TECHNICAL MANUAL

RealTime-Glo™ Extracellular ATP Assay

Instructions for Use of Products
GA5010, GA5011 and GA5012

Revised 4/22
TM652

Promega

RealTime-Glo™ Extracellular ATP Assay

All technical literature is available at: www.promega.com/protocols/
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1. Description

The RealTime-Glo™ Extracellular ATP Assay uses an optimized, bioluminescence detection chemistry to measure ATP that has been released into the cultured cell environment as a result of cell death, stress or activation (1–4; Figure 1). The nonlytic, homogeneous reagent is created by rehydrating the assay substrate with your preferred growth medium to either a 4X or 20X working concentration. The reagent is applied directly to cells at the time of dosing or treatment (Figure 2) to assess continuous ATP release during stimulus exposures of up to 24 hours (Figure 3). Longer exposure times can be addressed using a staggered reagent addition protocol. The real-time format eliminates the need for periodic and laborious cell-free supernatant sampling regimens while providing exquisite resolution of dose-dependent, ATP-release responses. Real-time extracellular ATP (eATP) can be measured in a "hands-off" kinetic mode three ways: Using atmospherically controlled plate readers, using heated readers without atmospheric controls by growing cells in CO_2-independent medium formulations, or manually collecting readings at scheduled time points with conventional incubators and a luminescence plate reader (Figure 4). The reagent is well-tolerated by a wide variety of both suspension and attachment-dependent cell types during standard experimental exposures and does not adversely affect cell health when used at the recommended concentrations. Additionally, the reagent can be used to measure total ATP at an exposure endpoint to assess the relative health of the cell population.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
RealTime-Glo™ Extracellular ATP Assay	**200 assays**	**GA5010**

For Laboratory Use. Contains sufficient reagent for 200 assays in a 96-well-plate format, or 800 assays in a 384-well-plate format. Includes:

- 1 vial RealTime-Glo™ Extracellular ATP Assay Substrate

PRODUCT	SIZE	CAT.#
RealTime-Glo™ Extracellular ATP Assay	**2,000 assays**	**GA5011**

For Laboratory Use. Contains sufficient reagent for 2,000 assays in a 96-well-plate format, or 8,000 assays in a 384-well-plate format. Includes:

- 1 vial RealTime-Glo™ Extracellular ATP Assay Substrate

PRODUCT	SIZE	CAT.#
RealTime-Glo™ Extracellular ATP Assay	**10 × 200 assays**	**GA5012**

For Laboratory Use. Contains sufficient reagent for 2,000 assays in a 96-well-plate format, or 8,000 assays in a 384-well-plate format. Includes:

- 10 × 1 vials RealTime-Glo™ Extracellular ATP Assay Substrate

Storage Conditions: For long-term storage, store the lyophilized RealTime-Glo™ Extracellular ATP Substrate at –10°C to –30°C. See product label for expiration information. Store reconstituted RealTime-Glo™ Extracellular ATP Reagent (mixed with growth medium) at room temperature, shielded from ambient light, for up to 24 hours, or at 4°C for at least 1 week with minimal loss of activity. To store the reconstituted RealTime-Glo™ Extracellular ATP reagent longer than 1 week, dispense into single thaw-and-use aliquots and freeze at –20°C.

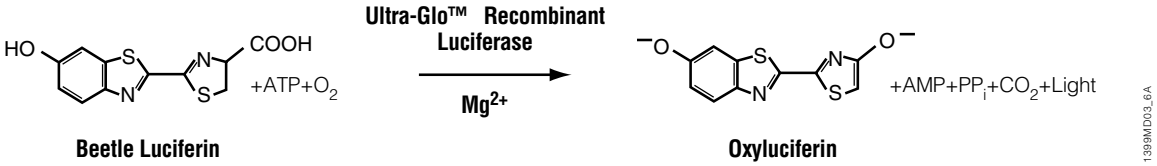

Figure 1. The bioluminescent extracellular ATP detection chemistry. Reactants in the reagent have been optimized to generate a nearly simultaneous luminescent response proportional to current eATP levels present in a cell culture for up to 24 hours.

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

3. Performing the RealTime-Glo™ Extracellular ATP Assay

Materials to Be Supplied by the User:

- opaque-walled 96- or 384-well tissue culture plates (clear or solid bottom) compatible with measuring luminescence
- multichannel pipettor and tips or liquid dispensing robot
- reagent reservoirs
- multiwell luminometer or multimodal plate reader (e.g., GloMax® Discover System, Cat.# GM3000)
- vortex and orbital shaker
- cells and culture medium
- positive control ATP release inducers (e.g. doxorubicin, idarubicin, mitoxantrone, bortezomib, romidepsin or staurosporine)
- hemocytometer and trypan blue or automated cell counting device
- multiwell luminometer
- **optional:** Digitonin (Cat.# G9441)
- **optional:** gas permeable plate sealer (Breath-Easy®, Diversified Biotech)

3.A. Reagent Preparation

The RealTime-Glo™ Extracellular ATP Assay Reagent is mixed and coincubated with cells in culture for up to 24 hours. Therefore, use aseptic technique while creating the reagent (preferrably in a biosafety cabinet or flow hood) to minimize the chance of introducing bacterial, fungal or yeast contaminates.

1. Prewarm culture medium used for propagation of the choosen cell type.
2. Equilibrate the lyophilized bottle(s) of RealTime-Glo™ Extracellular ATP Assay Substrate to room temperature prior to use.

   Note: Safeguard against accidental contamination by misting a 70% (v/v) ethanol solution on the substrate bottle and your gloves prior to proceeding. Allow the ethanol solution to evaporate prior to proceeding.

3.A. Reagent Preparation (continued)

3. Remove the aluminum ring from the top of the bottle(s). Carefully remove the gray rubber stopper using aseptic practice. Discard gray rubber stopper.

   Note: Do not attempt to recap bottle with gray stopper as this can cause accidental microbiological contamination.

4. Transfer the appropriate volume of culture medium into the amber glass bottle containing the RealTime-Glo™ Extracellular ATP Assay Substrate. See Table 1 for culture medium volume guidance.

