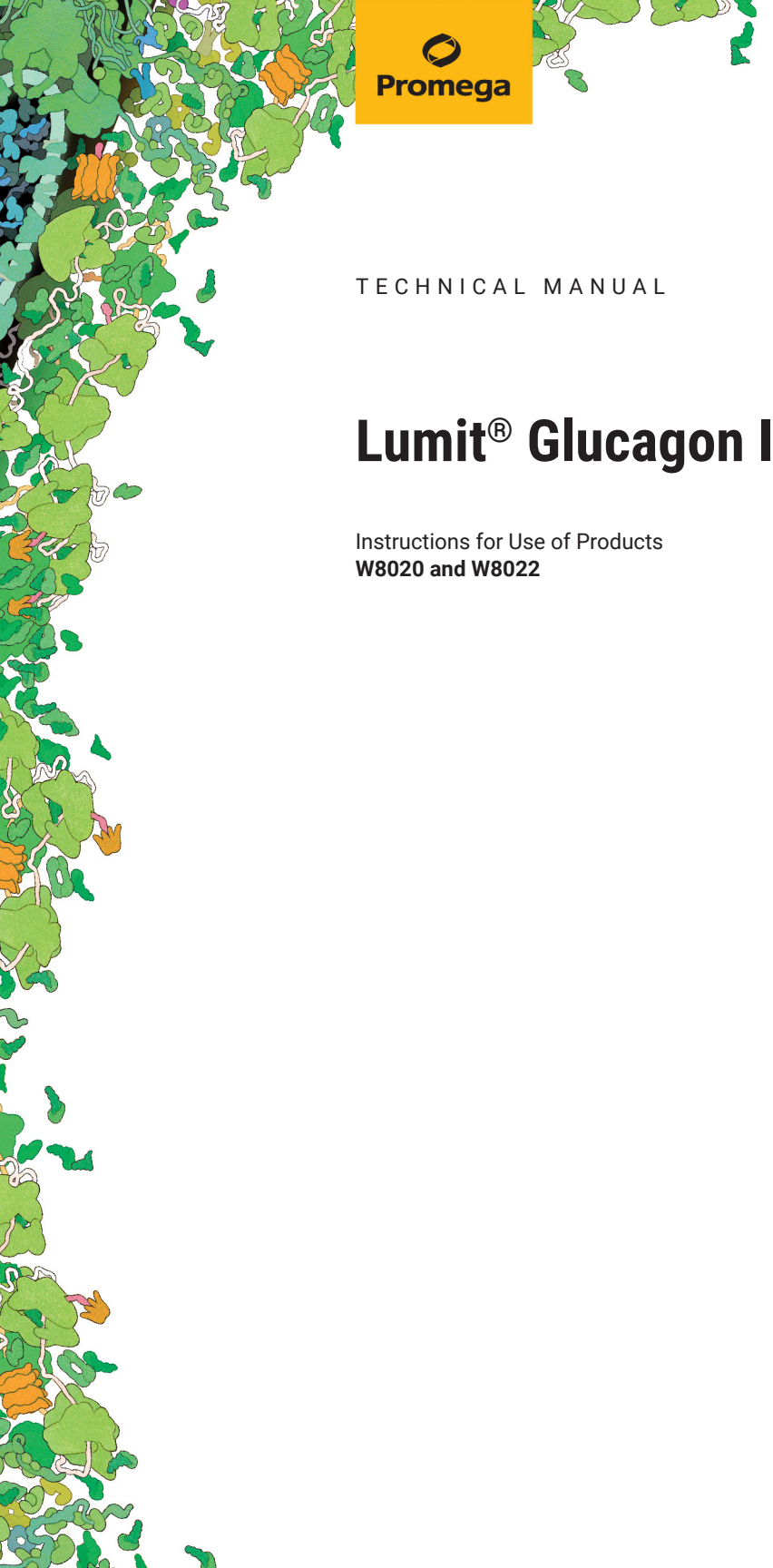


TECHNICAL MANUAL

Lumit® Glucagon Immunoassay

Instructions for Use of Products
W8020 and W8022

Revised 11/25
TM682

Promega

Lumit® Glucagon Immunoassay

All technical literature is available at: www.promega.com/protocols/
Visit the website to verify that you are using the most current version of this Technical Manual.
Email Promega Technical Services if you have questions on use of this system: techserv@promega.com

Promega

1. Description

The Lumit® Glucagon Immunoassay[a,b] is a homogeneous, bioluminescent assay for detecting glucagon in solution without the need for wash steps. This immunoassay has been developed for use with cell culture samples to measure glucagon secreted from cell lines or islets. The assay has a range of 2pM–2nM glucagon and is compatible with samples from human, mouse and rat cells. (See representative data in Section 6.)

Insulin and glucagon are small peptide hormones crucial for glucose homeostasis. Glucagon is synthesized by pancreatic islet alpha cells and also by intestinal and neuronal cells (1). It is processed from a longer proglucagon peptide into the mature 29 amino acid form. Glucagon functions to restore glucose levels in the blood stream by acting on the liver to induce gluconeogenesis and reduce glycogen synthesis. Though diabetes research has historically focused on insulin, it is increasingly recognized that glucagon has a role in diabetes as well (2).

