

Automated CytoTox-ONE™ Homogeneous Membrane Integrity Assay Protocol



Automated Protocol #EP016

**DESCRIPTION OF THE BECKMAN COULTER BIOMEK® 2000 and BIOMEK® FX METHODS
WITH PRODUCTS G7891 and G7892.**

Please visit the web site to verify that you are using the most current version of this Automated Protocol.
All technical literature is available on the internet at www.promega.com

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I. Description

This protocol describes automation of the CytoTox-ONE™ Homogeneous Membrane Integrity Assay^(a). Specific instructions are provided for the Beckman Coulter Biomek® 2000 and Biomek® FX Workstations. For information about available validated methods, please see: www.promega.com/automethods/

Please refer to the *CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin #TB306* to troubleshoot chemistry issues.

Note: All Promega Technical Bulletins are available at: www.promega.com/tbs/

II. Product Components

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	1,000–4,000 assays	G7891

Includes:

- 10 vials Substrate Mix
- 120ml Assay Buffer
- 0.5ml Lysis Solution
- 60ml Stop Solution
- 1 Protocol

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP	1,000–4,000 assays	G7892

Includes:

- 2 vials Substrate Mix
- 120ml Assay Buffer
- 0.5ml Lysis Solution
- 60ml Stop Solution
- 1 Protocol

Each 100ml of reagent is sufficient to perform 768 × 100µl assays in 96-well plates, or 3,072 × 25µl assays in 384-well plates using the Beckman Coulter Biomek® 2000 workstation. When using the Biomek® FX workstation, 100ml of reagent is sufficient to perform 384 × 100µl assays in 96-well plates or 1,536 × 25µl assays in 384-well plates. The single-plate Biomek® 2000 method includes a 2ml dead volume in the reagent trough, while the single-plate Biomek® FX method includes a 15ml dead volume in the reagent reservoir. The number of assays processed per 100ml of reagent will increase if multiplate methods are run and unused reagent is reused.

Storage Conditions: For long-term storage, store all components frozen at –20°C protected from light. The product is stable for 6 months when stored and handled properly. Reconstituted CytoTox-ONE™ Reagent may be stored without loss of activity for up to 3 days at room temperature, up to 1 week at 4°C, or 6–8 weeks at –20°C protected from light.

III. Before You Begin

Materials to Be Supplied by the User

- 96- or 384-well opaque white or black plate suitable for cell culture
- Fluorescent plate reader with excitation at 530–570nm and emission at 580–620nm.

A. Preparation of Solutions

Please read the following protocol carefully before using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on an automated workstation. Directions are given for performing the assay in a total volume of 250µl using 96-well plates, or in a total volume of 62.5µl using 384-well plates and a fluorescent plate reader. These volumes include the addition of Stop Solution to each assay well. However, the assay can be easily adapted to different volumes providing the 1:1 ratio of CytoTox-ONE™ Reagent volume to sample volume is preserved (e.g., 25µl of sample + 25µl CytoTox-ONE™ Reagent). If using Stop Solution in the assay, the 0.5:1 ratio of Stop Solution to sample volume must also be preserved.

CytoTox-ONE™ Reagent Preparation

Equilibrate the Substrate Mix and Assay Buffer to 22°C. A 37°C water bath may be used to thaw the Assay Buffer. Do not leave the Assay Buffer at 37°C longer than necessary. Prepare CytoTox-ONE™ Reagent by following the instructions provided on the product information sheet included with each system. Gently mix to dissolve the substrate. Protect the reagent from direct light. Unused portions of the CytoTox-ONE™ Reagent may be stored tightly capped at –20°C for 6–8 weeks.

B. Sample Preparation Before Automated Processing

1. Before starting the assay, prepare the CytoTox-ONE™ Reagent as described in Section III.A and mix thoroughly.
2. For best results, empirically determining the optimal cell number, induction treatment and incubation time for the cell culture system may be necessary.
3. Use identical cell numbers and volumes for assay and negative control samples. Wells that do not contain assay reactions or controls should contain a volume of liquid (water or medium) equal to that of assay and control wells.
4. The fluorescent product generated by the CytoTox-ONE™ Assay is proportional to the quantity of LDH. The enzymatic activity of LDH is influenced by temperature. We recommend that the temperature of the assay plate and the CytoTox-ONE™ Reagent be equilibrated to 22°C for 20–30 minutes before adding the CytoTox-ONE™ Reagent to initiate the reaction.