   Note: The appropriate volume will be dictated by the size of the vial and the concentration of working reagent desired. The reagent can be created at 4X or 20X. A 4X reagent is most suitable for a standard 24-hour experiment, whereas a 20X reagent may be indicated for use with parallel plates spanning multiple days of compound exposure.

Table 1. Diluting the Substrate to a Working Concentration.

		Volume of Medium to Add	
Cat.#	**Vial Part#**	**4X Reagent**	**20X Reagent**
GA5010, GA5012	GA501A	10ml	2ml
GA5011	GA501B	100ml	20ml

5. Mix by aspirating and dispensing the contents with a pipette to obtain a homogeneous solution. Avoid introducing bubbles in the reagent.

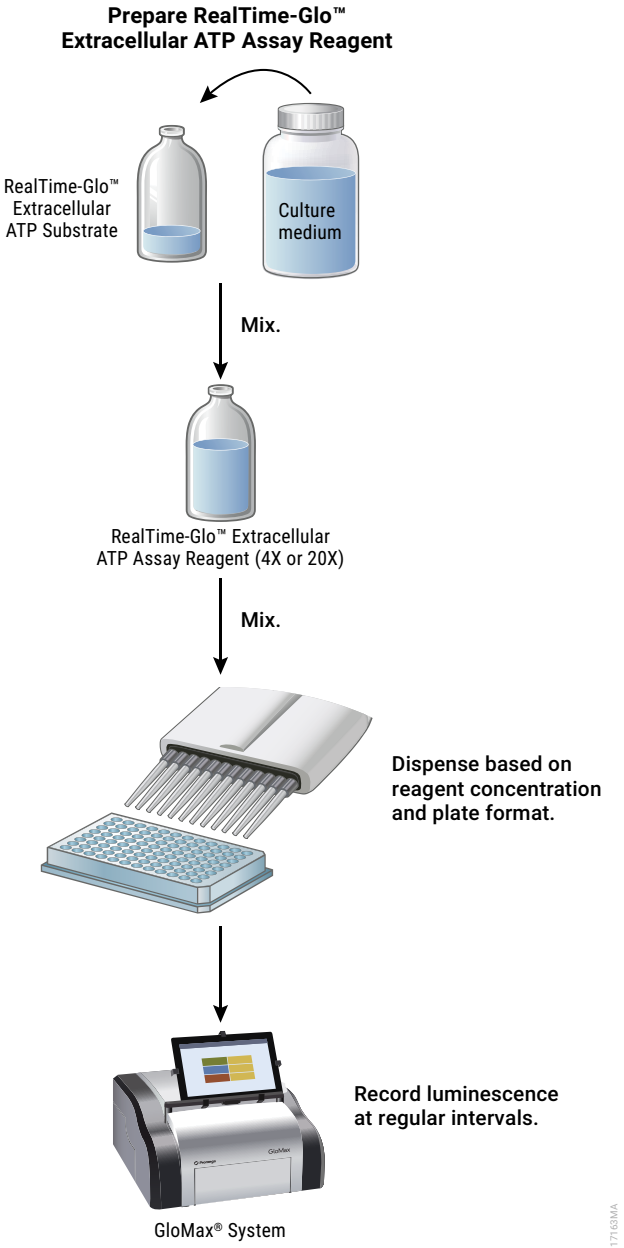

Figure 2. Flow diagram showing preparation and use of the RealTime-Glo™ Extracellular ATP Assay Reagent.

3.A. Reagent Preparation (continued)

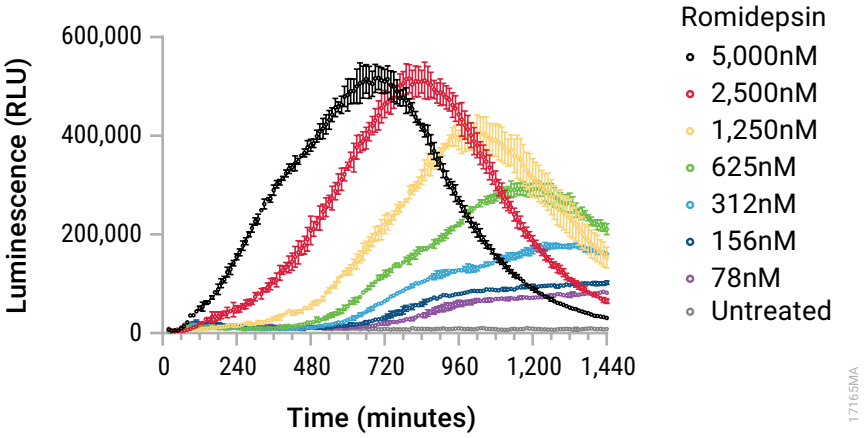

Figure 3. Real-time extracellular ATP release over a 24-hour exposure. A549 cells were seeded at a density of 10,000 cells per well in CO_2-Independent medium with 10% fetal bovine serum (FBS) and allowed to attach for 6 hours. The cells were dosed with serial dilutions of romidepsin and immediately followed by addition of the RealTime-Glo™ Extracellular ATP Assay Reagent. Luminescence measurements were collected in 10-minute intervals using a BMG POLARstar® Omega plate reader set at 37°C.

3.B. Protocol for Real-Time Detection of Extracellular ATP (Up to 24 Hours)

This method is most appropriate for measuring eATP release in standard 24-hour exposures. Refer to Section 4.A for considerations related to cell culture conditions, assay set up or other experimental objectives.

1. Harvest, wash and adjust test cells to a target concentration of 50,000–200,000 cells/ml using preferred culture medium, and add to a sterile, opaque assay plate.

   96-well format: Dispense 100µl per well for a density of 5,000–20,000 cells/well.

   384-well format: Dispense 25µl per well for a density of 1,250–5,000 cells/well.

2. Allow attachment-dependent cells to adhere to microtiter plate surface. Skip this step for suspension cell lines.

3. Program a luminometer or multimodal plate reader to measure luminescence kinetically at desired intervals (e.g., 5, 10 or 15 minutes) and temperature over the course of an exposure up to 24 hours or prepare for manual measurements at scheduled intervals. Refer to Section 4.A for other considerations related to instrumentation.

