

Miniaturized HTS Assays Using the Aurora Discovery BioRAPTR® FRD® Workstation

Automated Protocol #EP029

DESCRIPTION OF THE AURORA DISCOVERY BIORAPTR® FRD® WORKSTATION PROGRAM FOR PRODUCTS
G7570, G7571, G7572, G7573, G7790, G7791, G7792, G7890, G7891, G8090, G8091, G8092, V3771, V3772, V3773, V3774,
V9770, V9790, V9800, V9880, V9890.

All technical literature is available on the Internet at www.promega.com

Please visit the web site to verify that you are using the most current version of this Automated Protocol.

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I. Description

This document describes automation and miniaturization of the chemistries listed below. Specific instructions are provided for the Aurora Discovery BioRAPTR® FRD® workstation. The 1,536-well plate programs may be requested at:

www.promega.com/automethods/

- CellTiter-Glo® Luminescent Cell Viability Assay (Cat.# G7570, G7571, G7572 and G7573): An assay based on the quantitation of ATP in metabolically active cells.
- Caspase-Glo® 3/7 Assay (Cat.# G8090, G8091 and G8092): A luminescent method to detect caspase-3/7 activity.
- Apo-ONE® Homogeneous Caspase-3/7 Assay (Cat.# G7790, G7791 and G7792): A fluorescent method for the measurement of active caspase-3 and -7 activity.
- CytoTox-ONE™ Homogeneous Membrane Integrity Assay (Cat.# G7890 and G7891): A fluorometric method for estimating the number of nonviable cells.
- P450-Glo™ Screening Systems (Cat.# V9770, V9790, V9800, V9880 and V9890): A complete homogeneous, luminescent method for measuring cytochrome P450 activity.
- Kinase-Glo® Plus Luminescent Kinase Assay (Cat.# V3771, V3772, V3773 and V3774): A homogeneous method to determine purified kinase activity by quantifying the amount of ATP remaining following a kinase reaction.

II. Assay Systems Technical Literature

Detailed descriptions of the assay chemistries and reagents, as well as troubleshooting information are provided in the technical literature supplied with the product. Custom reagent volumes are also available. The Technical Bulletin for each system is listed below. All Promega technical literature is available at: www.promega.com/tbs/

- *CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288*
- *Caspase-Glo® 3/7 Assay Technical Bulletin #TB323*
- *Apo-ONE® Homogeneous Caspase-3/7 Assay Technical Bulletin #TB295*
- *CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin #TB306*
- *P450-Glo™ Screening Systems Technical Bulletin #TB340*
- *Kinase-Glo® Plus Luminescent Kinase Assay Technical Bulletin #TB343*

III. Before You Begin

Materials to Be Supplied by the User

CellTiter-Glo®, Caspase-Glo® 3/7, Apo-ONE® and CytoTox-ONE™ Assays

- 1,536-well opaque white plates suitable for cell culture (Aurora Discovery Part# 00018708)
- multimode luminometer, CCD camera or imaging device capable of reading luminescence and fluorescence in multiwell plates in high-density formats

P450-Glo™ Screening Systems

- 1,536-well opaque white plates suitable for biochemical assays (Aurora Discovery Part# 00018903)
- luminometer or CCD-based imaging device capable of reading luminescence in multiwell plates in high-density formats

Kinase-Glo® Plus Luminescent Kinase Assays

- ATP
- kinase substrate
- kinase of interest
- 1,536-well opaque white plates suitable for biochemical assays (Aurora Discovery Part# 00018903)
- luminometer or CCD-based imaging device capable of reading luminescence in multiwell plates in high-density formats

III.A. Preparation of Buffers and Solutions

Please read the following protocols carefully before beginning an assay. Directions are given for performing the assays in 1,536-well plate format. However, each assay can be easily adapted to different volumes if the ratio of assay components is kept constant across the range of volumes tested.

1. Before beginning each assay, prepare each reagent or assay component according to the instructions listed in the Technical Bulletin supplied with the product.
2. Equilibrate each reagent to room temperature, where appropriate, before beginning the assay.