Assay Principle

The Lumit® Glucagon Immunoassay is based on NanoLuc® Binary Technology (NanoBiT®). NanoBiT is a luminescent structural complementation system designed for biomolecular interaction studies (3,4). The system is composed of two subunits, Large BiT (LgBiT; 18kDa) and Small BiT (SmBiT; 11 amino acid peptide), that were optimized for stability and minimal spontaneous association. In this assay, a sample is incubated with a pair of anti-glucagon monoclonal antibodies covalently labeled with SmBiT or LgBiT. When the labeled antibodies bind to glucagon, the complementary LgBiT and SmBiT are brought into proximity, thereby reconstituting NanoBiT® enzyme and generating luminescence in the presence of the Lumit® substrate. The luminescent signal is directly proportional to the amount of glucagon present in the sample (Figure 1).

Assay Format

The no-wash, in-solution protocol for this immunoassay (Figure 2) offers flexibility in terms of both the number of data points that can be assayed in an experiment and the volume of sample that can be used. It is compatible with various multiwell formats (e.g., 96- and 384-well) and multiple sample volumes (e.g., 5–100µl).

Customize the assay format to your needs, provided you maintain the ratio of sample to reagent volume. The number of data points that can be collected using several sample volumes are listed in Table 1. For example, one kit (Cat.# W8020, 100–400 assays) contains sufficient reagents for 100 assays in 96-well plates (50µl sample volume) or 400 assays in 384-well plates (12.5µl sample volume).

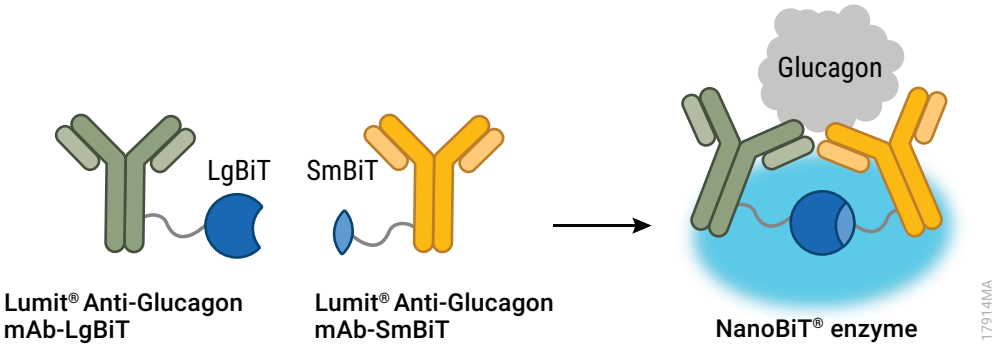


Figure 1. Assay principle. Primary monoclonal antibodies to glucagon are labeled with SmBiT and LgBiT. In the presence of glucagon, SmBiT and LgBiT are brought into proximity, forming active NanoBiT® enzyme. When Lumit® Detection Reagent B is added, a bright luminescent signal is generated.

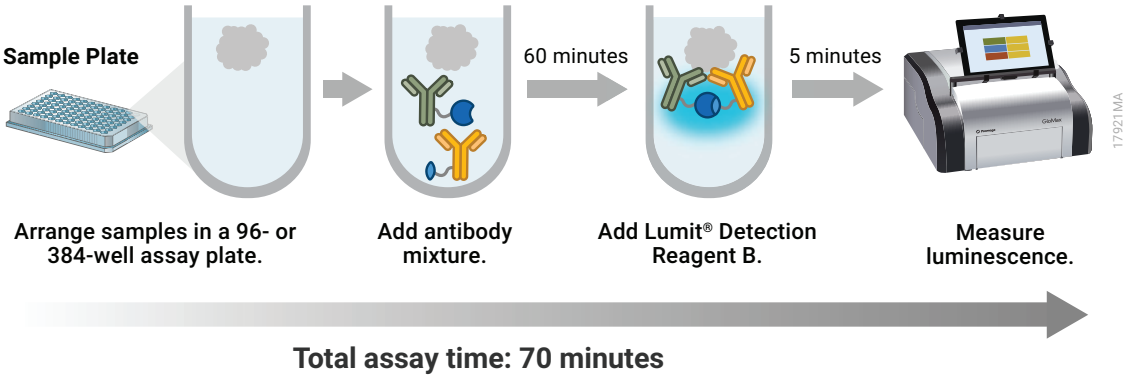


Figure 2. Assay protocol. The Lumit® Glucagon Immunoassay is performed using samples containing glucagon in a 96- or 384-well assay plate. The Lumit® Immunoassay protocol does not require wash steps and is complete in 70 minutes.

Promega

1. Description (continued)

Table 1. Multiwell Assay Formats.