4. Serially dilute test agents in cell culture medium.

   Note: Table 2 provides an example dilution scheme and template for sample placement. The initial dilution of test agent should be 4X the desired final concentration due to volume contributions from cells and reagent. Doxorubicin, idarubicin, mitoxantrone, bortezomib, romidepsin or staurosporine can be used at a 4X dose of 20–50µM (5–12.5µM final in assay) to release ATP as a positive control.

Table 2. Example Dilution Scheme and Template for Sample Placement.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Test	Agent	#1	Test	Agent	#2	Test	Agent	#3	Test	Agent	#4
B	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2	1:2
C	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4	1:4
D	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8	1:8
E	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16	1:16
F	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32	1:32
G	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64	1:64
H	UT Ctrl	UT Ctrl	UT Ctrl	UT Ctrl	UT Ctrl	UT Ctrl	Pos Ctrl	Pos Ctrl	Pos Ctrl	Pos Ctrl	Pos Ctrl	Pos Ctrl

5. Add serially diluted test material to assay plate. Include volume-matched untreated vehicle controls (row H, wells 1–6) and a positive control ATP-releasing stimulus (row H, wells 7–12).

   96-well format: Dispense 50µl per well.

   384-well format: Dispense 12.5µl per well.

6. Add 4X RealTime-Glo™ Extracellular ATP Assay Reagent to all wells. Replace the lid, or carefully add a gas-permeable membrane (e.g., Breathe-Easy® film, Diversified Biotech) to the plate.

   96-well format: Dispense 50µl of 4X RealTime-Glo™ Extracellular ATP Assay Reagent.

   384-well format: Dispense 12.5µl of 4X RealTime-Glo™ Extracellular ATP Assay Reagent.

   Note: Adding other volumes of cells, test material and 4X RealTime-Glo™ Extracellular ATP Assay Reagent are possible, but 4X RealTime-Glo™ Extracellular ATP Assay Reagent should not exceed 25% of the final volume.

7. Mix plate by orbital shaking (300–500rpm) for 30–45 seconds to ensure homogeneity.

8. Place the plate in the reader and initiate the ATP measurement program. Alternatively, place the plate in a conventional incubator and measure ATP at desired intervals by manually removing and inserting into a plate reader, then returning the plate to the incubator.

   Note: Total ATP (intracellular and extracellular) can be measured at the exposure endpoint by adding a pore-forming agent like digitonin to release remaining cellular ATP. Total ATP measured in treated wells can reflect the relative health of the cell population relative to the untreated wells at the end of the exposure period.

3.B. Protocol for Real-Time Detection of Extracellular ATP (Up to 24 Hours; continued)

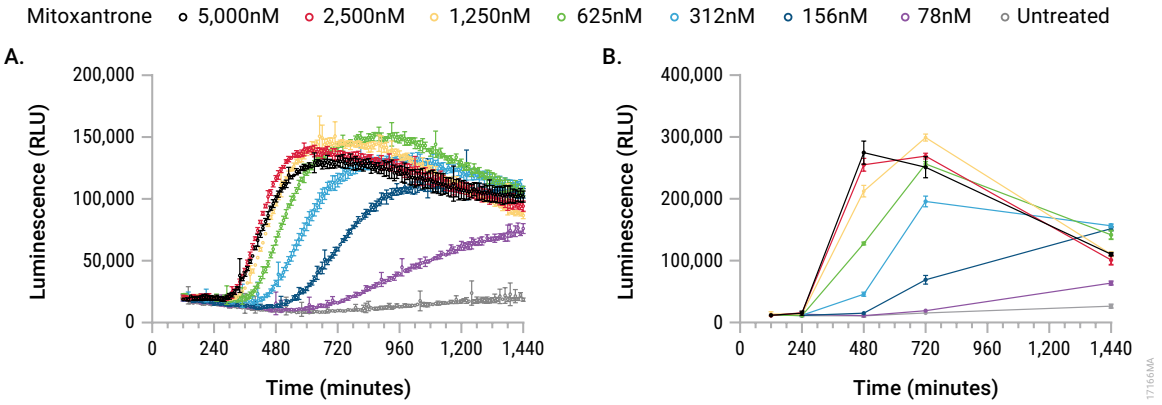

Figure 4. Luminescent measurements can be made in either automated or manual modes. U937 cells were seeded at 10,000 cells per well in RPMI 1640 + 10% FBS and dosed with serial dilutions of mitoxantrone in the presence of RealTime-Glo™ Extracellular ATP Assay Reagent in parallel plates. **Panel A.** Automated measurements were made every 10 minutes in a BMG CLARIOstar® plate reader equipped with an Atmospheric Control Unit. **Panel B.** Manual measurements were made by removing the plate from a cell culture incubator at scheduled intervals and measuring luminescence with a GloMax™ Discover System plate reader. The plate was promptly returned to the incubator after each measure.

Optional: Measuring Total ATP

9. Prepare a working solution of Digitonin (Cat.# G9441) by adding 40µl of the 20mg/ml solution to 1,960µl of cell culture medium. Mix by vortexing.

10. Add the digitonin working solution to each well of the assay plate. Mix by orbital shaking at 300–500rpm for 1 minute.

    96-well format: Dispense 20µl per well of working solution.

    384-well format: Dispense 5µl per well of working solution.

11. Measure endpoint luminescence within 5 minutes using luminometer settings from Step 3.

    Note: The optional total ATP endpoint measure must be made as soon as possible (and within 5 minutes) because the RealTime-Glo™ Extracellular ATP Assay detection chemistry is not formulated to produce an extended glow signal. The signal half-life is about 5 minutes.