III.B. Sample Preparation Before Automated Processing

For best results using Promega cell-based assay chemistries, empirical determination of the optimal cell number, treatment conditions and incubation time for the cell culture system may be necessary. Use identical cell numbers and volumes for test and control samples. For cell-based assays, equilibrate the sample plate and its contents to room temperature for approximately 30 minutes prior to performing the assay. See the Technical Bulletin supplied with each system for specific recommendations.

IV. Automated Processing Requirements

IV.A. Instrument Requirements

The following is a list of parts and their corresponding part numbers required for use of Promega cell-based and biochemical assays on the BioRAPTR® FRD® workstation.

Part Description	Quantity	Aurora Discovery Part Number
BioRAPTR® FRD® Workstation with the 4-Tip, 1536 Dispense Head	1	00018490
Sartorius Balance with Draft Shield	1	00019034
ChemLib MicroPlate, 1536-Well, White, Nonsterile, Untreated, With Black Lid	1	00018903
ChemLib Plate, 1536-Well, White St/Tr Sterile, Treated, With Black Lid	1	00018708

Other flanged and nonflanged plates are available from Aurora Discovery.

IV.B. Instrument Setup for Assays in a 1,536-Well Plate

The BioRAPTR® FRD® workstation is a bulk reagent dispenser. A single reagent bottle is connected to one dispensing tip. One or all tips can dispense to the same assay well sequentially. Each tip is capable of delivering reagent to any well on the plate. Thus assay sample composition can be varied according to the number of reagents available to the BioRAPTR® FRD® workstation and the volume of each reagent dispensed to each well. Multiple assay components may be dispensed during the same robotic program. Tables 1, 2 and 3 describe which assay reagent is placed into each bottle to perform the assay. The volume of reagent added to each well of the plate is also given. Tables 1 and 2 demonstrate how multiple chemistries can be run on separate sections of the same 1,536-well assay plate.

CellTiter-Glo®, Caspase-Glo® 3/7, Apo-ONE® and CytoTox-ONE™ Assays

In this application, all four cell-based assays can be performed in separate areas (e.g., rows or columns) of the same 1,536-well assay plate.

Table 1. Cell Culture and Reagent Volumes for Each Well of a 1,536-Well Plate Using the CellTiter-Glo[®], Caspase-Glo[®] 3/7, Apo-ONE[®] and CytoTox-ONE[™] Assays.

	Reagent	Bottle 1	Bottle 2	Bottle 3	Bottle 4
Day 1	Cultured cells	2.0µl			
	Culture medium		2.0µl		
Day 2	CellTiter-Glo [®] Reagent	4.0µl			
	Caspase-Glo [®] 3/7 Reagent		4.0µl		
	Apo-ONE [®] Reagent			4.0µl	
	CytoTox-ONE [™] Reagent				4.0µl
	CytoTox-ONE [™] Stop Solution				2.0µl

P450-Glo[™] Screening Systems

Promega offers five P450-Glo[™] Screening Systems, which can be used in a 1,536-well plate format.

Table 2. Reagent Volumes for Each Well of a 1,536-Well Plate Using the P450-Glo[™] Assay System.

Reagent	Bottle 1	Bottle 2	Bottle 3	Bottle 4
4X CYP1A2/KPO ₄ /Substrate Reaction Mixture	1.0µl			
4X CYP2C9/KPO ₄ /Substrate Reaction Mixture		1.0µl		
4X CYP3A4/Substrate Reaction Mixture			1.0µl	
4X CYP2C19/KPO ₄ /Substrate Reaction Mixture				1.0µl
4X CYP2D6/KPO ₄ /Substrate Reaction Mixture	1.0µl			
2X NADPH Regenerating System	2.0µl			
2X NADPH CYP3A4 Regenerating System		2.0µl		
Luciferin Detection Reagent for CYP1A2, 2C9 and 3A4 assays	4.0µl			
Luciferin Detection Reagent for CYP2C19 and 2D6 assays		4.0µl		

Kinase-Glo® Plus Assays

The Kinase-Glo® Plus Luminescent Kinase Assay, which measures kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction, can be performed in a 1,536-well plate.

