⟨123⟩ GLUCAGON BIOIDENTITY TESTS

INTRODUCTION

Glucagon is a peptide hormone that increases blood glucose levels via release of liver glycogen stores. A robust and precise physicochemical chromatographic procedure is used in the glucagon assay to assign potency on a mass basis. Bioidentity is still required in *Glucagon for Injection*, and two procedure options are presented here: an in vivo procedure based on release of glucose from freshly prepared rat liver cells (hepatocytes) stimulated with glucagon ex vivo, or production of cyclic adenosine monophosphate (cAMP) in vitro in response to glucagon stimulation of the glucagon receptor cell line. To meet the acceptance criteria of the bioidentity test, only one of these bioidentity tests is required.

PROCEDURE

• A. Primary Liver Cell Bioidentity Test

[NOTE—All buffers are oxygenated, prepared with either *Sterile Water for Injection* or *Sterile Water for Irrigation*, warmed to 37°, and adjusted to a final pH of 7.4 unless otherwise indicated. At least two independent assays (replicates) must be performed utilizing two rat livers for each lot of glucagon. *Figure 1* demonstrates the process used to generate one replicate value. A minimum of two replicates are combined according to the *Calculations* section. The concentration range of the *Standard preparations* and *Assay preparations* may be modified to fall within the linear range of the *Assay*, and the calculations can be adjusted accordingly. Alternatively, full curve analysis using validated nonlinear statistical methods can be used, provided that similarity is demonstrated when analysts compare the responses of the *Standard preparations* and *Assay preparations*.]