	Glucagon Sample Volume	Anti-Glucagon Antibody Mix Volume	Lumit® Detection Reagent B Volume	Total Reaction Volume	Recommended Multiwell Plate	Cat.# W8020 Number of Assays	Cat.# W8022 Number of Assays
	100µl	100µl	50µl	250µl	96-well	50	250
*	50µl	50µl	25µl	125µl	96-well	100	500
	25µl	25µl	12.5µl	62.5µl	96-well half area	200	1,000
*	12.5µl	12.5µl	6.25µl	31.1µl	96-well half area 384-well	400	2,000
	10µl	10µl	5µl	25µl	96-well half area 384-well	500	2,500
	5µl	5µl	2.5µl	12.5µl	384-well, low volume	1,000	5,000

Note: In addition to the formats specified in the kit description (* rows), the assay setup is highly flexible and can be scaled up or down. The assay can be adapted to many sample volumes provided the 1:1:0.5 ratio of Sample Volume: Anti-Glucagon Antibody Mix:Lumit® Detection Reagent B is maintained. See Section 8.A for more information about multiwell plates.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Lumit® Glucagon Immunoassay	**100–400 assays**	**W8020**

Sufficient for 100 assays in 96-well plates or 400 assays in 384-well plates. Includes:

- 15µl Lumit® Anti-Glucagon mAb-SmBiT, 400X
- 15µl Lumit® Anti-Glucagon mAb-LgBiT, 400X
- 5.5ml Lumit® Antibody Dilution Buffer B
- 160µl Lumit® Detection Substrate B
- 3.2ml Lumit® Detection Buffer B

PRODUCT	SIZE	CAT.#
Lumit® Glucagon Immunoassay	**500–2,000 assays**	**W8022**

Sufficient for 500 assays in 96-well plates or 2,000 assays in 384-well plates. Includes:

- 5 × 15µl Lumit® Anti-Glucagon mAb-SmBiT, 400X
- 5 × 15µl Lumit® Anti-Glucagon mAb-LgBiT, 400X
- 5 × 5.5ml Lumit® Antibody Dilution Buffer B
- 5 × 160µl Lumit® Detection Substrate B
- 5 × 3.2ml Lumit® Detection Buffer B

Storage Conditions: Store all components at –30°C to –10°C, with the following exceptions. Do not freeze-thaw components more than three times.

Prepared Reagents Storage Conditions: After thawing, store the Lumit® Antibody Dilution Buffer B at +2°C to +10°C or –30°C to –10°C. Store the Lumit® Detection Buffer B at +15°C to +30°C.

3. Before You Begin

Preparing a Glucagon Positive Control

A glucagon positive control can be used to assess the assay performance as well as measure the glucagon amount in samples. We recommend preparing a solution of glucagon that can be included as a positive control in each experiment. Instructions for preparing and diluting a stock of glucagon positive control are provided in Section 4.B.

Reagent Preparation

The Lumit® Antibody Dilution Buffer B and Lumit® Detection Buffer B must be thawed and equilibrated to room temperature for the experiment. The Lumit® Detection Buffer B can be thawed overnight at room temperature and stored at room temperature once opened. The Lumit® Antibody Dilution Buffer can be thawed overnight at 4°C and then equilibrated to room temperature on the day of the experiment. Store at either +2°C to +10°C or –30°C to –10°C once opened. Please note that the Lumit® Antibody Dilution Buffer B is sensitive to contamination; be careful to maintain reagent sterility.

To conserve components, prepare only the amount of anti-glucagon antibody mixture and Lumit® Detection Reagent B needed for the number of reactions in the experiment. When calculating the amount of anti-glucagon antibody mixture and Lumit® Detection Reagent B needed for the number of samples that will be tested, add some extra (e.g., one to two assays) to compensate for pipetting variability. Also include glucagon positive and negative control samples in your calculations. For negative controls, use wells containing only buffer that can be used to measure assay background (background controls).

Prepare the anti-glucagon antibody mixture, Lumit® Detection Reagent B and glucagon positive control dilutions fresh on the day of use. Do not store and reuse these preparations.

Promega

3. Before You Begin (continued)

Sample Buffer Considerations

The assay is compatible with commonly used PBS and Krebs-Ringer Bicarbonate (KRB) buffer formulations. KRB often forms the basis for buffers used to collect secreted insulin and glucagon samples in glucose-stimulated insulin secretion (GSIS) or pancreatic islet secretion experiments. These sample buffers typically include BSA. To avoid interference with Lumit® Immunoassay chemistry, the recommended BSA concentrations are 0.1–0.4%, with 0.1% BSA being the preferred concentration. An example sample buffer formulation can be found in Section 8.A.

As the exact buffer composition and BSA concentration will affect the absolute value of the relative light units (RLU), all samples and dilutions of the glucagon positive control should be prepared in the same buffer.

Use personal protective equipment and follow your institution's safety guidelines and disposal requirements when working with biohazardous materials such as cells and cell culture reagents.

Materials to Be Supplied by the User

- white, multiwell assay plates compatible with a luminometer (solid white or white with clear bottom; also see Section 8.A.)
- multichannel pipette
- pipette tips, preferably with aerosol filters
- multichamber, dilution reservoir (e.g., Dilux® D-1002) or tubes for dilutions
- reagent reservoir trays (e.g., Thermo Fisher Scientific Cat.# 8095)
- plate shaker for mixing multiwell plates
- luminometer capable of reading multiwell plates (e.g., GloMax® Discover Microplate Reader, Cat.# GM3000)

4. Protocol for Measuring Glucagon

This protocol describes the assay of samples containing glucagon in multiwell assay plates based on a glucagon sample volume of 50µl assayed in a 96-well plate. For this sample volume, the reaction includes: 50µl of glucagon sample + 50µl of antibody mixture + 25µl of Lumit® Detection Reagent B.

However, other sample volumes and plates can be used if the 1:1:0.5 volume ratio of glucagon sample:antibody mixture:Lumit® Detection Reagent B is maintained, e.g., 12.5µl + 12.5µl + 6.25µl in 384-well plates. The volumes of antibody mixture and Lumit® Detection Reagent B needed for additional sample volumes can be found in Table 1.