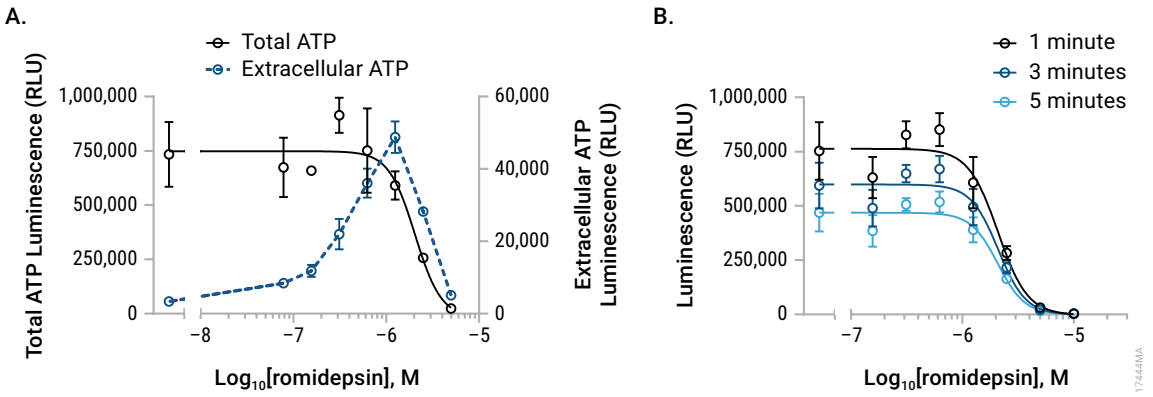

Figure 5. Correlation between extracellular and total ATP measurements at exposure endpoint. U937 cells were treated with serial dilutions of romidepsin prepared in CO_2-Independent medium in the presence of the RealTime-Glo™ Extracellular ATP Reagent. Luminescence data was collected every 10 minutes for 16 hours using a BMG POLARstar® plate reader. At 16 hours, digitonin was added to all wells, mixed briefly, and luminescence values recorded in 2 minutes intervals for 5 minutes. **Panel A.** Extracellular ATP and total ATP luminescence values at 16 hours. **Panel B.** Signal decay after adding digitonin.

3.C. Protocol for Real-Time Detection of Extracellular ATP (Beyond 24 Hours)

To successfully implement this method, you need to add the reagent to subsequent plates 3 hours prior to collecting data. This preincubation allows the reagent reactants and eATP to reach a steady-state. This method is most appropriate for staggered additions of reagent to parallel plates over a multiday exposure (Figure 6). Refer to Section 2 for reagent stability and storage recommendations, and read Section 4.A for other considerations related to cell culture conditions, assay set up or other experimental objectives.

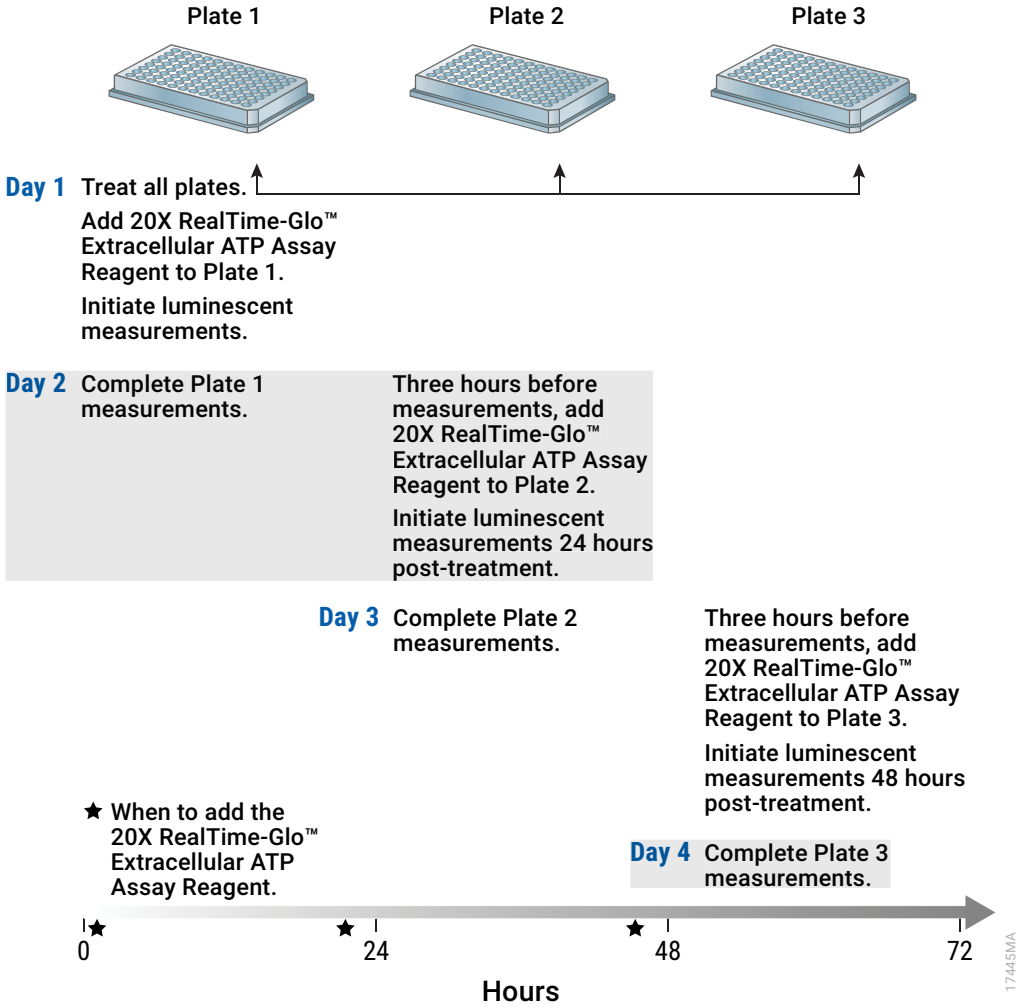

Figure 6. Staggering reagent additions can extend the RealTime-Glo™ Extracellular ATP Assay coverage beyond 24 hours.

1. Harvest, wash and adjust test cells to a target concentration of 37,000–143,000 cells/ml with your choice of culture medium, and add to a sterile, opaque assay plate.