**IV. Automated Processing Requirements:
Beckman Coulter Biomek® 2000 Workstation**

A. Instrument Requirements for the Biomek® 2000 Workstation

Description	Quantity	Beckman Coulter Part#
Biomek® 2000 Workstation, 50/60 Hz, 100–120V	1	609000
Biomek® 2000 Controller NT	1	609875
BioWorks™ 3.2 for Beckman Coulter Computer	1	609983
MP200 Multi Channel Tool	1	609025
Gripper Tool System with disposal option	1	609001
DPC MicroMix® 5 Shaker	1	380560
DPC MicroMix® 5 Integration kit	1	380561
Tip Rack Holder	1	609121
Gray Labware Holder	2	609120
Frame for Reservoirs	1	372795
Quarter Reservoir, Divided by Length	1	372788
Quarter Reservoir	1	372790

B. Labware Requirements for the Biomek® 2000 Workstation

Description	Quantity	Ordering Information
Biomek® P250 tips (rack)	1	Beckman Coulter Part# 372655
Costar® 96-well clear-bottom plate, white, polystyrene or equivalent (for 96-well assay)	1	Corning Part# 3610
Costar® 384-well clear-bottom plate, white, polystyrene or equivalent (for 384-well assay)	1	Corning Part# 3707

C. Initial Deck Layout for 96-Well Assay on the Biomek® 2000 Workstation

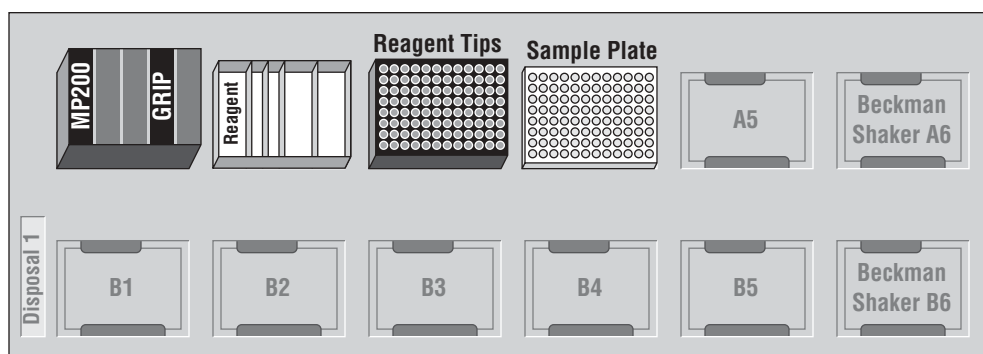


Figure 1. Deck layout for 96-well assay using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on the Biomek® 2000 workstation. This is an example of a CytoTox-ONE™ 96-well assay deck layout on the Biomek® 2000 workstation.

Position Name	Part Sitting on Deck Position
A1	Tool rack: 1) MP200 Multi Channel Tool; 2) Empty; 3–5) Gripper tool
A2	Frame for Reservoir: Quarter reservoir, containing 12ml CytoTox-ONE™ Reagent ; Quarter reservoir, divided by length, left side containing 7ml Stop Solution
A3	P250 tips
A4	96-well assay plate containing 100µl/well of sample, negative control or blank
A5	Empty
A6	Beckman Coulter shaker integration plate holder
B1–B5	Empty
B6	Beckman Coulter shaker integration plate holder