4.A. Preparing Samples

This assay has been developed for use with cell culture samples to measure glucagon secretion from cell lines or islets.

Assay performance with additional sample types must be determined by the user.

1. Collect glucagon samples in a buffer compatible with the assay (see Sample Buffer Considerations above).
2. Dilute samples into the assay range (2pM–2nM) if needed.

4.B. Preparing and Diluting a Glucagon Positive Control

A glucagon positive control can be used to assess the assay performance as well as measure the glucagon amount in samples. Before beginning experiments, a stock of glucagon positive control can be prepared, aliquoted and frozen. We prepare a 500µM stock of glucagon using commercially available glucagon powder (e.g., Sigma Cat.# G2044, synthetic glucagon). Resuspend glucagon in 0.01N HCl by adding 0.575ml of 0.01N HCl to 1mg of glucagon. Dispense into tubes (e.g., 25 to 50µl per tube) and store at –30°C to –10°C for up to 3 months. Do not freeze-thaw more than three times.

To measure the amount of glucagon in samples, prepare a titration curve using the glucagon positive control. The glucagon should be diluted to at least 2nM to establish the upper limit of the titration curve. Two- or threefold serial dilutions can then be prepared for an 8- or 12-point curve. This protocol is for generating a 12-point curve with twofold dilutions starting with a 500µM stock of glucagon. Representative data are shown in Figure 4 and Table 2.

As different buffers and their components, such as BSA, can affect light output, it is important that the glucagon dilutions be prepared in the same buffer as the samples.

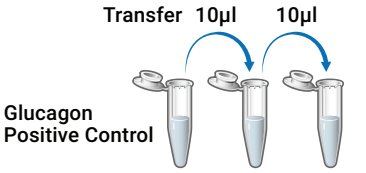
Wells containing only buffer should be included in all experiments as background controls. These wells are used to measure the assay background signal and to calculate the signal-to-background and signal-to-noise ratios.

1. If previously prepared and frozen, thaw the glucagon positive control immediately before use.
2. Briefly centrifuge the tube to collect all contents at the bottom of the tube before opening.
3. Mix by gently vortexing.
4. Prepare an initial concentration of 5µM glucagon by diluting the glucagon positive control 100-fold into the same buffer solution as the test samples. Follow this with an additional 100-fold dilution and 25-fold dilution. Recommended volumes are below (see Figure 3).
   - Prepare 5µM glucagon by adding 10µl of glucagon positive control to 990µl of buffer.
   - Prepare 50nM glucagon by adding 10µl of 5µM glucagon to 990µl of buffer.
   - Prepare 2nM glucagon by adding 40µl of 50nM glucagon to 960µl of buffer.

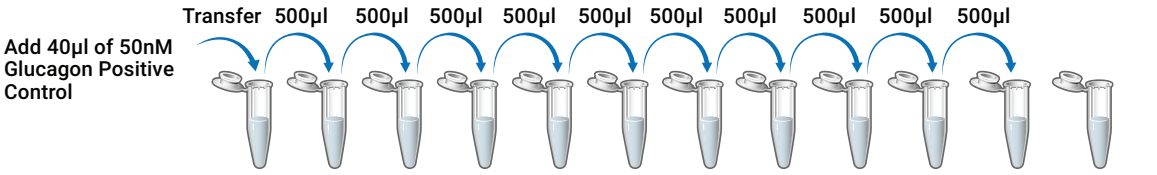
   Note: Mix thoroughly after each dilution. Change pipette tips between each dilution to avoid glucagon carryover and use aerosol filter tips. The range of the assay is large, so carryover from high to low concentrations can compromise the dilution series linearity.
5. Continue to prepare ten twofold serial dilutions of the insulin in tubes or a multichamber dilution reservoir. The last tube or chamber should contain only buffer for the background control.

   Note: Mix each dilution thoroughly before moving to the next tube or chamber. Be careful not to contaminate the background control with glucagon.

4.B. Preparing and Diluting the Glucagon Positive Control (continued)



Buffer		990µl	990µl
Glucagon (µM)	500	5	0.05
Glucagon (nM)	500,000	5,000	50



Buffer	960µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl	500µl
Glucagon (nM)	2.0	1.0	0.5	0.25	0.125	0.0625	0.0313	0.0156	0.0078	0.0039	0.00195	0
Glucagon (pM)	2,000	1,000	500	250	125	62.5	31.2	15.6	7.8	3.9	1.95	0

17922MA

Figure 3. Glucagon positive control dilution series.

4.C. Adding the Samples

1. Add 50µl of each glucagon experimental sample to the wells of a 96-well plate.
2. Add 50µl of each glucagon positive control dilution and background control to the wells of the 96-well plate.

   Note: We recommend assaying each sample, dilution and background control in duplicate or triplicate. If possible, assay samples and controls on the same plate.

4.D. Adding the Anti-Glucagon Antibody Mixture

After adding samples and controls to the assay plate, prepare the anti-glucagon antibody mixture.