   96-well format: Dispense 140µl per well for a density of 5,000–20,000 cells/well.

   384-well format: Dispense 35µl per well for a density of 1,250–5,000 cells/well.

2. Allow attachment-dependent cells to adhere to microtiter plate surface. Skip this step for suspension cell lines.

3. Program a luminometer or multimodal plate reader to measure luminescence kinetically at desired intervals (e.g., 5, 10 or 15 minutes) and temperature over the course of an exposure up to 24 hours or prepare for manual measurements at scheduled intervals. Refer to Section 4.A for other considerations related to instrumentation.

4. Serially dilute test agents in cell culture medium.

   Note: Table 2 provides an example dilution scheme and template for sample placement. The initial dilution should be 4X the desired final concentration due to volume contributions from cells and reagent. Doxorubicin, idarubicin, mitoxantrone, bortezomib, romidepsin or staurosporine can be used at a 4X dose of 20–50µM (5–12.5mM final in assay) to release ATP.

5. Add serially diluted test material to assay plate. Include volume-matched untreated vehicle controls (row H, wells 1–6) and a positive control ATP-releasing stimulus (row H, wells 7–12).

   96-well format: Dispense 50µl per well.

   384-well format: Dispense 12.5µl per well.

6. Add 20X RealTime-Glo™ Extracellular ATP Assay Reagent to all wells. Replace the lid, or carefully add a gas-permeable membrane (e.g. Breathe-Easy® film, Diversified Biotech) to the plate.

   96-well format: Dispense 10µl of 20X RealTime-Glo™ Extracellular ATP Assay Reagent.

   384-well format: Dispense 2.5µl of 20X RealTime-Glo™ Extracellular ATP Assay Reagent.

   Note: Other volumes of cells, test material and 20X RealTime-Glo™ Extracellular ATP Assay Reagent are possible, but 20X RealTime-Glo™ Extracellular ATP Assay Reagent should not exceed 5% of the final volume.

7. Mix plate by orbital shaking (300–500rpm) for 30–45 seconds to ensure homogeneity.

8. Place the plate in the reader and initiate the ATP measurement program. Alternatively, place the plate in a conventional incubator and measure ATP at desired intervals by manually removing and inserting into a plate reader, then returning the plate to the incubator.

   Notes:

   1. Total ATP (intracellular and extracellular) can be measured at the exposure endpoint by adding a pore-forming agent like digitonin to release remaining cellular ATP. Total ATP measured in treated wells can reflect the health of the cell population relative to the untreated wells at the end of the exposure period.
   2. Prewarmed reagent should be added to successive plates 3 hours prior to the actual collection of data.

Optional: Measuring Total ATP

9. Prepare a working solution of Digitonin (Cat.# G9441) by adding 40µl of the 20mg/ml solution to 1,960µl of cell culture medium. Mix by vortexing.

3.C. Protocol for Real-Time Detection of Extracellular ATP (Beyond 24 Hours; continued)

10. Add the digitonin working solution to each well of the assay plate. Mix by orbital shaking at 300–500rpm for 1 minute.

    96-well format: Dispense 20µl per well of working solution.

    384-well format: Dispense 5µl per well of working solution.

11. Measure endpoint luminescence within 5 minutes using luminometer settings from Step 3.

    Note: The optional total ATP endpoint measure must be made as soon as possible (and within 5 minutes) because the ATP detection chemistry is not formulated to produce an extended glow signal. The signal half-life is about 5 minutes.

3.D. Data Analysis and Interpretation

The magnitude and kinetics of an eATP release response are dose- or treatment-intensity dependent and shaped by the mechanism of action of the inducing agent on the cell type of interest. Extracellular ATP responses are often also transient due to underlying changes in cellular biology or metabolic health, the progression toward cell death or both. Therefore, raw luminescence data is most effectively plotted as a function of dose or treatment intensity versus time (Figure 7, Panel A).

Note: Exercise care when implementing dose versus response curve-fitting software due to the transient nature of signal often observed in early high-dose responses, leading to skewed estimates of potency (Figure 7, Panel B).

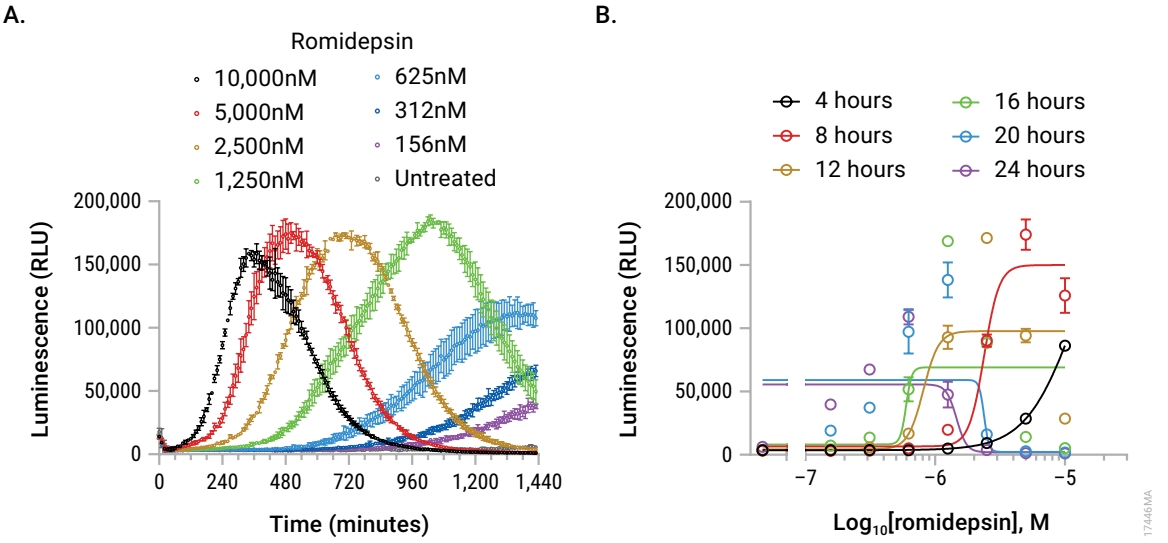

Figure 7. Extracellular ATP release events are dose-dependent and often transient. U937 cells were seeded into a 96-well plate at a density of 10,000 cells per well in CO_2-Independent medium + 10% FBS. The cells were dosed with romidepsin in the presence of the RealTime-Glo™ Extracellular ATP Assay Reagent. **Panel A.** The kinetics of eATP release are most effectively graphed as a function of luminescence intensity versus time for each treatment variable. **Panel B.** Standard dose versus response plots may result in poor fits as the exposure progresses due to extracellular ATP instability.

