Quantifying Insulin and Glucagon in Human Serum with LANCE Ultra TR-FRET

Introduction

Insulin and glucagon are small polypeptide hormones that are secreted by the pancreas in response to changes in blood glucose levels. Insulin is released when blood glucose rises and is responsible for switching from a glycolytic metabolism to gluconeogenesis, while glucagon is released as blood glucose drops. These key hormones can be decoupled from blood sugar changes in many metabolic diseases such as type 2 diabetes and autoimmune diseases such as type 1 diabetes. As preclinical biomarkers, insulin and glucagon can be found in various biological samples such as plasma, serum, and tissue homogenates. These sample types usually contain high concentrations of proteins that can interfere with homogeneous assay technologies and often need to be analyzed with tedious, wash-based assays to remove these extraneous components.

The LANCE® Ultra TR-FRET assay format affords a simple, no wash strategy to detect and quantify key proteins. This technology uses detection antibodies coupled to fluorescence dyes that have specific excitation and emission wavelengths designed to yield a robust signal, while the time-resolved emission reading removes background from any interfering fluorescence molecules (Figure 1). A modified protocol was developed to utilize serum samples in the LANCE Ultra TR-FRET insulin and glucagon assays. This modified protocol uses two-thirds less sample volume than the recommended assay conditions, which saves on costly and precious biological samples. Further, interference from serum components could be mitigated by diluting samples with LANCE Ultra HiBlock buffer, as this diluent yields a well-fit sigmoidal analyte standard curve and is shown to be a proper match for serum samples.
Figure 2. Workflow for 5 µL serum sample with the LANCE Ultra TR-FRET insulin and glucagon assays. The incubation times are specific for each assay (insulin: 18 hours, glucagon: 1 hour).

Materials and Methods

Reagents

- LANCE Ultra TR-FRET Insulin Detection Kit (PerkinElmer, #TRF1204)
- LANCE Ultra TR-FRET Glucagon Detection Kit (PerkinElmer, #TRF1312)
- LANCE Ultra 5X HiBlock Buffer (PerkinElmer, #TRF1011C)
- LANCE 10X Detection Buffer (PerkinElmer, #CR97-100C)
- Normal Single Donor Human Serum (BioreclamationIVT)
- Diseased Single Donor Human Serum (BioreclamationIVT, type 2 diabetes and chronic kidney disease)
- Pooled Normal Human Serum (Sigma, #H4522)
- OptiPlate™-384 (PerkinElmer, #6007290)
- TopSeal™-A Plus (PerkinElmer, #6050185)

Data Collection and Analysis

All LANCE Ultra TR-FRET assays were read on the EnVision® multimode plate reader equipped with the TRF laser module. All data shown are the ratiometric measurements of 665 nm emission/615 nm fluorescent emission multiplied by 10,000 to normalize for the Europium fluorophore. Insulin data are reported as µU/mL where 1.36 Units (U) is equivalent to 1 International Unit (IU). All data were interpolated from a standard curve diluted in LANCE Ultra HiBlock Buffer. LANCE Ultra TR-FRET kits use the following equations to define the lower limits of the assay:

Lower detection limit (LDL) = Interpolated value of the average measurement of 12 background wells + 2× standard deviation of 12 background wells

Lower limit of quantification (LLOQ) = Interpolated value of the average measurement of 12 background wells + 10× standard deviation of 12 background wells

Modified Sample Volumes for Human Serum

The LANCE Ultra TR-FRET insulin assay and LANCE Ultra TR-FRET glucagon assay have a recommended sample volume of 15 µL with a 5 µL addition of Europium- and ULight-labeled antibodies as a 4X mix. To reduce sample requirements and unnecessary large dilutions of sample, the protocol was modified to use a 5 µL serum sample with a 1.3X mix of Eu- and ULight-labeled antibodies in 15 µL (Figure 2). Using this new protocol, the analyte standard curve was run with the modified sample volume (5 µL) while preserving recommended concentrations of detection antibodies (Figure 3). With these modifications, the insulin assay has a LDL and LLOQ of 6.05 and 20.9 µU/mL respectively, while the glucagon assay has a LDL and LLOQ of 36.8 and 195.8 pg/mL respectively. Decreasing the volume of analyte in the standard curve will lessen the sensitivity of the assay, however it remains well within the range to detect analyte in biological samples. This assay protocol uses the same concentration of antibody as directed in the original technical data sheet (0.3 nM Eu-labeled antibody and 3 nM ULight-labeled antibody) and maintains the recommended incubation time of 18 hours for the insulin assay and 1 hour for the glucagon assay.
Figure 3. LANCE Ultra TR-FRET insulin (A) and glucagon (B) standard curves following the lower volume (5 µL) sample protocol with analyte diluted in LANCE Ultra HiBlock buffer.