1. Use the Lumit® Antibody Dilution Buffer B that has been thawed and equilibrated to room temperature (see Section 3, Reagent Preparation).
2. Remove the Lumit® Anti-Glucagon mAb-SmBiT and Lumit® Anti-Glucagon mAb-LgBiT antibodies from –30°C to –10°C immediately before use and hold on ice.
3. Briefly centrifuge the tubes to collect all contents at the bottom of the tube before opening.
4. Gently vortex the tubes to mix.

5. Immediately prior to use, prepare the anti-glucagon antibody mixture by diluting both antibodies into the Lumit® Antibody Dilution Buffer B. Calculate the amount needed using the table below. The dilution ratio is 2.5µl of each antibody to 1ml of Lumit® Antibody Dilution Buffer B.

Reagent	Volume per Well	Volume per 100 Wells
Lumit® Antibody Dilution Buffer B	50µl	5ml
Lumit® Anti-Glucagon mAb-SmBiT	0.125µl	12.5µl
Lumit® Anti-Glucagon mAb-LgBiT	0.125µl	12.5µl

6. Thoroughly mix the anti-glucagon antibody mixture by gently vortexing.
7. Add 50µl of the antibody mixture to the wells containing glucagon test samples, background controls and positive controls.
8. Gently shake the plate to mix. **Optional:** Briefly mix with a plate shaker (e.g., 20 seconds at 250–350 rpm).
9. Incubate for 60 minutes at room temperature.

4.E. Adding Lumit® Detection Reagent B

Prepare the Lumit® Detection Reagent B while the samples are incubating with the anti-glucagon antibody mixture (Section 4.D). Begin preparations approximately 10 minutes before the end of the incubation period.

1. Use the Lumit® Detection Buffer B that has been thawed and equilibrated to room temperature (see Section 3, Reagent Preparation).
2. Remove the Lumit® Detection Substrate B from –30°C to –10°C storage, mix and briefly centrifuge.
3. Prepare Lumit® Detection Reagent B by making a 20-fold dilution of Lumit® Detection Substrate B in Lumit® Detection Buffer B. Prepare enough volume of Lumit® Detection Reagent B for the number of wells to be assayed.

Reagent	Volume per Well	Volume per 100 Wells
Lumit® Detection Buffer B	23.75µl	2.375ml
Lumit® Detection Substrate B	1.25µl	125µl
Total Volume	25µl	2.5ml

Note: Prepare the reagent just before needed. Once reconstituted, Lumit® Detection Reagent B will lose 10% activity in approximately 3 hours at +10°C to +30°C.

4. Transfer the Lumit® Detection Reagent B to a reagent reservoir tray for easy pipetting.
5. Add 25µl of Lumit® Detection Reagent B to each well.

   Note: Work quickly and efficiently using a multichannel pipette to minimize variability from well-to-well and across the plate.

4.E. Adding Lumit® Detection Reagent B (continued)

6. Gently shake the plate to mix.
   Optional: Briefly mix with a plate shaker (e.g., 20 seconds at 250–350rpm).
7. Incubate 3–5 minutes at room temperature.
8. Read luminescence.

Note: If there is more than one plate in the experiment, add the Lumit® Detection Reagent B to the first plate and read that plate, before adding reagent to the second plate. Continue in this way until all plates have been read. For more information, see Signal Stability in Section 8.A, General Considerations.

5. Assay Controls and Data Analysis

Assay performance can be evaluated using positive controls prepared from the glucagon positive control and negative background controls consisting of wells containing buffer only. For this analysis, a glucagon dilution series is prepared and each concentration assayed in duplicate or triplicate. The data from replicate wells is averaged and plotted. The data are used to define the linear range of the assay and calculate parameters such as signal-to-background ratios, signal-to-noise ratios and sensitivity.

When signals from experimental samples fall within the linear range of the assay, there is a linear relationship between RLU and glucagon concentration, and samples can be directly compared. Results can be described simply in terms of RLU or a sample-to-sample ratio, such as “fold increased glucagon secretion” calculated from a treated and untreated sample. When working with signals close to the background control, first subtract the background control signal from all samples.

Glucagon concentration in experimental samples can be calculated using the linear equation generated by plotting the titration curve of the glucagon positive control. When planning the dilution series, select concentrations that encompass the range of your experimental samples. Each dilution should be assayed in duplicate or triplicate. The signals from replicate wells are averaged and plotted. The graph can then be used to estimate glucagon concentration in experimental samples.