Table 3. Reagent Volumes for Each Well of a 1,536-Well Plate Using the Kinase-Glo® Plus Luminescent Kinase Assay.

Reagent	Bottle 1	Bottle 2	Bottle 3	Bottle 4
Kinase/Kinase Substrate Mixture	2.0µl			
ATP		1.0µl		
Kinase-Glo® Plus Reagent	5.0µl			

V. Description of Cell-Based and Biochemical Assays in a 1,536-Well Plate

This overview describes the general liquid-handling steps required to perform assays in a 1,536-well plate. Each protocol assumes that the test compound has been previously dispensed to the plate. The assays can be adapted to different volumes if the 1:1 ratio of reagent volume to cell or reaction volume is preserved. These protocols are written for optimized base programs. They can be modified to meet your specific needs.

V.A. CellTiter-Glo®, Caspase-Glo® 3/7, Apo-ONE® and CytoTox-ONE™ Assays

Prior to beginning the program, dispense vehicle or test compounds into the appropriate wells of the assay plate. The volume of test compound or vehicle added to each well of the assay plate is 2.0µl for the 1,536-well plate program. The bottles and tips used for each dispensing step are described in Section IV.B, Table 1.

1. The BioRAPTR® FRD® workstation dispenses mammalian cells in culture medium to the appropriate wells of the assay plate. Culture medium without cells is dispensed to wells designated as negative control reactions. The optimal number of cells per well should be determined empirically.
2. Incubate the plate at 37°C in a humidified, 5% CO₂ atmosphere for the desired amount of time.
Note: The incubation time between dispensing the cells and performing the assay will vary depending on the type of cells and the nature of the assay being performed.
3. The workstation dispenses the CellTiter-Glo®, Caspase-Glo® 3/7, Apo-ONE® and CytoTox-ONE™ Reagents to the appropriate wells of the assay plate. The reagents are dispensed in the order shown in Table 1.
4. Rinse line 4 with deionized water.
5. Replace bottle 4, which contains the CytoTox-ONE™ Reagent, with a bottle that contains the appropriate volume of CytoTox-ONE™ Stop Solution.
6. After a 10-minute delay, the workstation adds the CytoTox-ONE™ Stop Solution to wells containing CytoTox-ONE™ assays.

7. Incubate the assay plate at room temperature to stabilize the signal before measuring the fluorescence or luminescence. The minimum incubation times are as follows:

CytoTox-ONE™ Assay	10 seconds (up to 1 hour)
CellTiter-Glo® Assay	10 minutes
Caspase-Glo® 3/7 Assay	30 minutes
Apo-ONE® Assay	30 minutes (up to 18 hours)

Note: Due to extended signal stability of the assays, the assay plate can be incubated for 30 minutes after reagent addition. The luminescent and fluorescent signals can then be measured after this 30-minute incubation. The half-life of the CellTiter-Glo® Assay signal is 5 hours, the half-life of the Caspase-Glo® 3/7 Assay signal is 3 hours.

8. Measure the fluorescent or luminescent signal of each well using a multimode plate-reading imaging device.

Note: Filter sets are required to read the fluorescent Apo-ONE® and CytoTox-ONE™ Assays. We recommend using an excitation wavelength of $485 \pm 20\text{nm}$ and an emission wavelength of $530 \pm 25\text{nm}$ for Apo-ONE® Assays, and an excitation wavelength of 560nm and an emission wavelength of 590nm for CytoTox-ONE™ Assays.

V.B. P450-Glo™ Screening Systems

Prior to beginning the program, dispense 1.0µl of the vehicle or test compounds into the appropriate wells of the 1,536-well assay plate. The bottles and tips used for each dispensing step are described in Section IV.B, Table 2.

1. Add the 4X Cytochrome P450/Substrate Reaction Mixture for CYP1A2, 2C9, 3A4 and 2C19 to the appropriate wells of the assay plate.
2. Rinse line 1 with deionized water.
3. Add the 4X Cytochrome P450/Substrate Reaction Mixture for CYP2D6 to the appropriate wells of the assay plate.
4. Incubate plate for 10 minutes at room temperature. Rinse lines 1–4 with deionized water.
5. Add the 2X NADPH Regenerating Systems and 2X CYP3A4 NADPH Regenerating Systems to the appropriate wells of the assay plate.

