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# Automation of CyQuant LDH Cytotoxicity Assay using Biomek i7 Hybrid Automated Workstation to Monitor Cell Health

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## Abstract

Determination of cell health is a part of many cell-based screening assays. It is used both as a final end-point measurement and a simple method for cell culture quality control. There are a multitude of different microplate-based assays that can be used to quantify living, viable cells or the number of dead and dying cells. One commercially available cytotoxicity kit is the CyQuant LDH Cytotoxicity Fluorescence assay from Thermo Fisher. This is a relatively fast and straightforward method that is highly amenable to automation using liquid handlers, like the Biomek i7 Hybrid Automated Workstation. Here we show that the automated method allows quantification of cytotoxicity over a wide range of cell densities and can accurately quantify the potency of cytotoxic chemical agents. Additionally, the automated assay setup can be adapted to differentiate cytotoxic from cytostatic compounds. In summary, automated CyQuant LDH Fluorescent assay plate handling by the Biomek i7 Hybrid Automated Workstation performs equivalently to manually prepared plates, while increasing throughput, requiring less user hands-on time, and reducing the chance of user-introduced error.

## Introduction

Determination of cell viability and toxicity are common end points in many cell-based screening paradigms, such as the identification of new chemotherapeutic agents or the discovery of new biologics via monitoring antibody-dependent cellular cytotoxicity. Additionally, monitoring viability of the cells within a microplate well can be a quality control step to allow data normalization in assays that measure other cellular events, such as reporter genes, second-messenger signaling, or protein trafficking. A large number of plate-reader compatible assays have been developed to monitor cell health, and these can largely be divided into those that quantify cell viability (i.e. the number of living cells) and those that track cytotoxicity (i.e. the number of dead cells). Viability assays often measure common markers of health that are critical in cellular metabolic functions, like the ability to synthesize ATP (luciferase bioluminescence) or mitochondrial function through the oxidative reduction of cell-permeable dyes (MTT absorbance assay). Cytotoxicity assays generally employ stains that selectively label dead cells (Trypan blue exclusion) or monitor cell integrity through detection of membrane leakage of cellular components from dying cells.[1] One such component that is released by dying cells and can be readily measured is the enzyme lactate dehydrogenase (LDH).

LDH converts lactate to pyruvate in an $NAD^+$-dependent manner. This reaction generates NADH, which acts as a cofactor for the enzyme diaphorase which converts resazurin to the fluorescent product resorufin (Figure 1). This enzyme-coupled reaction scheme allows fluorescent measurement of the amount of resorufin that is generated, which is dependent on the amount of LDH that is released, which is proportional to the number of dying cells (Figure 1A).[2] The workflow for the CyQuant LDH Cytotoxicity Assay is outlined in Figure 1B. Briefly, the cell line of interest is cultured in 96-well microplates, and cells are treated with the test compound. Control wells are then treated with lysis solution (positive control) or media (negative control). Following treatment, a small aliquot of the resulting cell media is moved to an assay plate and mixed with the LDH substrate mixture to allow

resorufin formation, the reaction is quenched, and fluorescence is measured using a plate reader. This assay requires precise pipetting and timing steps to obtain high quality results, making this workflow a candidate for laboratory automation with the use of liquid handling robots, such as the Biomek i7 Hybrid Automated Workstation (Figure 2).

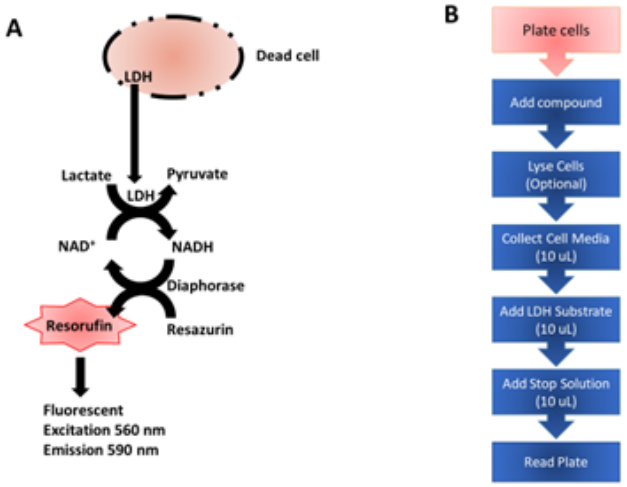


**Figure 1. CyQuant LDH Assay** Reaction scheme (A) and workflow (B) of fluorescent enzyme-coupled reaction scheme for CyQuant LDH assay. Steps that were performed offline of the Biomek i7 Hybrid Automated Workstation are highlighted in Red and automated steps in the workflow are indicated in blue.