6. Representative Data

6.A. Assay Performance

Titration of Glucagon Positive Control

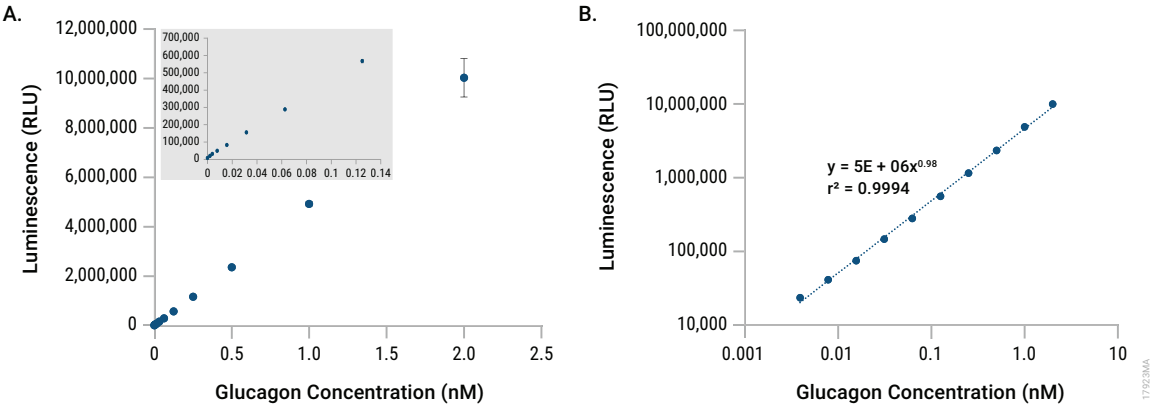


Figure 4. Titration curve for the Lumit® Glucagon Immunoassay. A glucagon positive control was prepared following the instructions in Section 4.B and serially diluted in PBS Buffer + 0.1% BSA. Fifty microliters of each concentration was assayed in triplicate in 96-well plates. The luminescence (RLU) was recorded using a GloMax® Discover System. The average RLU values are in Table 2. Error bars are ±1 standard deviation. **Panel A.** Linear-linear plot; the gray inset graph is an expanded view of the points close to 0. **Panel B.** Log-Log plot with background-subtracted RLU.

Note: These are representative titration curves. Absolute RLU values vary based on many experimental factors. Therefore, they should not be used for interpolation of unknowns. Generate a titration curve for each experiment to interpolate experimental samples.

6.A. Assay Performance (continued)

Table 2. Titration Curve for the Lumit® Glucagon Immunoassay.

	Glucagon (nM)	Glucagon (pM)	Glucagon pg/ml[a]	Average RLU	Standard Deviation	Percent CV	Signal-to-Noise[b]	Signal-to-Background[c]
1	2	2,000	6,960	10,019,750	780,410	7.8	20,600	1,198
2	1	1,000	3,480	4,921,250	88,808	1.8	10,109	588
3	0.5	500	1,740	2,357,000	57,695	2.4	4,833	282
4	0.25	250	870	1,165,500	27,197	2.3	2,381	139
5	0.125	125	435	567,600	10,231	1.8	1,151	68
6	0.0625	63	218	288,275	5,200	1.8	576	34
7	0.0313	31	109	155,150	2,977	1.9	302	19
8	0.0156	16	54	83,132	1,251	1.5	154	10
9	0.00781	8	27	49,512	1,023	2.1	85	6
10	0.00391	4	14	31,895	447	1.4	48	4
11	0.00195	2	7	20,370	324	1.6	25	2
12	0	0	0	8,366	486	5.8	0	1

[a]Calculated using a value of 1nM = 3,480pg/ml.

[b]The signal-to-noise ratios were calculated using the formula: (average signal – average signal of the background control)/ standard deviation of the background control.

[c]Signal divided by signal of the background control (0nM sample).

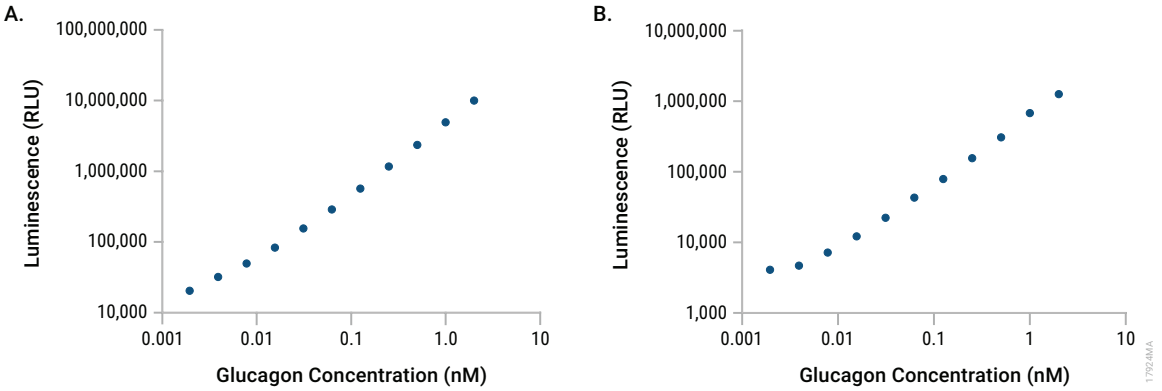


Figure 5. Titration curve for the Lumit® Glucagon Immunoassay using lower assay volumes. A glucagon positive control was serially diluted in PBS Buffer + 0.1% BSA and assayed in quadruplicate in either 96- or 384-well plates. **Panel A.** Assayed 50µl of each dilution in 96-well plates. **Panel B.** Assayed 12.5µl of each dilution in 384-well plates. The luminescence (RLU) was recorded using a GloMax® Discover System. Error bars are ±1 standard deviation.

6.B. Example Data with Islets

Perifusion of Mouse Islets

Perifusion is a powerful method for studying insulin and glucagon secretion over time and in response to sequential treatments. The time-resolved data provides important insights into the islet function. Samples are collected frequently (e.g., every minute) over an extended time (e.g., >1 hour). To obtain the most useful information from the data set, assay all samples. However, the large sample number can make this prohibitive when using traditional ELISA methods. The Lumit® Immunoassay solution-based approach can facilitate analysis. Many samples can be assayed quickly and in 384-well formats. In addition, because minimal sample volumes are needed, collected samples can be split into two wells to assay for both insulin and glucagon using the Lumit® Insulin and Lumit® Glucagon Immunoassay, respectively. For more information on Lumit® Immunoassays, see **www.promega.com/products/immunoassay-elisa/lumit-immunoassays/metabolic-target-research-with-lumit-immunoassays/**

An example of using Lumit® Insulin and Lumit® Glucagon Immunoassays to test mouse islet perifusion samples is shown in Figure 6. The same samples were analyzed for both hormones, allowing the two data sets to be superimposed.