Raw luminescence values collected in kinetic mode are typically saved as microplate results views at each time point (Figure 8, Panel A), in a collated table representing luminescence collected during the entire exposure (Figure 8, Panel B) or both. Manually collected data will be saved in microplate view for each time point.

A.

uminescence, Iteration #88

Emission Filter:	None
Integration Time(s):	0.3
Reading:	1

	1	2	3	4	5	6	7	8	9	10	11	12
A	5.96E+04	5.60E+04	5.95E+04	1.21E+05	1.12E+05	1.23E+05	9.70E+04	1.07E+05	1.07E+05	1.25E+05	1.27E+05	1.25E+05
B	5.99E+04	5.91E+04	5.88E+04	4.35E+04	5.20E+04	3.80E+04	1.14E+05	1.18E+05	1.33E+05	1.24E+05	1.25E+05	1.28E+05
C	5.81E+04	5.82E+04	5.96E+04	1.05E+04	1.23E+04	1.28E+04	1.36E+05	1.47E+05	1.45E+05	1.21E+05	1.25E+05	1.25E+05
D	6.16E+04	4.57E+04	5.37E+04	5.26E+03	4.55E+03	6.75E+03	1.12E+05	1.10E+05	1.15E+05	1.13E+05	1.21E+05	1.20E+05
E	5.67E+04	5.31E+04	5.31E+04	4.56E+03	4.61E+03	6.42E+03	7.89E+04	8.29E+04	8.93E+04	7.34E+04	8.63E+04	9.23E+04
F	5.36E+04	5.23E+04	5.19E+04	4.15E+03	3.80E+03	5.42E+03	7.54E+04	7.73E+04	8.30E+04	2.61E+04	3.06E+04	3.41E+04
G	5.02E+04	5.53E+04	5.27E+04	4.68E+03	4.84E+03	6.37E+03	7.93E+04	9.05E+04	8.53E+04	9.42E+03	1.26E+04	9.15E+03
H	4.55E+03	5.52E+03	5.23E+03	6.17E+03	4.15E+03	3.75E+03	6.46E+03	7.39E+03	7.43E+03	5.60E+03	5.07E+03	6.82E+03

B.

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Protocol: LUMI KINETIC

Result: LUMI KINETIC 2020.08.05 09:43:23

Read Type	Read Number	Row time (sec)	Row time (min)	A:1	A:2	A:3	A:4	A:5	A:6	A:7	A:8	A:9	A:10	A:11	A:12
Luminescence	1	0.000	0.000	2.43E+04	9.90E+03	1.09E+04	8.31E+03	1.10E+04	2.80E+04	2.37E+04	3.17E+04	2.96E+04	2.32E+04	2.74E+04	4.36E+04
Luminescence	2	899.092	14.985	8.40E+03	4.92E+03	7.32E+03	4.82E+03	6.84E+03	1.11E+04	1.03E+04	1.17E+04	1.02E+04	9.87E+03	9.13E+03	1.72E+04
Luminescence	3	1799.126	29.985	3.79E+03	2.69E+03	3.67E+03	3.65E+03	3.24E+03	6.00E+03	4.52E+03	4.38E+03	3.85E+03	4.41E+03	3.94E+03	5.51E+03
Luminescence	4	2699.162	44.986	2.93E+03	2.23E+03	3.23E+03	2.69E+03	2.59E+03	3.33E+03	3.30E+03	3.76E+03	2.80E+03	3.23E+03	2.70E+03	3.67E+03
Luminescence	5	3599.176	59.986	2.74E+03	2.15E+03	2.84E+03	2.43E+03	2.93E+03	3.45E+03	2.85E+03	3.39E+03	2.67E+03	2.89E+03	2.70E+03	2.96E+03
Luminescence	6	4499.189	74.986	3.03E+03	2.27E+03	3.04E+03	2.49E+03	2.50E+03	4.19E+03	2.58E+03	3.04E+03	2.77E+03	2.43E+03	3.03E+03	3.15E+03
Luminescence	7	5399.189	89.986	3.02E+03	2.41E+03	2.73E+03	2.61E+03	2.46E+03	3.20E+03	3.02E+03	3.00E+03	2.94E+03	2.65E+03	2.87E+03	3.61E+03
Luminescence	8	6299.199	104.987	3.27E+03	2.57E+03	2.86E+03	2.85E+03	2.55E+03	2.99E+03	3.25E+03	3.18E+03	3.03E+03	4.15E+03	3.34E+03	3.55E+03
Luminescence	9	7199.218	119.987	3.09E+03	2.91E+03	3.05E+03	3.44E+03	2.92E+03	3.00E+03	3.20E+03	3.11E+03	3.22E+03	2.90E+03	3.17E+03	3.44E+03

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Figure 8. Example Data Sets collected using the GloMax® Discover System. Panel A. Results View represents the data collected from a specific time point. **Panel B.** Table View represent all the values collected during the entire experiment. **Note:** The example provided is a subset of the data collected from the entire plate (through H:12) and through 96 Read Numbers.

Most users will find value in either a kinetic representation of the data, or a response index at a given time or collection of time points:

1. For a kinetic representation of the average response of replicates, copy and paste luminescence data into a spreadsheet where dose replicates are deposited in groups representing the Y axis and time increments represented by minutes on the X axis. GraphPad Prism® or SigmaPlot software can be used to graph the average response at each dose or treatment condition vs time.