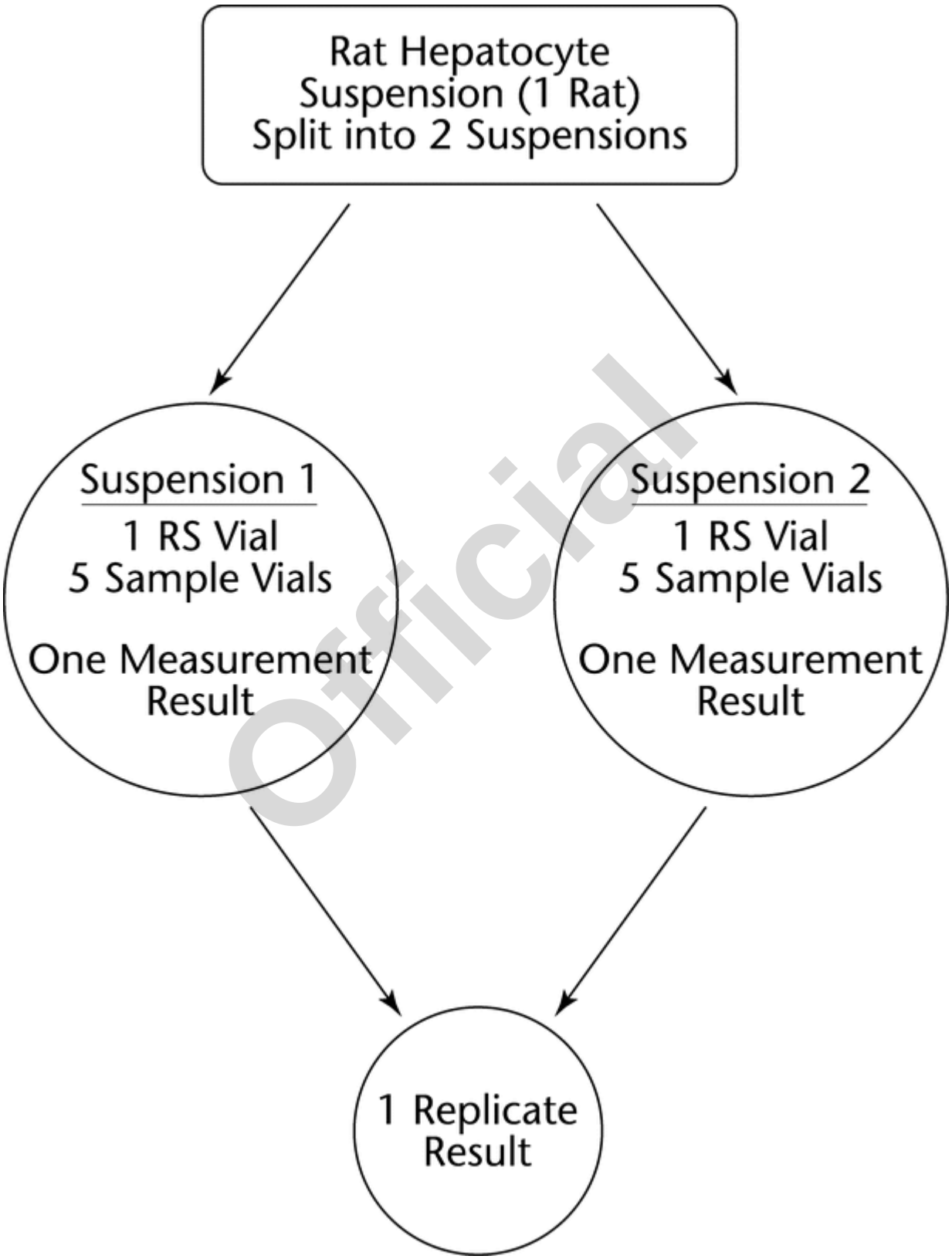

Figure 1. Rat hepatocyte assay method flow diagram (RS = Reference Standard).

Hepatocyte preparation

Calcium-free perfusion buffer with dextrose: Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 1.80 g/L of dextrose, 0.19 g/L of edetic acid (EDTA), and 2.38 g/L of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Oxygenate before use.

Collagenase buffer: Prepare a solution containing 3.62 g/L of sodium chloride, 23.83 g/L of HEPES, 0.35 g/L of potassium chloride, 0.52 g/L of calcium chloride, and 1.8 g/L of dextrose. Adjust to a pH of 7.6. Immediately before perfusion, dissolve a quantity of collagenase in this solution to obtain a concentration of 0.02%–0.05%. The exact concentration of collagenase is determined empirically for each new lot of enzyme and is the amount that can consistently dissociate the tissue within 10 min of buffer entry and produce a viable cell concentration of NLT 3×10^6 cells/mL.

Wash buffer: Prepare a solution containing 7.92 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.19 g/L of EDTA, 2.38 g/L of HEPES, 0.11 g/L of calcium chloride, and 0.06 g/L of magnesium sulfate.

Incubation buffer: Prepare a solution containing 6.19 g/L of sodium chloride, 0.35 g/L of potassium chloride, 0.22 g/L of calcium chloride, 0.12 g/L of magnesium sulfate, 0.16 g/L of monobasic potassium phosphate, 11.915 g/L of HEPES, and 10 g/L of bovine serum albumin (1% BSA). Adjust to a pH of 7.5.

Test animals: Male Sprague-Dawley rats are maintained on a standard rat chow diet, given water ad libitum, and allowed to adjust to their new housing before testing. On the morning of the test, select a healthy rat weighing approximately 300–400 g, and administer 100 units of *Heparin Sodium* subcutaneously.

Procedure: [NOTE—Conduct this procedure in the morning to ensure that the rat has optimal glycogen in its liver and so that the procedure can be completed in 1 day.] Anesthetize the rat with an appropriate anesthetic. Open the abdominal cavity and isolate the portal vein. Insert an angiocatheter and tie into the portal vein at the general location of the lienal branch and then connect to a perfusion pump. Start the perfusion (25 mL/min) in situ with the previously warmed, oxygenated, *Calcium-free perfusion buffer with dextrose*. As the liver enlarges, cut the inferior vena cava to allow pressure equilibrium. [NOTE—About 300 mL of the perfusate is needed to clear the liver of red blood cells at a flow rate of 25–60 mL/min.] Then circulate *Collagenase buffer* at an appropriate flow rate so that the liver leaks perfusate out of the lobes in approximately 10 min (typically 25–60 mL/min). When the liver significantly increases in size, changes color and consistency, and starts to leak perfusate out of the lobes, change the system to the oxygenated prewarmed *Wash buffer*. About 100 mL of *Wash buffer* is needed to wash the liver of collagenase at a flow rate of 25 mL/min. Surgically remove the liver from the animal, and place in a prewarmed Petri dish containing a small amount of oxygenated *Wash buffer* (37°). Gently comb the liver with a stainless steel, fine-toothed comb to free the hepatocytes. Filter and wash the hepatocytes with *Wash buffer*, through prewetted cheesecloth (three layers thick, or through a 150-µm mesh polyethylene net) into a beaker. Transfer the cells to two centrifuge tubes and spin for about 1 min at 600 rpm. Discard the supernatant fractions and resuspend the two pellets in *Incubation buffer*. Combine the two pellets in a suitable container and add sufficient *Incubation buffer* to make 150 mL.