The Biomek i7 Hybrid Automated Workstation is an automated liquid handler that is capable of efficiently performing the complex liquid handling steps of cell-based screening workflows. This minimizes the number of required user interactions and increases walk away time, freeing the operator to attend to other laboratory tasks. The multichannel pod can be equipped with a 96-well head that can accurately pipette 1 to 1200 µL or a 384-well head that is accurate over the range of 0.5 to 60 µL. Additionally, the 8-channel Span 8 pod is accurate from 1 to 1000 µL. The Biomek i7 Hybrid Automated Workstation supports 45 deck positions and can be directly fitted with orbital shakers, heating/cooling Peltiers, and tip-washers for plate and sample processing. Further, the Biomek i7 Hybrid Automated Workstation supports integration with other instruments, such as thermocyclers, automated incubators, barcode readers, plate washers, plate readers, centrifuges, and more, depending on user and workflow needs. Here we show that automated processing of the CyQuant LDH Cytotoxicity Assay using a Biomek i7 Hybrid Automated Workstation provides results equivalent to manually performed assays. The automated workflow reduces the hands-on time and reduces the chances of user handling errors.


**Figure 2**. Biomek i7 Hybrid Automated Workstation

## Methods

### Cell Culture

Lenti-X 293T cells (Takara) were maintained in DMEM supplemented with 10% FBS and 1% antibiotic/antimycotic (Gibco) at 5% $CO_2$ and 37°C. One day prior to running LDH assay, cells were washed with DPBS, harvested with trypsin, pelleted at 300 xg, and resuspended in OptiMEM (Gibco). Cells were counted and diluted in OptiMEM to achieve desired cell density. For digitonin and colchicine concentration-response experiments, 10,000 cells per well were used. Cells were plated in 96-well tissue-culture treated plate (CytoOne) in 100 µL total volume in OptiMEM and incubated at 5% $CO_2$ and 37°C for 24 hours.

### Compound Treatment

For colchicine time course experiments, 10 µL of 10X concentrated compound was added at indicated time point using Biomek i7 Hybrid Automated Workstation, and plate was returned to the incubator. For digitonin concentration response experiments, compound was added at the same time as Lysis solution. Prior to performing the assay, 10 µL of 10X concentrated digitonin, lysis solution (positive control), or media (negative control) were added to separate wells as appropriate. Plates were incubated for 40 min at 37 °C either in incubator (manual) or on Biomek i7 Hybrid Automated Workstation deck equipped with heating Peltier (automated).

### LDH Assay

Prior to running assay, kit components were assembled according to CyQuant LDH Assay manual.[2] 12 mL of Reporter Mix were combined with 1 mL Reagent Mix to make Reagent Stock, and extra was flash frozen in liquid nitrogen and stored at -20 °C for future use. LDH assays were then performed according to manufacturer instructions.[2] The deck layout for the automated Biomek method is shown in Figure 3. Following 40 min plate incubation with controls (lysis solution, media alone, or digitonin), 10 µL of resulting cell media was transferred to a black, clear bottom 384-well plate (Corning), and spun at 300 xg for 1 min either in Beckman Coulter Allegra X-12R (Manual) or Biomek i7 Hybrid Automated Workstation deck using integrated Agilent VSpin centrifuge (Automated). 10 uL of Reagent Stock was then added to each well, plate was spun at 300 xg for 1 min, and then incubated in the dark for 10 minutes. The LDH reaction was then quenched by addition of 10 uL of Stop Solution, plate was spun at 300 xg for 1 min, and incubated in the dark for 15 minutes. Finally, fluorescence (Ex 560 nm/Em 590 nm) was measured using a SpectraMax i3x plate reader (Molecular Devices). Data was analyzed using Prism 8 software (GraphPad). All data represents N ≥ 2 experiments, each performed with duplicate wells.