2. The response index can be calculated from the formula:

$$\textbf{Release response index} = \frac{[\text{Average RLU of replicates of stimulated response}]}{[\text{Average of replicates of unstimulated response}]} \times 100 - 100$$

Note: Although many criteria can be applied for the significance of a stimulus-driven eATP release response, any sustained response that reaches >150% of an unstimulated response (response index of >50%) at any given time should be considered a minimally measurable eATP response (5). In practice, users can expect much larger, dose-dependent stimulation indices, provided the inducer and cell type are able to produce an eATP release event.

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4. Appendix

4.A. Other Considerations

Assay Medium Choice

The live-cell, kinetic assay format requires maintaining pH within the cell culture over the entire test exposure period. Standard medium formulations require 5–10% (v/v) CO_2 environments to control changes in pH. Therefore, only use standard medium formulations with atmospheric control-equipped multimode plate readers or standard cell culture incubators in the manual transfer-and-measure mode. For all other luminometers with kinetic and heating control functionality at 37°C, use CO_2-independent pH control. CO_2-Independent Medium (GIBCO Cat.# 18045-088) and Leibovitz (GIBCO Cat.# 11415-064) have been successfully employed with the assay. If CO_2-independent medium formulations are used, verify that these media have no deleterious effects on cell health relative to standard medium formulations.

Mode of eATP Release

If you need to differentiate between active or passive release of eATP, set up parallel assay plates with identical cell numbers, treatment volumes and dilutions without the RealTime-Glo™ Extracellular ATP Assay Reagent. Orthogonal real-time, cell health chemistries for cytotoxicity, viability determinations or both can be measured using CellTox™ Green Cytotoxicity Assay (Cat.# G8741) or RealTime-Glo™ MT Cell Viability Assay (Cat.# G8711), respectively. Consult the *CellTox™ Green Cytotoxicity Assay Technical Manual* #TM375 and *RealTime-Glo™ MT Cell Viability Assay Technical Manual* #TM431 for instructions on applying the reagents and measuring the responses from these assays. Alternatively, LDH-Glo™ Cytotoxicity Assay (Cat.# J2380) can be used to measure loss of membrane integrity from supernatants collected over the exposure. (See Technical Manual #TM548 for more information on using the LDH-Glo™ Assay.)

Spontaneous eATP Release from Cells

Every cell type has a different basal level of eATP release. However, physical manipulation of cells during test setup (harvesting, pipetting and adding volumes of test compound or detection reagent) often cause a significant spontaneous eATP release event (6). Endogenous ATPases (CD39 and those present in serum supplementation) will degrade excess eATP and allow levels to return to an unstimulated baseline within 30 minutes to 2 hours. The RealTime-Glo™ Extracellular ATP Assay Reagent will measure both the initial release and subsequent decay events. Users can choose whether or not to include these events in their stimulation-dependent graphs.

Collating eATP Release Data Beyond 24 Hours

Staggered additions of the RealTime-Glo™ Extracellular ATP Assay Reagent can define eATP release kinetics and dynamics during exposures beyond 24 hour periods, but should not be considered to be seamless with respect to absolute luminescence values. For instance, by implementing the suggested protocol, plates 2, 3 etc., will be exposed to a slightly higher concentration of inducer in the period prior to reagent addition. Further, a 3-hour preincubation is required to allow reagents and eATP to reach equilibrium. Failure to preincubate the reagent may lead to poorly stitched data sets.

Spectral Interferences

Positive control inducers such as doxorubicin, idarubicin and mitoxantrone absorb light in the visible spectrum and can quench luminescence in a dose-dependent manner. Consider normalizing data sets to basal release values for each dose.

3D Cell Models

The protocols described in Section 3 can be used without modification for microtissues and spheroids. Porous extracellular matrices (Matrigel®, etc.) allow uniform reagent access, but may cause optical interferences. You can expect a reduction in luminescence values when using black microplates with rounded bottoms.

Instrumentation Parameters

Instrument interfaces and functionality vary greatly depending upon vendor and instrument model. Familiarize yourself with proper usage of the instrument with particular attention to the following aspects.

- **Integration Time:** In general, increasing integration intervals to at least 0.5 seconds may reduce replicate variation relative to shorter periods.
- **Photomultiplier Adjustment:** Some luminometers or multimodal plate readers offer an automatic gain adjustment feature, which adjusts the photomultiplier tube (PMT) based on selected wells during a preread. This feature should not be used because initial basal luminescence may be artificially high due to physical manipulation of cells during the seeding and dosing setup. Luminescence saturation of the detector is unlikely using the RealTime-Glo™ Extracellular ATP Assay. Therefore, a manual setting that is 85–90% of the highest PMT or gain setting should be used. Alternatively, a vendor-installed program (e.g., GloMax® instruments) should be used as a default.
- **Temperature:** If using automated measure mode, adjust temperature control to 37°C and allow to reach target temperature. If using atmospherically controlled instruments, initiate gas equilibration per the manufacturer's instruction manual. If using manual time-point method, minimize exposure away from the 37°C incubator. When employing manual measures, be aware that ATP release is often dose- and exposure-dependent and subject to kinetic transience. Fewer timepoint measures can affect the quality of the data set collected and underestimate the magnitude of ATP release.

4.A. Other Considerations (continued)

High Throughput Validation

Z´factor analysis of control data sets are often used to evaluate the performance and suitability of an assay for high-throughput screening (7). Because the biology of eATP release is often a relatively transient phenomenon, peak luminescence values collected during a control compound response should be used to assess the dynamic range of the assay chemistry (Figure 9).