System suitability of cell preparation: The cell yield may vary because of the collagenase activity and the viability of the hepatocytes. To check cell viability and to determine viable cell concentration, dilute a 100-µL aliquot of the cell suspension with 400 µL of *Wash buffer* and 500 µL of isotonic 0.4% trypan blue solution. Load aliquots of the cell suspension into both chambers of a hemocytometer and count all eight quadrants. To meet system suitability of the cell preparation method, a viable cell concentration of 3×10^6 cells/mL (acceptable range of 2.5×10^6 to 3.4×10^6 cells/mL) must be obtained to proceed with the bioassay. If the viable cell concentration exceeds the upper limit, additional *Incubation buffer* may be added to the cells to adjust the concentration to 3×10^6 cells/mL. In this case, the cells are counted again in a hemocytometer, as described above to verify the concentration. [NOTE—Viable cells are those cells that exclude the trypan blue.]

Glucose determination

Negative control solution: Prepare a solution containing 0.5% BSA using *Sterile Water for Injection* or *Sterile Water for Irrigation*.

Incubation flasks: Use specially prepared 25-mL conical flasks, the bottoms of which have been heated and pushed inward to form a conically raised center, or similar flasks that allow sufficient mixing when swirling. Place the *Incubation flasks* in an orbital shaker water bath at 35°.

Standard preparations: On the day of the assay, dissolve two vials of USP rGlucagon RS in 0.01 N hydrochloric acid or other suitable diluent (volume based on the potency of the Reference Standard lot) to obtain two solutions each containing 1 USP rGlucagon Unit/mL. All dilutions thereafter are made using *Negative control solution*. Accurately dilute measured volumes of each solution with *Negative control solution* to obtain an intermediate concentration of 400 µU/mL, and then dilute the intermediate to produce five concentrations: 200, 100, 50, 25, and 12.5 µU/mL (*Standard preparations*). Pipet 0.1 mL of each *Standard preparation* into separate *Incubation flasks*. Pipet 0.1 mL of *Negative control solution* into each of two flasks (*Negative control solutions 1* and *2*).

Assay preparations: Using accurately weighed quantities of glucagon samples, proceed as directed for *Standard preparations* or, if testing *Glucagon for Injection*, reconstitute 10 vials by slowly adding the contents of the accompanying prefilled syringes containing an appropriate glucagon diluent. Gently mix each vial until the glucagon is dissolved. Using the same syringes, withdraw the contents of five vials and place the solutions in a 25-mL volumetric flask. Repeat for the second five vials, transferring the contents to a second 25-mL volumetric flask. Dilute each flask with 0.01 N hydrochloric acid to volume. Dilute an accurate amount of each solution with 0.5% BSA to yield a concentration of 400 µU/mL, and dilute the intermediate to produce five *Assay preparation* concentrations: 200, 100, 50, 25, and 12.5 µU/mL. Then proceed as directed for the *Standard preparations*.

Reference stock solution: Dry USP Dextrose RS, and then transfer 1.0 g, accurately weighed, to a 100-mL volumetric flask. Dissolve in and dilute with saturated benzoic acid solution to volume.

Reference solutions: Transfer suitable quantities of *Reference stock solution* to four flasks, and dilute with saturated benzoic acid solution to obtain *Reference solutions* having concentrations of 100, 500, 1000, and 1500 mg/L.

Potassium ferrocyanide solution: Dissolve 1.25 g of trihydrate potassium ferrocyanide in 125 mL of *Sterile Water for Injection*, or use an appropriate commercial source.