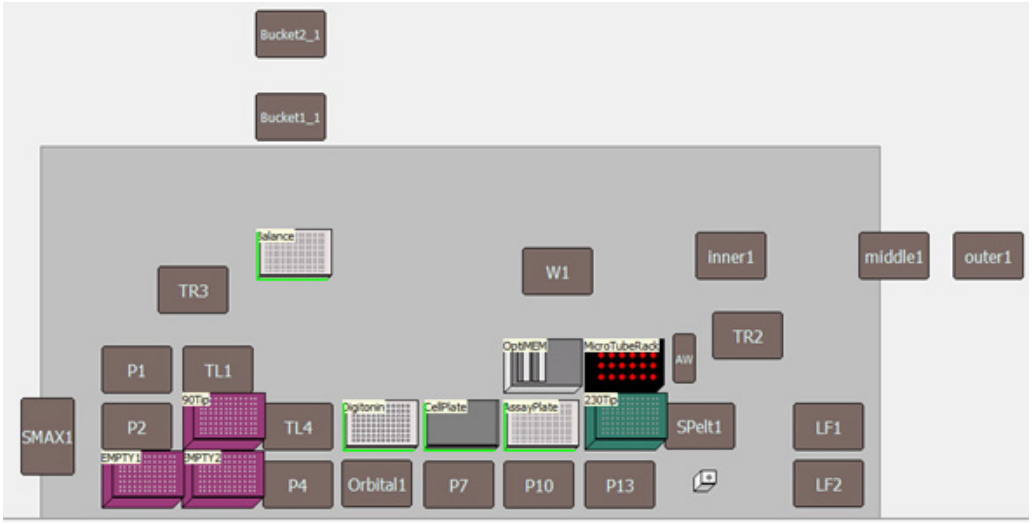


**Figure 3**. Deck layout for Biomek i7 Hybrid Automated Workstation CyQuant LDH method. The automated method used the following components: 3 tip-loading ALPs, 6 1X1 ALPs, 1 trash bin, a heating Peltier, a microplate centrifuge (Agilent), and microplate reader (Molecular Devices SpectraMax i3x).

## Results and Discussion

CyQuant LDH Cytotoxicity Fluorescence kit is a relatively fast and simple way to assess cellular cytotoxicity through the release of LDH from dead and dying cells. To develop an automated method for this kit, assay plates prepared manually were compared to plates prepared using a Biomek i7 Hybrid Automated Workstation. The Biomek was equipped with an integrated heating Peltier, Agilent VSpin microplate centrifuge, and SpectraMax i3x plate reader, which allowed walk away plate processing following initial deck set up. Additionally, we sought to miniaturize the assay from a volume from 150 µL in 96 well format to a total volume of 30 µL in 384-well format. The first set of experiments was designed to determine the linear range of the CyQuant LDH assay kit by measuring LDH release at multiple cell densities in the presence and absence of the lytic control reagent. As can be seen in Figure 4, similar results were obtained for manual and automated methods. In each case, excellent linearity was observed over HEK293 cell densities ranging from 1,000 to 20,000 cells per well. For cells that were subjected to the lytic positive control, LDH-dependent fluorescence increased linearly with the number of cells with an $R^2$ value of 0.99 using both manual and automated workflows. Additionally, there was a cell-density dependent increase in the spontaneous LDH activity in wells that were treated with media vehicle (negative control). Excellent linearity was observed for manual and automated assay plates, as the observed $R^2$ values were 0.98 and 0.95, respectively. This proved that the Biomek-automated CyQuant plate processing method was equivalent to manual, while requiring significantly less user input following deck setup.

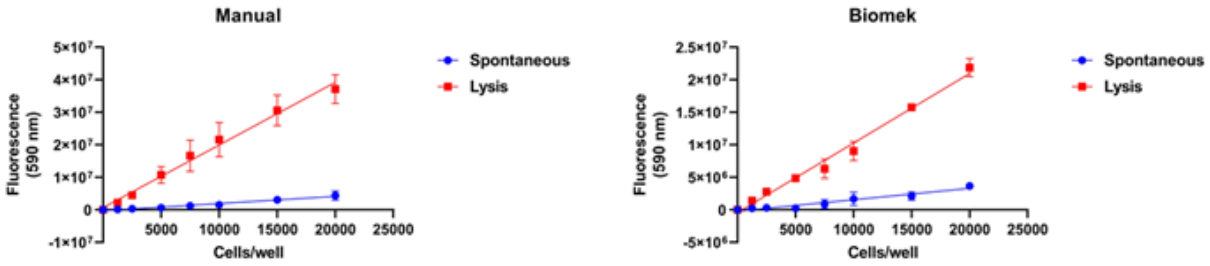


**Figure 4**. LDH activity from varying cell densities of living (Spontaneous) and dead (Lysis) HEK293 cells from assay plates prepared manually and with Biomek i7 Hybrid Automated Workstation.

