reads)
- AlphaPlex Tb-Eu dual mirror block (PerkinElmer #2101-5900)
- Alpha filter for Europium (PerkinElmer #5100-2090)
- Alpha filter for Terbium (PerkinElmer #2101-5930)
- TopSeal®-A (PerkinElmer #6050185)
- EnVision® multilabel plate reader with Alpha standard module and filter options (model 2101, 2103, 2104)*

*EnVision systems that are not already equipped with Alpha or AlphaPlex detection technology can be upgraded. Please contact your local sales or service representative for details.

Protocol

1. Preparation of 1X AlphaLISA HiBlock Buffer:
- Add 1 mL of 10X AlphaLISA HiBlock Buffer to 9 mL H2O.

2. Preparation of Insulin and/or Glucagon analyte standard dilutions:
- Insulin and Glucagon analytes are provided at 100 µg/mL. The first point of the curve is 1 µg/mL, so a 100X dilution of the stock solution is required.
- Prepare standard dilutions as follows (change tips between each standard dilution) in 1X AlphaLISA HiBlock Buffer or sample-matching matrix of choice (such as charcoal-stripped fetal bovine serum).

3. Preparation of 5X AlphaLISA Anti-Glucagon Acceptor beads and AlphaPlex Anti-Insulin Acceptor beads mix (50 µg/mL):
- Add 15 µL of 5 mg/mL AlphaLISA Anti-Glucagon Acceptor beads and 15 µL of 5 mg/mL AlphaPlex Anti-Insulin Acceptor beads to 1470 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.
4. Preparation of 5X AlphaLISA Biotinylated Anti-Glucagon Antibody (15 nM) and Biotinylated Anti-Insulin Antibody (5 nM):
   • Add 45 µL of 500 nM Biotinylated Anti-Glucagon Antibody and 15 µL of 500 nM Biotinylated Anti-Insulin Antibody to 1440 µL of 1X AlphaLISA HiBlock Buffer. Prepare just before use.

5. Preparation of 2X Streptavidin (SA) Donor beads (80 µg/mL):
   Keep the beads under subdued laboratory lighting.
   • Add 48 µL of 5 mg/mL SA-Donor beads to 2952 µL of 1X AlphaLISA HiBlock Buffer.

6. Samples: If applicable, dilute samples to be tested in diluent (e.g. 1X AlphaLISA HiBlock Buffer).
   • Add 48 µL of 5 mg/mL SA-Donor beads to 2952 µL of 1X AlphaLISA HiBlock Buffer.

7. In a 96- or 384-well microplate:
   - Add 5 µL of each analyte standard dilution or 5 µL of sample
   - Add 10 µL of (freshly prepared) Alpha anti-analyte Acceptor bead mix (10 µg/mL final of each bead)
   - Incubate 30 min
   - Add 10 µL of biotinylated anti-analyte antibody mix (glucagon at 3 nM final; insulin at 1 nM final)
   - Incubate 60 min at 23 °C in the dark
   - Add 25 µL of 2X SA-Donor beads (40 µg/mL final)
   - Incubate 30 min at 23 °C in the dark
   - Read using EnVision Alpha Reader

**Figure 3. Flowchart for Alpha duplexing assay.**

**Programming the EnVision Plate Reader for Duplex Operation**

There are two options available to read AlphaPlex depending on the EnVision model. On the one-detector model (EnVision XCite), only the sequential mode is available. On the two detector model (EnVision HTS), it is also possible to read both colors simultaneously for faster measurement. In the simultaneous mode, a special dichroic mirror is needed, whereas the standard AlphaScreen mirror can be used in the sequential mode. Suitable emission filters are needed independent of the measuring mode.

Instructions for installing the mirrors and filters, and setting up the detection protocols, can be found in the EnVision AlphaPlex Instrument Set-Up Guide.

**Data Analysis**

A spreadsheet is used to analyze the data. This worksheet can define the optical crosstalk between the Europium anti-glucagon beads and the Terbium anti-insulin beads. The worksheet can also be used once the crosstalk has been defined to correct the data from any experiment both for standard curve and samples.

Once the crosstalk is measured and the data corrected, the following procedure is used:

- Calculate the average count value for the background wells.
- Generate a standard curve by plotting the AlphaLISA counts versus the concentration of analyte. A log scale can be used for either or both axes. No additional data transformation is required.
- Analyze data according to a nonlinear regression using the 4-parameter logistic equation (sigmoidal dose-response curve with variable slope) and a 1/Y2 data weighting (the values at maximal concentrations of analyte after the hook point should be removed for correct analysis).
- Calculate LDL by interpolating the average background counts (12 wells without analyte) + 3 x standard deviation value [average background counts + (3xSD)] on the standard curve.
- The LLOQ as measured here is calculated by interpolating the average background counts (12 wells without analyte) + 10 x standard deviation value [(average background counts + (10 x SD)] on the standard curve. Alternatively, the true LLOQ can be determined by spiking known concentrations of analyte in the matrix and measuring the percent recovery, and then determining the minimal amount of spiked analyte that can be quantified within a given limit (usually +/- 20% or 30% of the real concentration).
- Interpolate the concentration of analyte contained in the samples from the standard curve.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

**Results**

The first experiments performed are intended to ensure that the kits are selective for their own target and do not recognize the other analyte. If that was to occur, it would make calculation of crosstalk and correction factors very difficult. Each kit was tested using standard curves for only their target or only the other target (Figure 4).