6.B Example Data with Islets (continued)

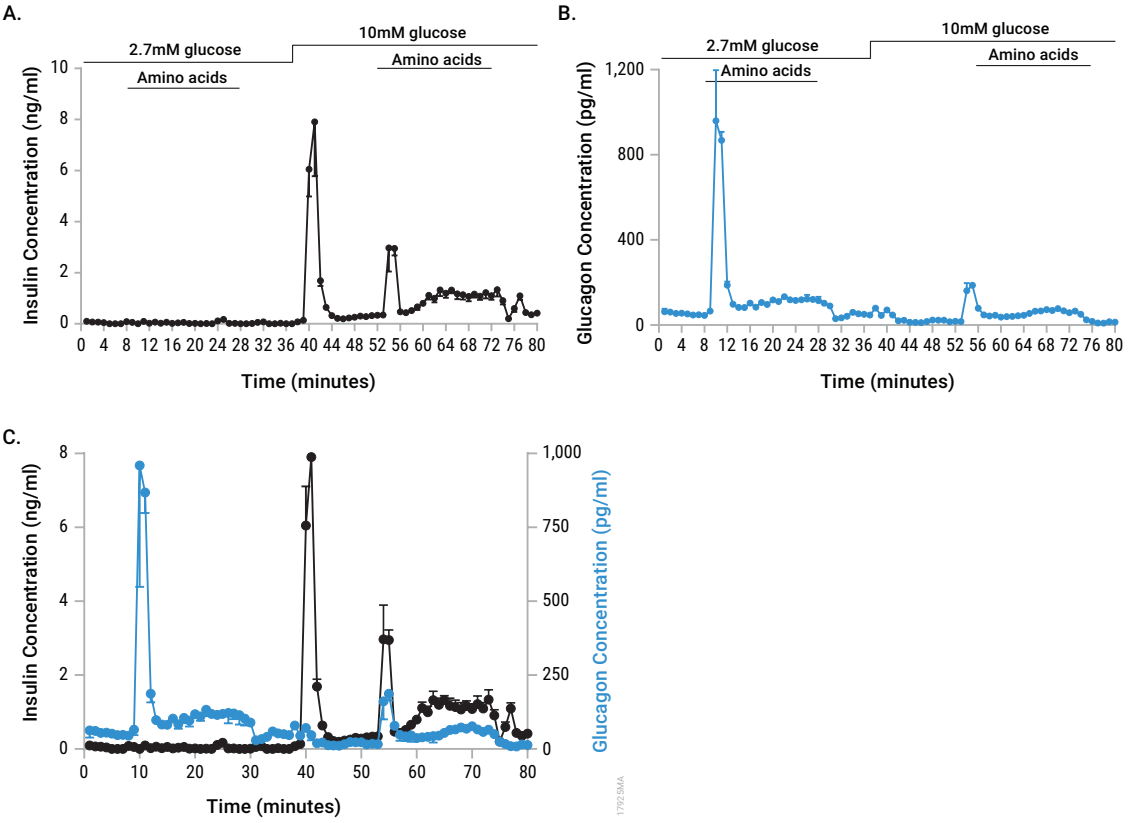


Figure 6. Perifusion experiments: Measuring insulin and glucagon. Insulin and glucagon secretion were measured in samples collected during perifusion experiments. This data was kindly provided by Drs. Hannah Foster and Matthew Merrins, University of Wisconsin VA Hospital, Madison, WI. The samples were collected according to published protocols (5). Briefly, 80 mouse islets were placed in triplicate chambers of a perifusion instrument (Biorep, Miami Lakes, Florida). The islets were treated with 2.7mM glucose and then 10mM glucose, in a combination with an amino acid mixture. Perifusate (100µl) was collected every minute. Ten microliters of each sample were transferred into wells of a 384-well plate and assayed for either insulin (**Panel A**) or glucagon (**Panel B**). **Panel C.** The superimposed data sets. The data is the average of triplicate chambers and the error bars are plus or minus 1 standard deviation.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. Email: **techserv@promega.com**