Results

Serum Samples Diluted in LANCE Ultra HiBlock Buffer Give Dilutional Linearity

To be able to accurately quantify serum samples with the LANCE Ultra insulin and LANCE Ultra glucagon kits, a diluent must be chosen for both the analyte standard curve and to dilute samples to ensure they are within the range of quantification. While human serum is a protein-rich matrix, LANCE Ultra HiBlock buffer acts as a suitable diluent for such samples. As a 75 µU/mL insulin sample in serum is serially diluted 2-fold, the interpolated values from the standard curve give a linear response (Figure 4A). Identical steps were performed with a 3 ng/mL glucagon spiked serum sample and diluted 2, 4, 8, and 16-fold in LANCE Ultra HiBlock buffer (Figure 4B). It is important to note the pooled human serum had quantities of the desired analytes below or near the assay LDL, so the detection of analyte was solely from the intended spike. Dilutional linearity testing is best performed with an analyte-depleted sample or matrix without high levels of analyte to test how non-analyte components affect the assay performance. To ensure absolute linearity, an $R^2$ of $>0.995$ calculated from a linear regression is recommended. From the data in Figure 4, it is recommended that serum samples used for measuring insulin and glucagon be diluted in 2-fold (equal volume) with LANCE Ultra

Figure 4. Dilutional Linearity for the LANCE Ultra insulin (A) and glucagon (B) assays. Serum spiked with 75 µU/mL insulin or 3 ng/mL glucagon was subsequently diluted 2-fold with LANCE Ultra HiBlock buffer. A linear regression to give an $R^2$ value of $>0.995$ is in blue; the undiluted sample is in red and is excluded from the linear fit.
Recovery Percentages of Spiked Analyte Samples in Pooled Normal Serum

Mock samples were created by spiking the analyte into pooled normal human serum and diluting with the recommended diluent: LANCE Ultra HiBlock buffer. These samples were then diluted 2-fold, 4-fold, and 8-fold and subsequently run in the LANCE Ultra detection kits to determine if the quantification would correlate to the known concentration. LANCE readings were interpolated from the standard curve, multiplied by the dilution factor, and then corrected for the endogenous (no spiked analyte) baseline sample (Figure 5). All serum samples that were diluted 2-fold or greater fell within the accepted range of 70-130% recovery of observed analyte spike in diluent. Undiluted spiked serum samples systematically show greater than the expected values. This can be due to components in the serum that interfere with the assay or a significant level of analyte in the pooled normal serum. To ensure all serum samples can be quantified properly, it is recommended to dilute serum samples 2-fold before running in the LANCE Ultra TR-FRET insulin and glucagon kits.

![Figure 5. Recovery of spiked insulin (A) and glucagon (B) in pooled normal serum and diluent as a control. Three different concentrations of analyte were spiked into pooled normal serum and diluted with the recommended diluent (LANCE Ultra HiBlock buffer). To be considered a good diluent, recovery must fall between 70-130% of the observed concentration (shaded red area). When compared to a spiked analyte sample in diluent, samples that were diluted 2-fold gave the best recovery.](image-url)
Insulin and Glucagon Detection in Patient Serum Samples

Human serum was sourced from healthy volunteers and diseased volunteers that are type 2 diabetics (T2D) with or without chronic kidney disease (CKD). All samples were diluted 2-fold in LANCE Ultra HiBlock buffer then assayed in triplicate using the modified protocol of the LANCE Ultra TR-FRET insulin and glucagon kits (Figure 6). Insulin or glucagon concentrations were interpolated from the standard curve and then were corrected for the dilution factor. All individuals with T2D had elevated serum insulin of > 80 µU/mL, whereas healthy individuals showed a basal insulin level between 26.7-43.9 µU/mL. Elevated insulin levels were seen in both samples from male and female T2D individuals.

Glucagon is only elevated under fasting conditions or due to severe pathological impairments. Individuals with T2D were found to have levels of glucagon below the assay LDL, suggesting blood sugar levels could be high. One of the patients with chronic kidney disease had a glucagon concentration of 521.7 pg/mL, while the other had a glucagon level that fell within the range of the healthy volunteers. Healthy individuals had a wide range of glucagon levels (184.5 - 406.6 pg/mL), which shows how vastly different glucagon levels can range without standardized fasting prior to sample collection.

Figure 6. Insulin (A) and glucagon (B) levels in healthy volunteers, type 2 diabetics, and type 2 diabetics with chronic kidney disease.
Conclusion

The detection of insulin and glucagon can be a prevalent readout for many metabolic and endocrinial diseases. As a biomarker that can be easily changed by blood glucose levels, these metabolites can shed insight on the function of the pancreas under fasting or non-fasting conditions. Utilizing the LANCE Ultra TR-FRET insulin and glucagon kits provides a fast approach to quantify analyte levels in human serum. With reduced sample volume requirements for human serum samples, precious material can be saved for other purposes such as additional analyte testing. From the data presented, it is recommended serum samples be diluted 2-fold with LANCE Ultra HiBlock buffer to reduce any interference from the serum components when quantifying insulin or glucagon. This will ensure proper interpolation of values from the analyte standard curve run in the same diluent. The homogeneous, no wash LANCE Ultra TR-FRET technology gives superb detection of high value protein biomarkers.

References

2. PerkinElmer LANCE Ultra Insulin Technical Data Sheet.