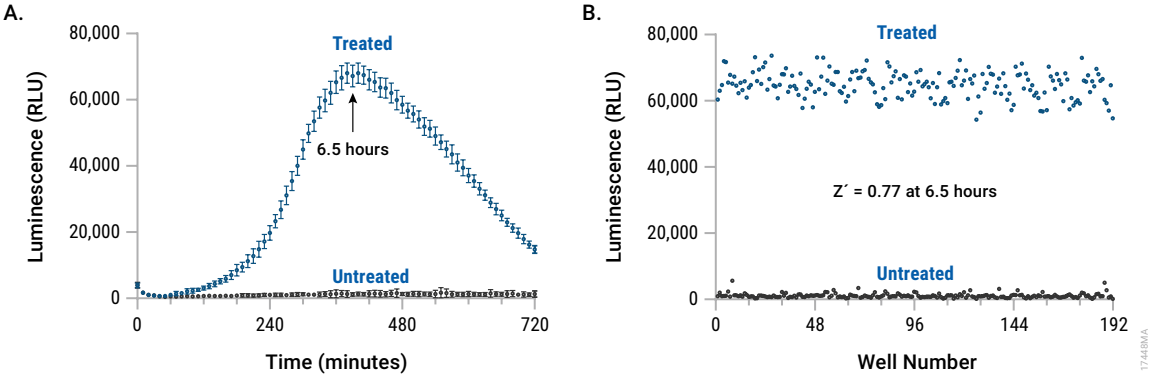

Figure 9. Statistical evaluations of the assay for high throughput suitability. U937 cells were seeded into a 384-well plate at density of 4,000 cells per well in CO_2-Independent medium + 10% FBS and dosed with 10µM romidepsin or left untreated in the presence of the RealTime-Glo™ Extracellular ATP Assay Reagent. Luminescence measures were made using a BMG POLARstar® Omega Plate Reader. **Panel A.** The averaged RLU kinetic responses of the positive and negative control values demonstrate a peak eATP response at ~6.5 hours. **Panel B.** The scatter plot associated with each data point collected, and the resulting Z´ calculation.

4.B. Troubleshooting

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

Symptom	Causes and Comments
High initial signal that decreased with time	High signal with variability in replicates is likely due to spontaneous ATP release as a result of physical manipulation, experimental stresses or both. This signal is independent of test agent or treatment and will typically reach a stable basal release level within 30 minutes to 2 hours. Some spontaneous release can be mitigated by prewarming test agents and reagents and by careful mixing. Mechanical perturbation of cells will exacerbate the phenomenon.
Low sustained signal	Low signal can result from inappropriate reagent dilution, inappropriate luminometer data collection parameters or unresponsive cells. Refer to reagent dilution suggestions or luminometer collection parameters for more guidance. Positive eATP inducer compounds or the optional digitonin lysis protocol serve as reagent controls.
Time-dependent increase in untreated signal	Modest increases in eATP release rate (≤2-fold) between initial stable basal and 24 hours basal release) are to be expected during an exposure due to normal proliferation. The relative health of untreated cells is often linked to density per well and the culture medium employed. Metabolic stress can increase basal eATP release so cell model refinement/adaptation should be conducted prior to employing the reagent. Last, never use the final concentrations of the reagent at levels that exceed recommendations.

4.C. References

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4. Young, C.N.J. and Górecki, D.C. (2018) P2RX7 purinoceptor as a therapeutic target- The second coming? *Front. Chem.* **6**, 248.
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7. Zhang, J.H., Chung, T.D. and Oldenburg, K.R. (1999) A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4**, 67–73.

4.D. Related Products

Cell Viability Assays

Product	Size	Cat.#
CellTiter-Glo® 2.0 Cell Viability Assay	10ml	G9241
CellTiter-Glo® Cell Viability Assay	10ml	G9681
RealTime-Glo™ MT Cell Viability Assay	100 reactions	G9711

Additional sizes available.

Cytotoxicity Assays

Product	Size	Cat.#
LDH-Glo™ Cytotoxicity Assay	10ml	J2380
CytoTox-Glo™ Cytotoxicity Assay	10ml	G9290
CellTox™ Green Cytotoxicity Assay	10ml	G8741

Additional sizes available.

Inflammation Assays

Product	Size	Cat.#
Lumit™ HMGB1 (Human/Mouse) Immunoassay	100 assays	W6110
Caspase-Glo® 1 Inflammasome Assay	10ml	G9951
Lumit™ FcRn Binding Immunoassay	100 assays	W1151
Lumit™ Human IL-1β Immunoassay	100 assays	W6010
Lumit™ Mouse IL-1β Immunoassay	100 assays	W7010
Lumit™ IFNγ (Human) Immunoassay	100 assays	W6040
Lumit™ IL-2 (Human) Immunoassay	100 assays	W6020
Lumit™ IL-4 (Human) Immunoassay	100 assays	W6060
Lumit™ IL-6 (Human) Immunoassay	100 assays	W6030
Lumit™ IL-10 (Human) Immunoassay	100 assays	W6070
Lumit™ TNF-α (Human) Immunoassay	100 assays	W6050

Additional sizes available.

Apoptosis Assays

Product	Size	Cat.#
Caspase-Glo® 3/7 Assay System	2.5ml	G8090
Caspase-Glo® 3/7 3D Assay System	10ml	G8981
RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay	100 assays	JA1011

Additional sizes available.

Energy Metabolism Assays

Product	Size	Cat.#
NAD/NADH-Glo™ Assay	10ml	G9071
NADP/NADPH-Glo™ Assay	10ml	G9081
Glucose Uptake-Glo™ Assay	5ml	J1341
Lactate-Glo™ Assay	5ml	J5021
Glucose-Glo™ Assay	5ml	J6021
Glutamine/Glutamate-Glo™ Assay	5ml	J8021
Glycerol-Glo™ Assay	5ml	J3150
Triglyceride-Glo™ Assay	5ml	J3160
Cholesterol/Cholesterol Ester-Glo™ Assay	5ml	J3190

Additional sizes available.

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4.D. Related Products (continued)

Luminometers

Product	Size	Cat.#
GloMax® Discover System	1 each	GM3000
GloMax® Explorer System (Fully Loaded)	1 each	GM3500
GloMax® Explorer System (Luminescence and Fluorescence)	1 each	GM3510
GloMax® Navigator System	1 each	GM2000
GloMax® Navigator System w/Dual Injectors	1 each	GM2010

4.E. Summary of Changes

The following changes were made to the 4/22 revision of this document:

1. Changed the concentration in Section 3.B, Step 4 Note.
2. Updated Section 4.D.
3. Updated the cover page.