D. Initial Deck Layout for 384-Well Assay on the Biomek® 2000 Workstation

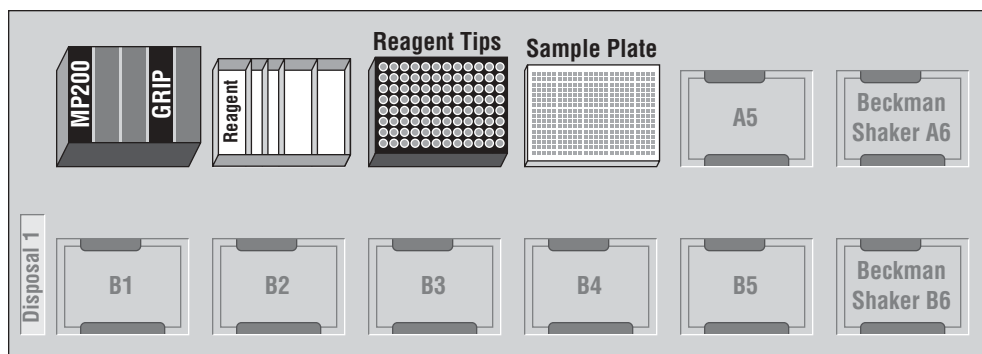


Figure 2. Deck layout for 384-well assay using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on the Biomek® 2000 workstation. This is an example of a CytoTox-ONE™ 384-well assay deck layout on the Biomek® 2000 workstation.

Position Name	Part Sitting on Deck Position
A1	Tool rack: 1) MP200 Multi Channel Tool; 2) Empty; 3–5) Gripper tool
A2	Frame for Reservoir: Quarter reservoir, containing 12ml CytoTox ONE™ Reagent; Quarter reservoir, divided by length, left side containing 7ml Stop Solution
A3	P250 tips
A4	384-well assay plate containing 25µl/well of sample, negative control or blank
A5	Empty
A6	Beckman Coulter shaker integration plate holder
B1–B5	Empty
B6	Beckman Coulter shaker integration plate holder

E. Pre-Run Biomek® 2000 Workstation-Specific Requirements

Instructions on importing Biomek® 2000 programs and instructions for integration of the DPC MicroMix® 5 Shaker on the Biomek® 2000 are available on Promega’s web site in the documents: *Importing Biomek® 2000 Programs* and *DPC MicroMix® 5 Shaker Integration: Biomek® 2000* (www.promega.com/automethods/beckman/biomek2000)

**V. Automated Processing Requirements:
Beckman Coulter Biomek® FX Workstation**
A. Instrument Requirements for the Biomek® FX Workstation

Any single-arm multichannel Biomek® FX is able to run this protocol. The protocol can also be adapted for a dual-arm Biomek® FX with at least one multichannel pod.

Part Description	Quantity	Beckman Coulter Part#
Minimum: Biomek® FX Software version 2.1		Contact Beckman Coulter
Minimum number of Labware Positions by 1 POD	5	Contact Beckman Coulter
Tip Loader ALP	1	719356
Orbital Shaker ALP	1	Contact Beckman Coulter
96-channel POD (for both 96-well and 384-well assays)	1	Contact Beckman Coulter

B. Labware Requirements for the Biomek® FX Workstation

Part Description	Quantity	Ordering Information
Requirements for 96-well assay		
Costar® 96-well clear-bottom plate, white, polystyrene or equivalent	1	Corning Part# 3610
AP96 P250 Tips	2	Beckman Coulter Part# 717251
96-well, pyramid-bottom reservoir, polypropylene	2	Innovative Microplate Part# S30014
Requirements for 384-well assay using 96-well tips (96-384-well format)		
Costar® 384-well clear-bottom plate, white, polystyrene or equivalent	1	Corning Part# 3707
AP96 P250 Tips	2	Beckman Coulter Part# 717251
96-well, pyramid-bottom reservoir, polypropylene	2	Innovative Microplate Part# S30014