The results show that both assay kits are very selective for their own target. Next, we verified that the measurement of one analyte when both kits were combined was not affected. To do this, the lower limit of detection of both kits were measured when used alone, or mixed with the components of the other kit (Table 2).

<table>
<thead>
<tr>
<th>Table 2. Lower limit of detection (LDL) for each analyte alone vs. mixed with reagents from the duplexing kit.</th>
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<tbody>
<tr>
<td></td>
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<tr>
<td>LDL (pg/mL) kit alone</td>
</tr>
<tr>
<td>LDL (pg/mL) both kits mixed</td>
</tr>
</tbody>
</table>
These results indicate that measurements from standalone kits do not change when the duplexing reagents are added. Experiments were then performed to measure optical crosstalk (i.e., the signal from one assay that generates a spurious signal in the other assay derived purely due to an optical signal being measured in the other channel). This originates either from “leaky” filters or from the presence of small emission at the measured wavelengths by the lanthanides used.

To perform these experiments, one standard curve for insulin and one standard curve for glucagon were performed in the presence of both detection Acceptor beads and both biotinylated antibodies. These assays were read at both wavelengths of emission (Figure 5).

These results show clearly that there is a small amount of optical crosstalk between the two channels. The results were then adjusted using the spreadsheet to measure the correction factor and applying the correction factor to the data. The results show that the correction factor for 545 channel leakage into the 615 channel is 0.62%, while the leakage of the 615 channel into the 545 channel is 3.22%. Results for the corrected curves are shown in Figure 6.
These data show that the crosstalk signal has been corrected. This eliminates the possibility that a high level of one analyte present in the assay is generating spurious signal for the other analyte.

To confirm that the crosstalk correction did not skew results, we performed a spike and recovery assay with insulin and glucagon in concentrations simulating four conditions:

1. High levels of both hormones (10 ng/mL of each)
2. High levels of insulin (10 ng/mL of insulin and 100 pg/mL of glucagon)
3. High levels of glucagon (100 pg/mL of insulin and 10 ng/mL of glucagon)
4. Low levels of both hormones (100 pg/mL of each)

The data were then plotted on standard curves to interpolate the amounts detected by the kits compared to the actual amounts (recovery). We observed that the amount of glucagon recovered at low concentrations is significantly affected by the signal of high amounts of insulin in the other channel (Table 3).

Using the corrected standard curves, the recovery rates are now as expected (Table 4). This demonstrates the importance of applying the correct correction factor when analyzing data.

A final set of experiments (data not shown) demonstrated that as long as a particular assay is performed using the same instrument and mirrors/filters, there is no change in the correction factor measured. Therefore, correction experiments can be performed only once, and the correction factor applied to all subsequent uses of the kit.

### Table 3. Spike-and-recovery results without applying correction factor.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Insulin Recovery (%)</th>
<th>Glucagon Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (10 ng/mL) / Glucagon (10 ng/mL)</td>
<td>89%</td>
<td>118%</td>
</tr>
<tr>
<td>Insulin (100 pg/mL) / Glucagon (10 ng/mL)</td>
<td>105%</td>
<td>104%</td>
</tr>
<tr>
<td>Insulin (10 ng/mL) / Glucagon (100 pg/mL)</td>
<td>87%</td>
<td>169%</td>
</tr>
<tr>
<td>Insulin (100 pg/mL) / Glucagon (100 pg/mL)</td>
<td>100%</td>
<td>102%</td>
</tr>
</tbody>
</table>

### Table 4. Spike-and-recovery results using correction factor.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Insulin Recovery (%)</th>
<th>Glucagon Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (10 ng/mL) / Glucagon (10 ng/mL)</td>
<td>126%</td>
<td>125%</td>
</tr>
<tr>
<td>Insulin (1 ng/mL) / Glucagon (10 ng/mL)</td>
<td>97%</td>
<td>76%</td>
</tr>
<tr>
<td>Insulin (10 ng/mL) / Glucagon (1 ng/mL)</td>
<td>106%</td>
<td>103%</td>
</tr>
<tr>
<td>Insulin (1 ng/mL) / Glucagon (1 ng/mL)</td>
<td>114%</td>
<td>117%</td>
</tr>
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### Conclusions

These data demonstrate duplexing of important targets in the metabolic pathways of glucose homeostasis using AlphaPlex technology. These results show that the assay can be performed easily with a short series of preliminary experiments on the EnVision multilabel plate reader, and illustrate the necessary protocols for Alpha duplexing using EnVision Alpha standard.