System suitability: Analyze the *Potassium ferrocyanide solution*, the *Reference solutions*, and an additional 5 replicates of either the 500- or 1000-mg/L *Reference solution* in an appropriate glucose analyzer. [NOTE—*Potassium ferrocyanide solutions* are only appropriate standards for glucose analyzers that measure glucose oxidase activity. The procedure can also be performed using alternative platforms.] Prepare a standard curve using the *Reference solutions* as directed for the *Standard preparations*. The square root of the residual error mean square from the regression divided by the average of the response multiplied by 100% (line %RSD) must be NMT 2.0%. In addition, the response of the *Potassium ferrocyanide solution* must be NMT 30 mg/L, and the relative standard deviation must be NMT 2.0% for the replicate analyses of the middle *Reference solution*.

Procedure: Dispense 5 mL of *Hepatocyte preparation* into the *Incubation flasks* in sequence from high glucagon concentration to low glucagon concentration, alternating the *Standard preparations* with the *Assay preparations*. Swirl the flasks in an orbiting water bath at 125 rpm at 30°–35° for approximately 30 min. Following incubation, remove 1.0-mL aliquots from each *Incubation flask*, transfer to labeled microcentrifuge tubes, and centrifuge at 13,000 rpm for 15 s. Place each supernatant fraction in a labeled sampling tube for a glucose analyzer, and determine the glucose concentration (mg/L) of each *Standard preparation* and *Assay preparation*. Measure the background reading of *Negative control solutions 1* and *2*, and calculate the average of the two responses.

To conform to the linear range of the instrument being used, analysts may find it necessary to adjust by dilution each of the *Standard preparations* and *Assay preparations*. Use a glucose analyzer that has demonstrated appropriate specificity, accuracy, precision, and linear response over the range of concentrations being determined. Determine the increase in glucose concentration for each *Standard preparation* and *Assay preparation* compared to the average value of the *Negative control solution*.

Calculations

Calculate the relative potency of the glucagon samples using statistical methods for parallel-line assays, comparing the Reference Standard curve (from the *Standard preparations*) to the glucagon sample curve (from the *Assay preparations*). No dose–response reversals may occur within a run for the 25, 50, or 100 µU/mL *Standard preparations* and *Assay preparations*. [NOTE—Either the low- or high-dose level, but not both, may be excluded from the calculation in order to meet linearity requirements.] Because a minimum of two valid assays (rats) are required, the estimated potencies are combined using the procedures in *Design and Analysis of Biological Assays* ⟨111⟩, *Combination of Independent Assays*, and the width, *L*, of a 95% confidence interval for the estimated logarithm of the relative potency is calculated. If *L* is NMT 0.1938, the results are valid. If *L* is >0.1938, additional assays may be performed and combined until a valid *L* term results, and the relative potency is then calculated from all valid independent runs. Calculate the potency of the glucagon samples in USP rGlucagon Units/mg by multiplying the relative potency result by the potency of USP rGlucagon RS.

Acceptance criteria: NLT 0.80 USP rGlucagon Units/mg

Change to read:

- **B. IN VITRO CELL-BASED BIOIDENTITY TEST**

Medium A: Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 mg/mL of D-glucose and sodium pyruvate, 4 mM L-alanine-L-glutamine[1] or L-glutamine, 10% (v/v) fetal bovine serum,[2] and 0.5 mg/mL G418 [3]

Buffer A: Hank's balanced salt solution[4] containing 5.3 mM potassium chloride, 0.4 mM potassium phosphate monobasic, 4.2 mM sodium bicarbonate, 137.9 mM sodium chloride, 0.3 mM sodium phosphate dibasic anhydrous, and 5.6 mM dextrose