C. Initial Deck Layout for 96-Well Assay on the Biomek® FX Workstation

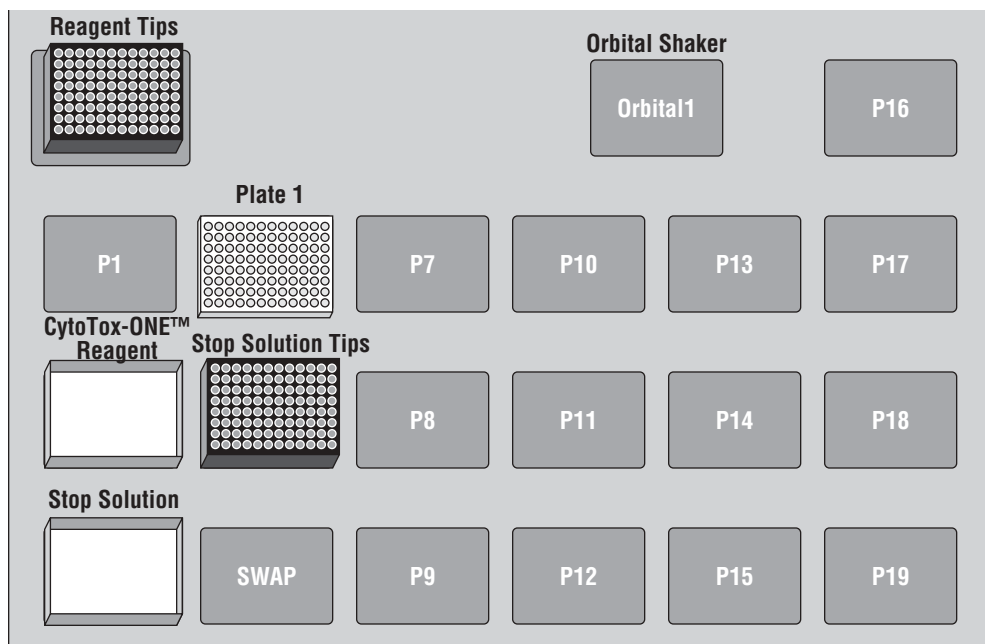


Figure 3. Deck layout for 96-well assay using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on the Biomek® FX workstation. This is an example of a CytoTox-ONE™ Homogeneous Membrane Integrity 96-well assay deck layout on the Biomek® FX workstation. Your specific deck layout may be different depending on your Biomek® FX configuration.

ALP Name	Part Sitting on ALP
Tip loader	AP96 P250 tips
P1	Empty
P2	Pyramid-bottom reservoir containing 25ml CytoTox-ONE™ Reagent
P3	Pyramid-bottom reservoir containing 20ml of Stop Solution
P4	96-well assay plate containing 100µl/well of sample, negative control or blank
P5	AP96 P250 Tips
P6	Swap Space
P7–P19	Empty
Orbital 1	Orbital shaker ALP

D. Initial Deck Layout for 384-Well Assay Using a 96-Channel POD on the Biomek® FX Workstation

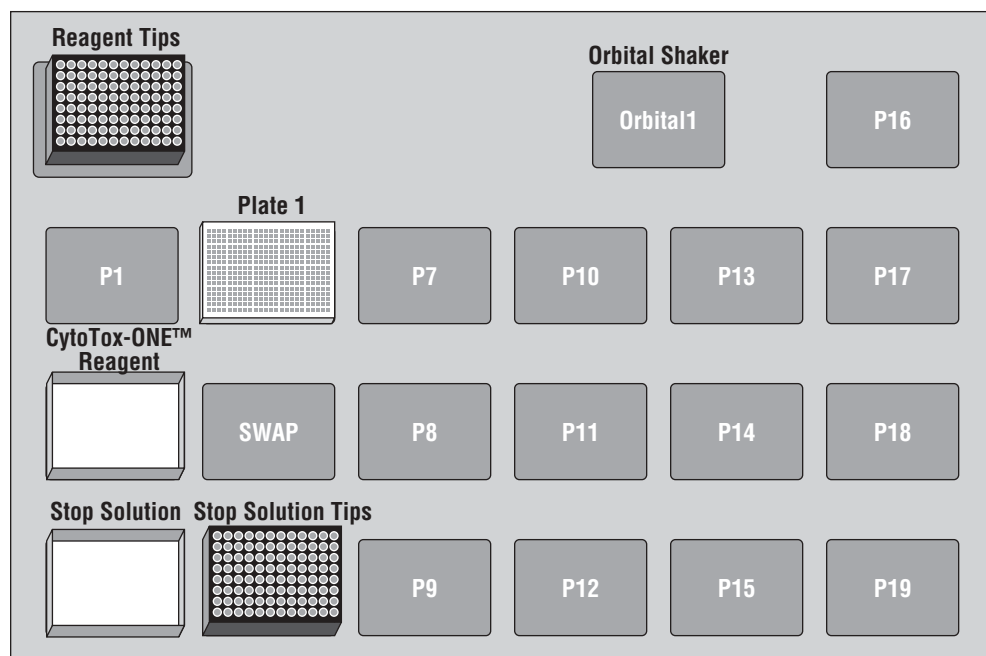


Figure 4. Deck layout for 384-well assay using a 96-channel POD and the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on the Biomek® FX workstation. This is an example of a CytoTox-ONE™ 384-well assay deck layout on the Biomek® FX workstation. Your specific deck layout may be different depending on your Biomek® FX configuration.