Note: For assays with CYP3A4, better results are obtained when the KPO_4 is withheld from the 4X Reaction Mixture and is added at a 2X concentration (400mM) as a component of the 2X CYP3A4 NADPH regenerating system (see *P450-Glo™ Screening Systems Technical Bulletin #TB340*).

6. Incubate the plate for 60 minutes at room temperature. Rinse lines 1–2 with deionized water.
7. Add the Luciferin Detection Reagent for CYP1A2, 2C9 and 3A4 to the appropriate wells of the assay plate. Add the Luciferin Detection Reagent for CYP2C19 and 2D6 to the appropriate wells of the assay plate.



These Luciferin Detection Reagents are not interchangeable, so be sure to use the correct reagent with a given cytochrome P450 enzyme assay. Label the reconstituted Luciferin Detection Reagent so that you know which buffer was used to reconstitute the lyophilized Luciferin Detection Reagent.

8. Incubate the plate for 20 minutes at room temperature.
9. Measure the luminescence of each sample using a plate-reading luminometer or CCD-based imaging device.

V.C. Kinase-Glo® Plus Luminescent Kinase Assay

Prior to beginning the program, dispense 2.0µl of the vehicle or test compounds into the 1,536-well assay plate. The bottles and tips used for each dispensing step are described in Section IV.B, Table 3.

1. Dispense the kinase/kinase substrate mixture to the appropriate wells of the assay plate.
2. Initiate the kinase reactions by dispensing ATP to the appropriate wells of the assay plate.
3. Incubate the plate at room temperature for 20 minutes. Rinse lines 1 and 2 with deionized water.
4. Add the Kinase-Glo® Plus Reagent.
5. Incubate the plate at room temperature for 20 minutes.

Note: The long half-life of the Kinase-Glo® Plus signal (greater than 4 hours) allows plates to be left longer at room temperature, if desired.

6. Measure the luminescence of each sample using a plate-reading luminometer or CCD-based imaging device.

VI. General Guidelines for Adaptation to the BioRAPTR® FRD® Workstation

VI.A. Calibration

Calibration activities on the BioRAPTR® FRD® workstation ensure dispensing accuracy. During calibration, a calibration file is created, verified, then written to a dispense head. Calibration should be performed if the properties of the reagent being dispensed deviate from those of water, a dispense valve is replaced, the dispense pressure is modified to accommodate reagent viscosity or dispense accuracy no longer meets acceptable specifications.

Once the BioRAPTR® calibration software is opened, the Auto Calibration tab is the primary tab used for priming activities, automated calibration operation and customized calibration operation (Figure 1).

When preparing for an automated calibration, the operator must select tips to be calibrated. To activate the tips, click the green buttons labeled with the appropriate tip number (Figure 2). The Plate Type control box allows the operator to select the microplate density associated with the dispense head. In this case, calibrate using the 1,536 dispense head. The Max Retries box allows the operator to define how many times a failed point will be repeated before moving to the next point. We recommend limiting the number of retries to 1. A test type can be selected from the pull-down menu. Three test types are available to the operator: Calibration and Volume, Calibration Only and Volume Only.

Once all parameters are set, the operator clicks the run test button to initiate the automated calibration. In the event of a problem, the pause button can be clicked to pause the calibration. Pause is usually used if the reagent bottles require refilling or if the waste reservoir is too full.

Once calibration has begun, a message box will appear to confirm the intent to clear existing data. Clicking “Yes” will clear the calibration file data for the selected tips (data for tips not selected will not be cleared). Tip calibration will begin using default timings, referred to as counts, which are used to equate the necessary length of time the valve is open to achieve accurate dispensing. A calibration cycle involves taring the balance, dispensing each count 200 times and recording the weight. The weight is automatically converted to a volume and displayed on the Calibration QC tab. The actions buttons (e.g., Volume Test, Mark All, etc.) will be dimmed indicating that they are not available during the calibration cycle. The calibration cycle is repeated for all counts.



Figure 1. The BioRAPTR® FRD® calibration software general user interface.