Cell culture preparation: Remove glucagon receptor cell line[5] from cryostorage and immediately thaw at 37° until the cell suspension has just thawed. Aseptically transfer the cell suspension from the cryotube into a sterile test tube containing 10 mL of warmed *Medium A*. Mix and then pellet the cells by centrifugation for 5 min at 125 × g. Remove the supernate and resuspend the cells in fresh, warmed *Medium A*. Measure the quantity of cells contained in an aliquot of the suspension by suitable methods and adjust the cell concentration with *Medium A* such that the cell suspension is 0.5–5 × 10^3 cells/cm^2 of tissue culture flask surface. Inoculate tissue culture flasks[6] and store in a humidified incubator at 37° containing 5% carbon dioxide. Cells should be passaged 2–3 times/week when they are NMT 90% confluent but never trypsinized 2 days in a row. If cells are not ready for passaging after 3 days of culture, the medium should be replaced with fresh *Medium A*. Cells are passaged by first removing the medium from the cell flasks, followed by adding sufficient *Buffer A* prewarmed to room temperature to cover the surface of the flasks. The flasks are gently rocked to wash the cells. This wash fluid is discarded and then sufficient trypsin[7] is added to the cell flasks to cover the surface, followed by gentle rocking and placement back in the incubator. After 3–5 min, the trypsinized cells are aseptically collected from the flasks and transferred to a sterile centrifuge tube, then a volume of *Medium A* is added that is a minimum of 2 times the volume of trypsinized cells, and an aliquot is counted. The cell suspension is further diluted with fresh *Medium A* to a final cell concentration of 0.5–5 × 10^3 cells/cm^2 of tissue culture flask surface. After a minimum of 8 passages post-thaw but NMT 25 passages, the cells can be used in the assay. The day before an assay, follow the cell passaging instructions above but resuspend the cells in fresh, warmed *Medium A* to a final cell concentration of 4–5 × 10^4 cells/mL. [NOTE—Three identical, independent 96-well white plates[8] are needed for measurement of two test samples ("A" and "B") analyzed on each. All wells must be loaded with cells within 40 min of resuspension following trypsinization and counting in order to keep a

[1] Invitrogen catalog #31966-021 or suitable equivalent.
[2] Gibco catalog #10082-147 or suitable equivalent.
[3] Calbiochem catalog #345812 or suitable equivalent.
[4] Invitrogen catalog #14175 or suitable equivalent.
[5] Available from ATCC.
[6] Corning catalog #3151 or suitable equivalent.
[7] Gibco catalog #12563-011 or suitable equivalent.
[8] PerkinElmer catalog #6005680 or suitable equivalent.

homogeneous cell suspension.] Using constant, gentle mixing without foaming, each well is loaded with 0.1 mL of cell suspension and incubated overnight at 37° and 5% carbon dioxide (approximately 24 h ± 4 h) prior to starting the *Procedure*. [NOTE—All remaining solutions should be prepared on the day of the *Procedure*.]

Medium B: Krebs' salt solution containing 0.3% (v/v) human serum albumin, 25 mM HEPES, 1.7 mM 3-isobutyl-1-methyl-xanthine, 0.2 mg/mL of glucose, 650 KIU/mL of aprotinin, and 0.0003% (v/v) polysorbate 80, pH 7.4

Medium C: Krebs' salt solution containing 0.3% (v/v) human serum albumin and 25 mM HEPES, pH 7.4

Standard stock solution: Reconstitute USP rGlucagon RS in *Water for Injection* or another suitable diluent to a concentration of 4 mg/mL by gently mixing on a rotator for 10 min or until completely clear. Dilute this material 1:1000 with *Medium B* to a concentration of ▲4▲ (ERR 1-Aug-2019) µg/mL and then 1:200 with *Medium B* to 20 ng/mL.

Standard solutions: Within an hour of use, dilute the 20 ng/mL *Standard stock solution* with *Medium B* to make a 1:5 concentration series of 4 ng/mL (R8), 0.8 ng/mL (R7), 160 pg/mL (R6), 32 pg/mL (R5), 6.4 pg/mL (R4), and 1.3 pg/mL (R3). A final dilution is made from R3 in *Medium B* to make 65 fg/mL (R2). [NOTE—Alternative suitable concentrations can be similarly prepared if necessary and validated.] R1 is a blank solution of *Medium B*. [NOTE—For these *Standard solutions*, as well as the *Sample solutions* and *cAMP standard solutions* described below, it may be helpful to prepare these dilutions in a dilution microplate that mimics the assay plate layout to easily and quickly transfer the materials to the wells of the assay plate.]

