

BioRobotics

HIGH-THROUGHPUT SCREENING IN 1536-WELL PLATES USING THE BIOMEK® FX LIQUID HANDLING SYSTEM INTEGRATED ONTO THE SAGIAN™ CORE SYSTEM

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Introduction

High-throughput screening is a crucial step in the drug discovery process for the pharmaceutical industry. Automation, miniaturization, and good assay development are key factors in achieving high-throughput screening. There is an increasing demand to perform high-throughput screening assays in miniaturized formats in order to screen a greater number of compounds in a cost-effective manner. The key to successful high-throughput screening in miniaturized formats is to have automation with consistent, low-volume pipetting.

This bulletin describes the automation of the screening process for effectors of apoptosis in a miniaturized format using the SAGIAN™ Core System from Beckman Coulter. We have screened a compound library for effectors of caspase 3 activity which is a key protease late in the apoptotic pathway. The screen was carried out in 1536-well plates in order to achieve high-throughput screening in low-volume format.

Materials and Methods

Automation of the high-throughput screening process for apoptotic effectors in a 1536-well format used the SAGIAN Core System from Beckman Coulter. The Core System includes an Acquest® reader from Molecular Devices Corp. and the Biomek® FX Liquid Handling System integrated with a 3-Meter ORCA® System. Data management and data analysis were accomplished using Firepower® Software (Exegetix, Inc.) in conjunction with Spotfire® Pro Software (Spotfire, Inc.). A compound library was screened for effectors of caspase 3 with a commercially available reagent kit.

I. Caspase 3 Assay

The assay utilized the EnzChek® Caspase 3 Assay Kit from Molecular Probes, Inc., which provides buffer and the substrate Asp-Glu-Val-Asp-7-Amino-4-methylcoumarin (Z-DEVD-AMC). Human recombinant caspase 3 was obtained from CN Biosciences, Inc. To achieve a functional primary screening assay in 1536-well plates, the assay volume was miniaturized to 10 µL/well.

The assay was optimized with various concentrations of caspase 3 in combination with various concentrations of the substrate Z-DEVD-AMC. As the final goal of the primary screening is to identify effectors of the caspase 3 enzyme, a known inhibitor, Ac-DEVD-CHO, was used to optimize the assay.

The assay was performed in Hi-Base, black, 1536-well plates from Greiner Bio-One, Inc. After the addition of all the reagents into the 1536-well plates, the assay plates were incubated at room temperature for 45 minutes. The assay plates were then read using the Acquest reader.

II. Reader

The Acquest reader was used to read fluorescent intensity. The Acquest has up to eight logs of linear dynamic range with simplified data handling and data reduction. The Acquest was equipped with a 360 nm excitation filter and a 485 nm emission filter to detect fluorescence intensity. In the plate



read protocol, the photomultiplier tube set up was set to digital and also to collect data points in fluorescence intensity mode.

III. Compound Library

A compound library was used for the high-throughput screening process. The compound library was supplied by Lexicon Pharmaceuticals (formerly Coelacanth, Inc.). A total of 44,880 compounds was supplied in a total of 561 bar-coded, half-height 96-well plates with 80 compounds per plate, columns 1 and 12 vacant. The compounds were synthesized from proprietary, medically relevant building blocks with large compound diversity and purity of >85%. The pharmaceutical property profiling (P3) includes aqueous solubility, metabolism, and intestinal transport. Of the total compounds in the library, 24,320 were used in the primary screening assay.

IV. Robotics

The integrated SAGIAN™ Core system for the caspase 3 screening assay in 1536-well plates consists of a 3-Meter ORCA® System, a Biomek® FX Liquid Handling System, two ambient-temperature Microplate Carousels, CO₂ Incubator with Microplate Carousel, Bar Code Reader, Microplate Print and Apply for bar coding the plates, and the Acquest plate reader. SAMI™ software was used to control the Core System. For compound library reformatting, the Biomek FX was equipped with a 96-Channel Disposable Tip Pipetting Head, Tip Wash 96-Channel. For screening, the 96-Channel Pipetting Head was replaced with a 384-Channel Disposable Tip Pipetting Head and the Tip Wash 96-Channel was replaced with the Tip Wash 384-Channel. A Positive Positioning ALP (Automated Labware Positioner) for 1536-well plates was also used for screening. The schematic of the SAGIAN Core System layout is shown in Figure 1.