We next sought to show that the automated method could accurately measure concentration-dependent cytotoxicity. Digitonin was selected as an acutely cytotoxic control compound, as it is often used as an agent for permeabilizing and solubilizing cell membranes.[3] Digitonin was serially diluted either by hand or using the Serial Dilution function on the Biomek. Cells were treated with compound for 40 minutes at 37 °C, and LDH release was then quantified as before. In both manual and automated assay plates, a robust, concentration-dependent increase in LDH activity was observed, indicative of cell death (Figure 5). Moreover, similar potency was observed between experimental conditions with $EC_{50}$ values of 7 and 9 uM for manual and automated plates, respectively. This further showed the utility of the Biomek-automated CyQuant plate processing method, as accurate determination of cytotoxic compound potency could be measured.

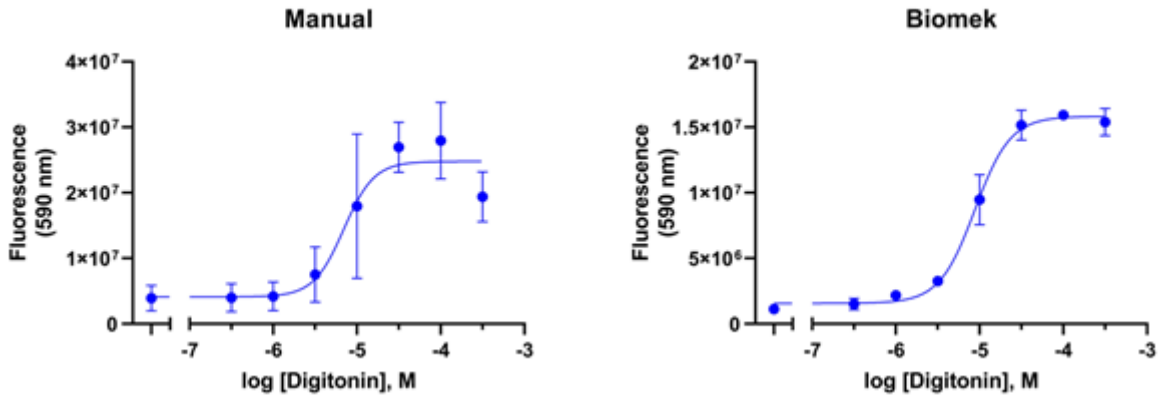


**Figure 5**. Concentration-dependent cytotoxicity of digitonin in manually and Biomek i7 Hybrid Automated Workstation prepared CyQuant LDH assay plates.

In addition to measuring the potency of acutely cytotoxic chemicals like digitonin, the CyQuant assay can also be used to determine if compounds are cytostatic, causing decreased cellular division and proliferation. One such compound is colchicine, which destabilizes microtubule formation, causing cell cycle arrest, which can ultimately lead to apoptosis depending on the cell line being interrogated.[4] Using the Biomek i7 Hybrid Automated Workstation, the effect of 10 µM colchicine on HEK293 cells was assessed following acute (1 hr) or prolonged (24 and 48 hr) treatment. Cytotoxicity was measured by examining LDH release in culture medium. The cells in each well were also lysed according to kit instructions, and LDH activity was measured to determine the number of cells in each well. These results are presented in Figure 6. As compared to vehicle control, colchicine did not cause an appreciable acute (1 hr) cytotoxic response in the spontaneous culture medium sample. Moreover, there was not an appreciable increase in cytotoxicity observed at 24 or 48 hr, either. In fact there was a slight increase in spontaneous LDH activity observed in untreated and acutely treated cells. This was likely due to the increased cell number in these wells, as the cell titration experiment presented in Figure 4 shows spontaneous activity increases with increasing cell density. Together, this indicated that colchicine was not appreciably cytotoxic to HEK293 cells. Conversely, colchicine did cause a time-dependent change in the amount of LDH signal observed following cell lysis. Cells treated with 10 µM colchicine for 48 hours only exhibited approximately 50% of the LDH signal as compared to vehicle treated-control cells, indicative of fewer living cells in the well. Similar results were observed for cells treated with colchicine for 24 hours, as only approximately 75% of the vehicle-treated signal is observed. Taken together, this indicated that at longer exposure times, less LDH was released following lysis because fewer living cells were present. This supports the conclusion that against HEK293 cells 10 µM colchicine is cytostatic rather than cytotoxic.[4]