Sample solutions: Reconstitute two independent preparations of glucagon from the same lot of material in *Water for Injection* or the same suitable diluent used to prepare the *Standard stock solution* to a concentration of 4 mg/mL. Prepare by diluting this material further with *Medium B* to prepare a concentration series similar to that suggested for the *Standard solutions*. One preparation series is *Sample solution* A8–A2 and the other preparation series is *Sample solution* B8–B2.

cAMP standard solutions: Prepare a 5 mM cAMP[9] solution in water, and then prepare further solutions by dilution with *Medium B* as shown in *Table 1*. [NOTE—To conform to the linear range of the instrument being used, analysts may find it necessary to adjust by dilution each of the *Standard solutions*, *cAMP standard solutions*, and *Assay solutions*. Alternative suitable concentrations can be similarly prepared if necessary and validated.]

Table 1. Preparation of cAMP Standard Solutions

Starting cAMP Solution	Fold Dilution with Medium B	Final cAMP Solution	Final cAMP Standard Solution Name
5 mM	1:10	0.5 mM	—
0.5 mM	1:20	25 µM	C1
25 µM	1:3.3	7.5 µM	C2
25 µM	1:10	2.5 µM	C3
7.5 µM	1:10	0.75 µM	C4
2.5 µM	1:10	250 nM	C5
0.75 µM	1:10	75 nM	C6
250 nM	1:10	25 nM	C7
75 nM	1:10	7.5 nM	C8
Medium B only	—	No cAMP	C9

Donor biotin-cAMP beads: Dissolve 10 nmol biotinylated cAMP in 0.5 mL of phosphate-buffered saline, pH 7.4. Add 80 µL of this biotinylated cAMP solution to 33.8 mL of a 5 mM HEPES lysis buffer containing 0.1% (w/v) BSA and 0.3% (v/v) polysorbate 20, pH 7.4.[9] Add 270 µL of donor beads and 2.4 mL of 120 mM $MgCl_2$ solution.

Procedure: Remove the plates seeded with cells the day before, and discard the *Medium A* in each well. Wash the cells with 350 µL/well of *Medium C*, then discard the wash solution. Rapidly add 20 µL/well of diluted 1× anti-cAMP-acceptor beads. Add 30 µL/well of each *Standard solution*, *Sample solution*, *Medium B*, or *cAMP standard solution* as indicated in the plate layouts shown in *Tables 2* and *3*. [NOTE—The white plate for the *cAMP standard solutions* does not contain cells.] Cover the plate,[10] protecting it from light and evaporation, and incubate for 35–60 min in an incubator at 37° with gentle shaking. Next, dispense 60 µL of *Donor biotin-cAMP beads* per well, mix well, then cover[9] the plate again and wrap it with foil. [NOTE—The beads are light sensitive so plates should be kept dark or under green light conditions.] Gently rotate the plate on a shaker for at least 30 min at room temperature. Keep the plate in the dark, at room temperature, without shaking for 16–30 h before measuring the top luminescence from the plate wells on a suitable plate reader[11] and detection mode (excitation wavelength of about 680 nm; emission of 520–620 nm).

Table 2. Schematic Representation of the Standard and Sample Assay Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	R1	R2	A2	B2	R3	A3	B3	R4	A4	B4	R8	—
B	R1	R2	A2	B2	R3	A3	B3	R4	A4	B4	R8	—
C	R1	R2	A2	B2	R3	A3	B3	R4	A4	B4	R8	—

[9] PerkinElmer catalog #6760635D or suitable equivalent.
[10] PerkinElmer catalog #6050195 or suitable equivalent.
[11] PerkinElmer catalog #2300-0000 or suitable equivalent.