V. Liquid Handling and Integrated Robotic Methods

Three categories of methods were developed:

1) Methods to validate pipetting performance of the Biomek FX Liquid Handling System; 2) Methods to test the assay performance; and 3) Methods to perform the screening.

1. Methods to Validate the Pipetting Performance of the Biomek FX Liquid Handling System

To validate the liquid handler's pipetting performance in 1536-well plates, experiments were conducted to determine the carryover from washing tips using the 384-channel tip wash station and the precision of the 384-channel disposable tip head pipetting into the 1536-well plate.

High-throughput screening assays consume a large number of tips in transferring and mixing the reagents. One way to conserve tips is to wash them between the various liquid transfers. This method carries with it the risk of cross contamination. The following tests were performed to test for reagent carryover by the tips during the screening assay. Two microliters of 5 μ M AMC, a fluorescence reference standard (from EnzChek Caspase-3 Assay Kit), were transferred from a 384-well plate into a 1536-well plate with 8 μ L of water. The tips were then washed and used for transferring water into a different quadrant of the same 1536-well plate. The tip washing was tested from one to six washes between the transfers. The Acquest reader was used for detecting the fluorescence intensity of the samples.

Reproducible pipetting is a key to miniaturizing an enzyme assay. In order to check the pipetting reproducibility of the 384-channel disposable tip head, a method was developed to pipette 3 μ L of the 5- μ M AMC reference standard and the remaining 7 μ L buffer added in aliquots corresponding to the other assay components from a 384-well plate into a 1536-well plate. In this method, the actual assay format was mimicked using the reference standard and buffer with tip washing (five cycles) in between the transfers. The fluorescence intensity of the 1536-well plates was read with the Acquest reader. The average, standard deviation and percent correlation coefficient (%CV values) were calculated and plotted for each of the columns and rows of the plate.

2. Methods to Validate the Miniaturized Assay Performance

After verifying the pipetting precision and the reader performance, the miniaturized assay performance was checked in 1536-well plates. The caspase 3 assay was performed in a total volume of 10 μ L in all the wells of the 1536-well plate following the optimized assay protocol. This assay is similar to the assay that was used for screening except that, instead of the library compounds, DMSO was added in order to observe the maximum activity in all the wells. The reagents were dispensed from 384-well source plates into the 1536-well assay plate in the order of 3 μ L buffer, 3 μ L caspase 3, 1 μ L DMSO,

and 3 μL substrate. The assay plate was incubated for 45 minutes and read. The average, standard deviation, and %CV values were calculated and plotted for each of the columns and rows of the plate.

3. Methods to Perform the Screening

Methods were developed to reformat the chemical library compounds and carry out the assay in 1536-well plates using the SAGIAN™ Core System with integrated Biomek® FX Liquid Handling System. Two automated methods were developed.

The first method was used for reformatting the compounds from 96-well plates into 384-well plates. The second method was used for performing the caspase 3 assay in 1536-well plates using the reformatting library compounds from the 384-well plates.

A. Compound Library Reformatting

The library compounds were supplied in 96-well plates. The screening assay for caspase 3 effectors was performed in 1536-well plates using the 384-channel disposable tip head for pipetting. The library compounds were first transferred from the supplied 96-well plates into 384-well plates prior to being used in the screen. The reformatting was done on the Biomek FX Liquid Handling System with the 96-Channel Disposable Tip Pipetting Head. During the reformatting, the compounds were also diluted 20 times to achieve 200 μM concentration. Each reformatting 384-well compound plate was assigned a unique bar code via the “Bar Code Assign” function using the Print and Apply module to enable sample tracking. Additionally, the four 96-well compound library plates were linked to one 384-well reformatting compound library plate via the