Symptoms	Causes and Comments
RLUs are not identical to those in this Technical Manual	Bioluminescent signal intensity, e.g., absolute RLU, will vary between laboratories due to several factors, such as specific experimental conditions (buffers, volumes), as well as plates and plate readers. Establish the assay performance in your lab with the sample buffer, plates and instruments you will be using. A glucagon positive control can be used to generate a titration curve that will help establish the parameters expected in your lab. Range and signal-to-background ratio are some parameters that can be noted.
Luminescence for the titration curve is variable	There may be some variation in absolute RLU due to experimental conditions, such as specific buffers used, temperature, etc. Include a titration curve for each experiment, run under the same conditions as the test samples, to measure glucagon concentrations. Though the RLU may vary, the assay range and signal-to-background should still be as previously observed.
The glucagon titration curve is not linear	Mix each dilution thoroughly before moving on to the next tube or chamber. The dilutions should be carefully prepared, avoiding carryover from a higher concentration. The sensitivity of the assay and the broad range (~3 logs) means that any carryover will compromise the linearity of the dilution series. We recommend changing pipette tips after each dilution step. Also, make sure that no glucagon contaminates the background control.
Dilutions at higher concentrations have lower light units than dilutions at lower concentrations	The dilutions at higher concentrations are above the range of the assay and need to be further diluted. This is due to the Hook Effect, which occurs when excess antigen prevents both antibodies from binding to a single antigen thereby preventing the LgBiT and SmBiT from complementing each other.
High background observed	Background can be increased by the spurious association of the antibodies over time. Another cause of high background is glucagon contamination of the buffer or background control wells. Avoid contamination by changing tips often and preparing the glucagon dilution series separately from the Anti-Glucagon Antibody Mixture. Do not create the antibody mixture too far in advance to prevent elevated background.

Promega

8. Appendix

8.A. General Considerations

Sample Buffer Formulations

This is an example of a buffer that is compatible with the Lumit® Glucagon Immunoassay:

Sample Buffer

136mM	NaCl
4.7mM	KCl
1.2mM	$MgSO_4$
1mM	$CaCl_2$
1.2mM	KH_2PO_4
5mM	$NaHCO_3$
10mM	HEPES (pH 7.5)
0.1%	BSA

Species Specificity

These antibodies recognize human, mouse and rat glucagon.

Cross-Reactivity

Cross-reactivity with GLP-1 and oxyntomodulin were determined. All were tested at a range of concentrations up to 2nM and the percent of glucagon light signal was calculated.

Peptide	Glucagon Signal Percent
GLP-1	not detectable over background
Oxyntomodulin	6%

Signal Stability

After the addition of the Lumit® Detection Reagent B, the light signal is stable with a half-life of approximately 1 hour.

There are two approaches to read multiple assay plates to allow for plate-to-plate light signal comparisons.

One approach is to add the Lumit® Detection Reagent B to one assay plate at a time and read after 3–5 minutes. In this way, the light units will be consistent from plate to plate.

The second approach is to add the Lumit® Detection Reagent B to all assay plates at once. The 1-hour signal half-life is compatible with this batch processing of multiple plates. To control for any decrease in light signal during the time it takes to read multiple plates, we recommend incorporating positive controls on each assay plate for normalization.

Temperature

The intensity and stability of the luminescent signal is temperature sensitive. For consistent results, equilibrate sample buffers, Lumit® Antibody Dilution Buffer and Lumit® Detection Buffer B to room temperature before use. Insufficient equilibration may result in increased well-to-well variability across the plate.

Assay Plates and Equipment

Use **opaque, white-walled multiwell plates** for the bioluminescent assay. Light signal is diminished in black plates. Increased well-to-well crosstalk is observed in clear plates.

The assay is compatible with various multiwell plate formats (96- and 384-well; Table 1) and multiple sample volumes (5–100µl; Table 1). Examples of plates are below. Choose plates that are compatible with your luminometer.

All standard plate readers capable of reading luminescence are suitable for this assay. An integration time of 0.25–1 second per well should be suitable. Some instruments might require optimizing the gain settings to achieve sensitivity and dynamic range. Consult the instrument manual for instrument settings.

The GloMax® Discover Microplate Reader has a pre-installed "Lumit Immunoassay" protocol under the 'Luminescence Protocols' tab that can be used. It has a 0.5 second integration time.

Note: The light signal values will vary depending on the specific plates and luminometers used to generate the data.

Plate Format	Plate Catalog Numbers
96-well	Corning® Cat.# 3912
96-well, half-area	Corning® Cat.# 3693
384-well, regular volume	Corning® Cat.# 3572
384-well, low volume	Corning® Cat.# 4512

8.B. References

1. Müller, T.D. *et al*. (2017) The new biology and pharmacology of glucagon. *Physiol. Rev.* **97**, 721–66.
2. Ojha, A. *et al*. (2019) Current perspective on the role of insulin and glucagon in the pathogenesis and treatment of type 2 diabetes mellitus. *Clin. Pharmacol.* **11**, 57–65.
3. Dixon, A.S. *et al*. (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. *ACS Chem. Biol*. **11**, 400–8.
4. Hwang, B. *et al*. (2020) A homogeneous bioluminescent immunoassay to probe cellular signaling pathway regulation. *Commun. Biol.* **3**, 8.
5. Lewandowski, S.L. *et al.* (2020) Pyruvate kinase controls signal strength in the insulin secretory pathway. *Cell Metab.* **32**, 736–50.

9. Summary of Changes

The following changes were made to the 11/25 revision of this document:

1. Updated Figure 3.
2. Updated one patent statement.
3. Made miscellaneous text edits including addition of Prepared Reagents Storage Conditions in Section 2.
4. Removed Related Products, Section 8.C.

[a]U.S. Pat. Nos. 9,797,889, 9,797,890, 10,107,800 and 11,493,504; European Pat. No. 2970412; Japanese Pat. Nos. 7280842 and 7532562; and other patents and patents pending.

[b]U.S. Pat. No. 8,809,529, European Pat. No. 2635582, Japanese Pat. No. 5889910 and other patents and patents pending.