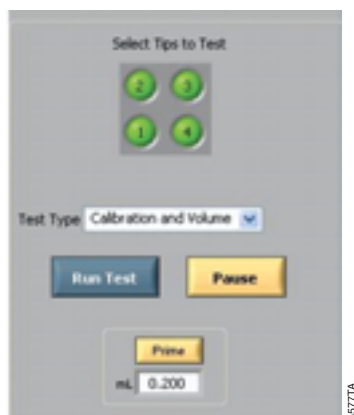


Figure 2. Automated calibration.

VI.B. Dispense Table

Dispense patterns on the BioRAPTR® FRD® workstation are extremely flexible and can be created on any computer with Microsoft® Excel. The workstation supports user-defined dispense patterns, where each well can be individually addressed by each tip. The control software uses Excel worksheets to assign dispense patterns and volumes for each of the reagents. An additional worksheet determines the total volume of reagent assigned to each well. Collectively, these worksheets are called a dispense table, which is an Excel-based plate map to allow users to identify dispense wells and set up dispense volumes in microliters (Figure 3). To create a new dispense table, open and modify an existing dispense table. Be sure to save the new dispense table using the “Save As” function.

Note: All Excel files, including the file used as the plate map file, and the Excel program must be closed before the BioRAPTR® FRD® program is used.

29		2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
30	H	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
31		2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
32		2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
		1				2			
		1	2	3	4	5	6	7	8
		Tip 1 /		Tip 2 /		Tip 3 /		Tip 4 /	
				Total /					

Figure 3. Dispense table section.

VI.C. Bottle Setup

The BIORAPTR® FRD® workstation is equipped with reagent reservoir bottles containing deionized water when not in use (Figure 4). Preparation entails replacing reagent reservoir bottles containing deionized water with those containing reagents, then executing a prime to ensure the fluid path is completely filled with the desired reagent. Cleaning of the lines is typically done through priming with deionized water.

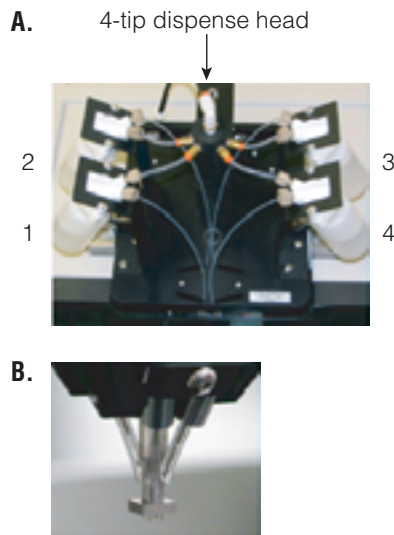


Figure 4. Reservoir orientation relative to dispense tips. Panel A. The position of the 4-tip dispense head and the four reagent bottles. Panel B. A close-up of the 4-tip dispense head.

To remove the reagent bottles from the BioRAPTR® FRD® workstation:

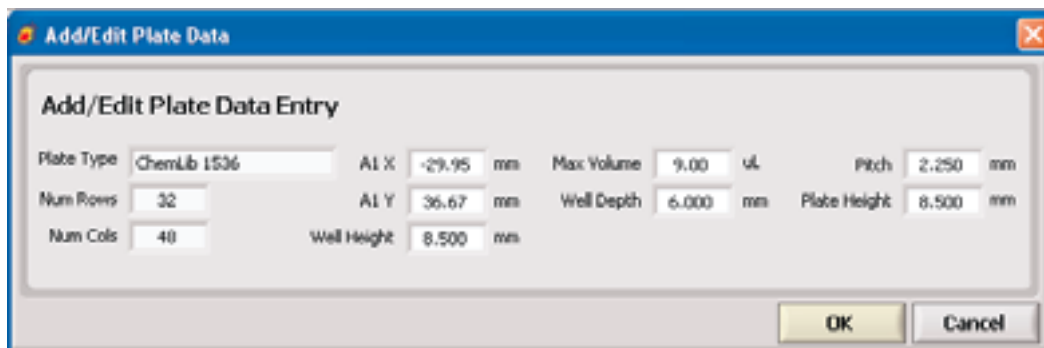
1. Verify that the BioRAPTR® FRD® control software is idle by checking the status bar on the Main tab of the software.
2. Depressurize the fluidics by releasing the quick-disconnect air valve to the dispense head.
3. On the dispense head, locate the reagent reservoir bottle that you intend to replace.
4. Hold the lid steady while unscrewing the reagent reservoir bottle from its lid.
5. Remove the reagent reservoir bottle containing deionized water from the dispense head. Hold the lid steady while screwing the reagent reservoir bottle tightly into the lid.