Table 2. Schematic Representation of the Standard and Sample Assay Plate *(continued)*

	1	2	3	4	5	6	7	8	9	10	11	12
D	R1	R2	A2	B2	R3	A3	B3	R4	A4	B4	R8	—
E	R5	A5	B5	R6	A6	B6	R7	A7	B7	A8	B8	C9
F	R5	A5	B5	R6	A6	B6	R7	A7	B7	A8	B8	C6
G	R5	A5	B5	R6	A6	B6	R7	A7	B7	A8	B8	C4
H	R5	A5	B5	R6	A6	B6	R7	A7	B7	A8	B8	C1

LEGEND:
A2–A8 = Dilution series of *Sample solution* A (same solution is used on three plates, with 4 ng/mL as starting concentration).
R1–R8 = Dilution series of *Standard solution* for each test plate, with 4 ng/mL as starting concentration; as a result, each *Sample solution* is only compared to the *Standard solution* on that plate.
B2–B8 = Dilution series of *Sample solution* B (same solution is used on three plates, with 4 ng/mL as starting concentration).
— = No glucagon (contains 30 µL *Medium B* instead), but cells are present.
C = cAMP standard controls, no glucagon. C1 contains 25 µM cAMP, C4 contains 0.75 µM cAMP, C6 contains 75 nM cAMP, and C9 contains no cAMP.

Table 3. Schematic Representation of the cAMP Standards Assay Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	—	—	—	—	—	—	—	—	—	—	—	—
B	—	—	—	—	—	—	—	—	—	—	—	—
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	—	—	—
D	C1	C2	C3	C4	C5	C6	C7	C8	C9	—	—	—
E	C1	C2	C3	C4	C5	C6	C7	C8	C9	—	—	—
F	—	—	—	—	—	—	—	—	—	—	—	—
G	—	—	—	—	—	—	—	—	—	—	—	—
H	—	—	—	—	—	—	—	—	—	—	—	—

C1–C9 = Dilution series of cAMP standard controls starting with C1, which contains 25 µM cAMP, through C9, which does not contain cAMP.

System suitability criteria: The lower asymptote of the 4-parameter logistic curves generated from *Sample solutions* A2–A8 and B2–B8 and *Standard solutions* R1–R8 must be above the lower limit (defined as C3) of the *cAMP standard solutions*. NLT 3-fold difference between the signals obtained in the wells of cells treated without glucagon (R1) versus those with the maximal amount of glucagon (R8). NMT 4 technical outliers may be omitted per standard curve. Any plate that fails one or more of these criteria is rejected and must be repeated. If either *Sample solutions* A or B fail one of these criteria, then only the passing series can be used for the calculations. [NOTE—The upper asymptotes of the 4-parameter logistic standard curves generated from *Sample solutions* A2–A8 and B2–B8 and *Standard solutions* R1–R8 should be approximately equal to or less than the C8 response of the *cAMP standard solutions* and within the linear range of the instrument used.]

Calculations: A series of two independent *Sample solution* preparations must be used for each test sample across three plates (assays). Outliers identified by Grubbs' test (see ⟨111⟩; but NMT 4/curve and NMT 1 data point from a replicate set) are omitted, and then the same number of *Standard solution* and *Sample solution* dose responses, including the 50% response (EC_{50}) of the standard/test sample within this range, are used to calculate the relative potency of the glucagon sample using statistical methods for parallel-curve analysis with a 4-parameter logistic fit using all replicate values. For each individual *Sample solution* compared to the *Standard solution*, the statistical tests for slope and parallelism must pass at the 95% level. Calculate the relative potency of the glucagon samples by comparing the Reference Standard curve (from the *Standard solutions*) to the glucagon sample curve (from the *Sample solutions*). Because a minimum of three valid assays are required, the estimated potencies are combined using the procedures in *Design and Analysis of Biological Assays* ⟨111⟩*, Combination of Independent Assays*, and the confidence interval is calculated using suitable statistical methods. If the confidence limits are between 64% and 156%, the results are valid. If not, up to two more assay plates may be performed and combined until a valid confidence interval results, and the relative potency is then calculated from all valid independent runs. Calculate the potency of the glucagon samples in USP rGlucagon Units/mg by multiplying the relative potency result by the potency of USP rGlucagon RS.

Acceptance criteria: NLT 0.80 USP rGlucagon Units/mg

ADDITIONAL REQUIREMENTS

- **USP REFERENCE STANDARDS** ⟨11⟩

  USP Dextrose RS
  USP rGlucagon RS