ALP Name	Part Sitting on ALP
Tip loader	AP96 P250 tips
P1	Empty
P2	Pyramid-bottom reservoir containing 25ml CytoTox-ONE™ Reagent
P3	Pyramid-bottom reservoir containing 20ml Stop Solution
P4	384-well assay plate containing 25µl/well of sample, negative control or blank
P5	Swap Space
P6	AP96 P250 Tips
P7–P19	Empty
Orbital 1	Orbital shaker ALP

E. Pre-Run Biomek® FX Workstation-Specific Requirements

The Biomek® FX workstation allows users the flexibility to configure the robot's deck according to need. Because of this flexibility, it is likely that the deck used for writing a Biomek® FX method will differ from an end-user's deck. Therefore, it is generally necessary to map an imported method onto an end-user's deck configuration. To map an imported method onto your deck, please follow the instructions provided in the document *Biomek® FX Deck Mapping* (www.promega.com/automethods/beckman/biomekfx/default.asp).

Before the first run of the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on the Biomek® FX, ensure that the deck has been properly framed. Failure to do so may result in tips bending during the method and potentially damaging the instrument.

VI. Description of the CytoTox-ONE™ Homogeneous Membrane Integrity Assay

This overview describes general liquid handling steps required for performing 25µl or 100µl assays in a 384-well or 96-well format. The assay can be adapted to a variety of automated liquid handling robots, as well as to different volumes provided the 1:1 ratio of CytoTox-ONE™ Reagent volume to sample volume is preserved. If using Stop Solution, the 0.5:1 ratio of Stop Solution to sample volume must also be preserved. For additional information for adaptation to liquid handling robots other than those referenced above, please see section VII, "General Guidelines for Adaptation to Alternative Robotic Platforms".

A. CytoTox-ONE™ Reagent Addition

96-Well Format: CytoTox-ONE™ Reagent (100µl) is transferred to the assay plate containing 100µl of blank, untreated control cells or treated cells in culture.

384-Well Format using 96-channel POD (96-384-well format): CytoTox-ONE™ Reagent (25µl) is transferred to the assay plate containing 25µl of blank, untreated control cells or treated cells in culture.

To avoid cross-contamination, do not allow the pipette tips to touch the material in the sample wells.

B. CytoTox-ONE™ Reagent and Sample Mix

1. Assay Plate Transfer. The assay plate is transferred to the orbital shaker.
2. Incubation Mix. The contents of the wells are mixed at 300–500rpm for 30–60 seconds.
3. Assay Plate Replacement. The assay plate is transferred back to its original position on the deck.

C. Sample Plate Incubation

The sample plate is incubated at 22°C for 10 minutes.

D. Stop Solution Addition (optional)

96-Well Format: Stop Solution (50µl) is transferred to the assay plate containing 100µl of blank, untreated control cells, or treated cells in culture and 100µl of CytoTox-ONE™ Reagent.

or

384-Well Format Using a 96-Channel POD (96-384-well format): Stop Solution (12.5µl) is transferred to the assay plate containing 25µl of blank, untreated control cells, or treated cells in culture and 25µl of CytoTox-ONE™ Reagent.

Manually assay samples using a fluorescent plate reader after the predetermined incubation time period. Shake plate for 10 seconds and record fluorescence with an excitation wavelength of 560nm and an emission wavelength of 590nm.

VII. General Guidelines for Adaptation to Alternative Robotic Platforms

To avoid cross-contamination of samples or introduction of bubbles into the wells, ensure that the tips do not touch the liquid in the wells during dispensing of the reagent. No tip touches are done on the sides of the wells. This makes it possible to aliquot reagent to more than one plate using a single box of tips.

If each 96- or 384-well assay plate is not filled completely with samples, we recommend that the empty wells be filled with water or media to a level that is equal to the sample volume.

(a)Patent Pending.

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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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