Note: Verify the lid is screwed on tightly to prevent system air leaks.

6. Screw a clean lid onto the reagent reservoir bottle containing deionized water, then set the bottle aside.
7. Connect the pressurized air valve.
8. Repeat Steps 3–7 for any additional reagent reservoir bottles required for the assay.

VI.D. Plate Data Files

The BioRAPTR® FRD® plate data file defines the various plate type parameters, such as well heights and well positions. Plates need to have their plate parameters registered in the BioRAPTR® FRD® control software prior to use (see Figure 5 for an example). Registering a microplate entails manually entering the parameters of the new plate, aligning the tips with the A1 well location and storing these values in the BioRAPTR® FRD® control software. After defining all plate types, we recommend performing a practice run to validate alignment and dispensing positional accuracy.



Add/Edit Plate Data Entry			
Plate Type	ChemLib 1536	A1 X	-29.95 mm
		Max Volume	9.00 uL
		Pitch	2.250 mm
Num Rows	32	A1 Y	36.67 mm
		Well Depth	6.000 mm
		Plate Height	8.500 mm
Num Cols	40	Well Height	8.500 mm

Figure 5. An example of plate data file specification parameters.

VI.E. Plate Carrier

The plate carrier, also known as the plate holder, is integrated with the positioning slides. It can hold one plate at a time. The plate sits on the plate carrier with the chamfered, A1 corner facing the far right part of the enclosure when viewed from the front of the workstation (Figure 6). A pneumatic-powered clamp secures the plate and is activated using the BioRAPTR® FRD® control software. A plate can be added or removed from the plate carrier when the clamp is retracted or when prompted by the software.

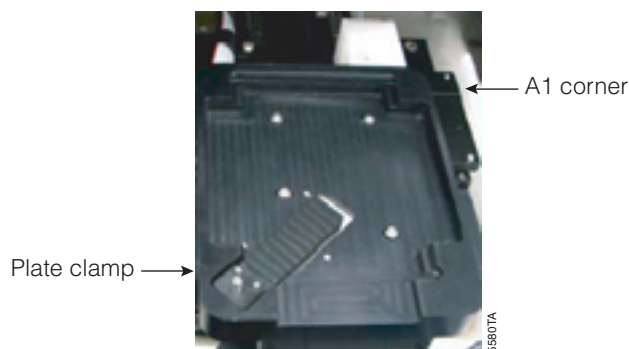


Figure 6. Plate orientation on the plate carrier.

VI.F. Dispense Velocity

The Velocity box on the BioRAPTR® FRD® software allows an operator to decrease the velocity of dispensing when necessary, although it is best to use the recommended velocity. After the appropriate dispense table has been selected, the software automatically inputs a dispense velocity that is appropriate for the volume to be dispensed and the chosen plate. The recommended velocity is specific to each BioRAPTR® FRD® workstation and the reagent being dispensed.

VI.G. Preparing the BioRAPTR® FRD® Workstation for Use

The BioRAPTR® FRD® workstation requires minimal preparation prior to operation. These activities typically take two to five minutes and include:

- Loading the dispense table by selecting “Load Dispense Table” and selecting the appropriate Excel file (Figure 7).
- Selecting the plate type into which you will be dispensing, and selecting the appropriate checkboxes for delidding, barcode read or evaporation wells.
- Placing the desired reagents into bottles, selecting appropriate tips and executing the prime by selecting “Prime”.
- Placing the plate onto plate carrier.
- Executing the dispense by selecting “Dispense”.



Figure 7. The Main tab of the BioRAPTR® FRD® software.

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All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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