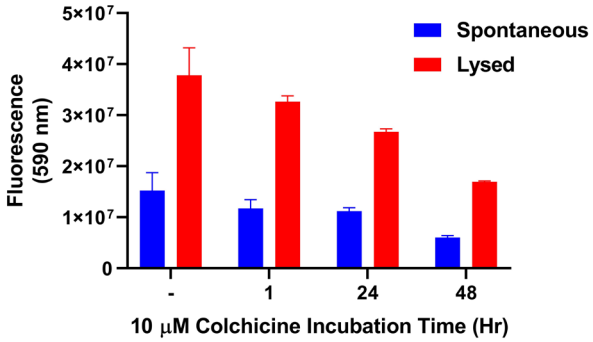


**Figure 6**. Time-dependent changes in LDH activity in Lenti-X 293T cells treated with vehicle (-) or 10 uM colchicine. Cytotoxicity was measured by assessing cell media directly (Spontaneous, Blue). Cells were also lysed to assess cell number in each well (Red).

## Summary

Determination of cell viability is an important part of many cell-based assay workflows, as both a final endpoint and a simple method for cell culture quality control. Many commercial methods are available to measure cytotoxicity, and one which has gained popularity is the CyQuant LDH Cytotoxicity Fluorescence assay. This is a relatively fast and straightforward method that is highly amenable to automation using liquid handlers, like the Biomek i7 Hybrid Automated Workstation. Here we show that the automated Biomek method performs favorably compared with manually prepared assay plates. In both cases, the kit was amenable to miniaturization to 30 uL assay volume, a five-fold reduction, allowing for cost savings on reagents and increasing throughput. Additionally, control experiments showed that the Biomek automated protocol displayed excellent linearity over the range of 1,000 to 20,000 cells per well, comparable to manual results. We also highlighted the utility of the automated method in determining the potency of a cytotoxic compound. The concentration response curve and $EC_{50}$ for the cytotoxic, small molecule digitonin were nearly identical between plates prepared by hand and using the Biomek i7 Hybrid Automated Workstation. Finally, we showed that the method could also be adapted to determine if compounds are cytostatic rather than cytotoxic. Together the data presented here highlights a miniaturized and automated LDH cytotoxicity workflow that reduces the hands-on time required by the user, freeing them to attend to other laboratory tasks. The automated method also reduces the chances of user handling errors, as no user input is required following initial deck setup.

## References

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## Materials

| Equipment | Manufacturer |
|---|---|
| **Allegra X-12R Centrifuge** | Beckman Coulter |
| **SpectraMax i3x** | Molecular Devices |
| **Biomek i7 Hybrid Automated Workstation** | Beckman Coulter |

**Table 1**. Instruments used

| Reagents | Manufacturer | Part Number |
|---|---|---|
| **Lenti-X 293T cell line** | Takara | 632180 |
| **DMEM, high glucose pyruvate** | Gibco | 11-996-065 |
| **Antibiotic/Antimycotic 100X** | Gibco | 15-240-062 |
| **Fetal Bovine Serum** | Gibco | 16-000-044 |
| **DPBS** | Gibco | 14-190-144 |
| **Trypsin** | Gibco | 25-200-072 |
| **OptiMEM I** | Gibco | 31-985-070 |
| **Digitonin** | TCI Chemicals | D0540 |
| **Colchicine** | Alfa Aesar | J61072 |
| **CyQuant LDH Cytotoxicity Assay-Fluorescence** | Invitrogen | C20302 |

**Table 2.** Reagents used.

| Consumable | # | Manufacturer | Part Number |
|---|---|---|---|
| **96-Well Tissue-Culture Treated Plate** | 1 | CytoOne | CC7682-7596 |
| **Biomek 96-well microplate,** | 1 | Beckman Coulter | 609844 |
| **384-well plate, black, clear bottom** | 1 | Corning | 3762 |
| **Biomek i-Series, 90 uL pipette, sterile** | 2 | Beckman Coulter | B85884 |
| **Biomek i-Series, 230 uL pipette, sterile** | 1 | Beckman Coulter | B85906 |

**Table 3.** Consumables used per 96 sample run



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