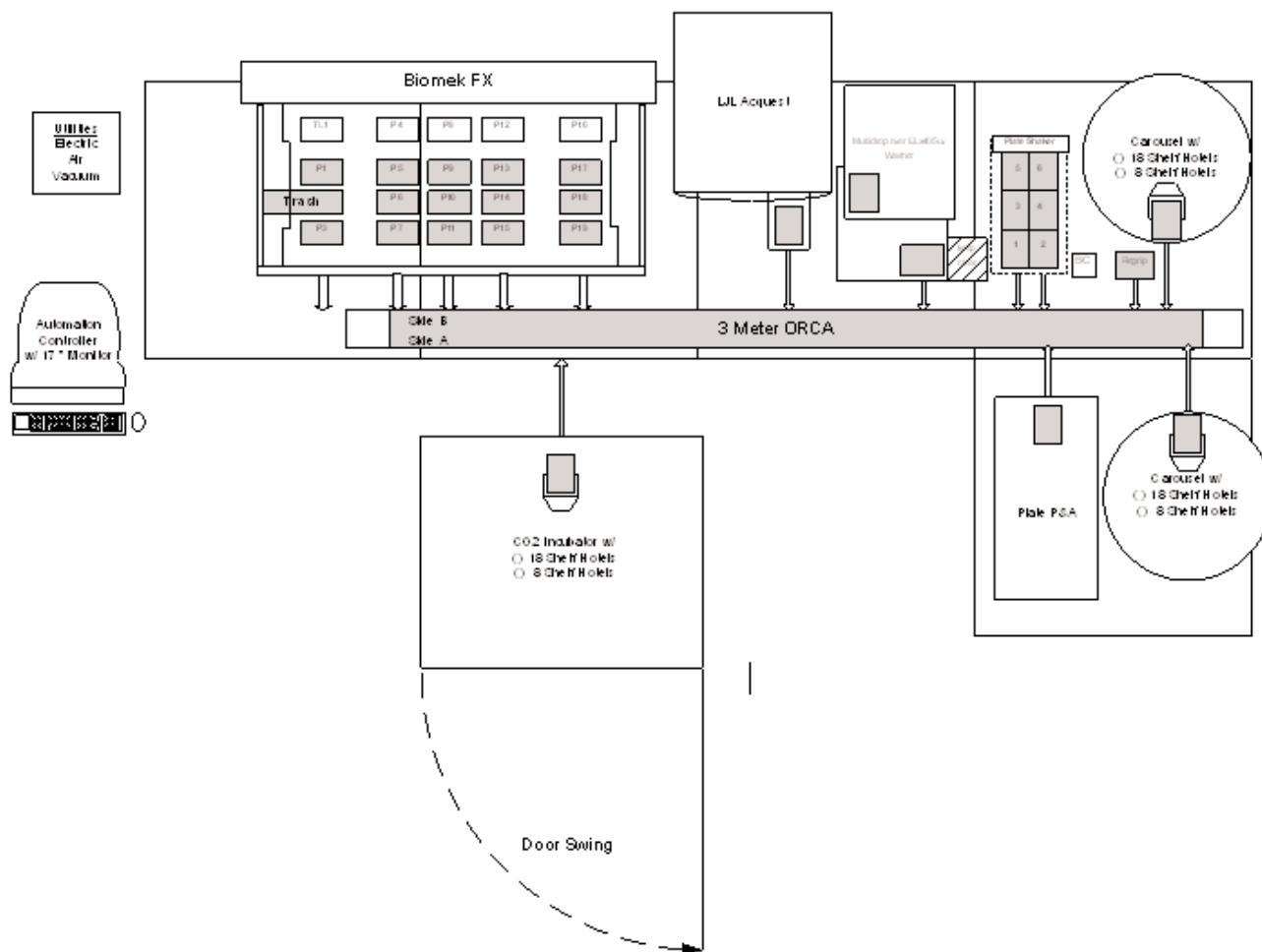


Figure 1. The overview of the Core System layout. The integrated Core System for caspase 3 screening assay in 1536-well plates consists of a 3-Meter ORCA® System, Biomek FX Liquid Handling System, two ambient-temperature Microplate Carousels, CO₂ Incubator with Microplate Carousel, Bar Code Reader, Microplate Print and Apply for bar coding the plates, and the Acquest plate reader from Molecular Devices, Corp. The Core System is under the control of SAMI™ software. The Biomek FX was equipped with 96-Channel Disposable Tip Pipetting Head, Tip Wash 96-Channel, 384-Channel Disposable Tip Pipetting Head, Tip Wash 384-Channel, and Positive Positioning ALP for 1536-well plates.

use of the Relationship Logger module. Later during the screening assay, the compounds from four 384-well reformatted compound library plates were transferred into one 1536-well assay plate and the bar codes were linked via the Relationship Logger module. All the compound library plates as well as the assay plates were bar coded to enable sample tracking from the 1536-well assay plate back to the original 96-well compound library plate. The reformatting scheme is shown in Figure 2.

B. 1536-Well Plate Assay Method

The reagents, enzyme, substrate, and buffer for the assay were prepared in 384-well plates using the Biomek® 2000 Laboratory Automation Workstation. These plates were then transferred to the Biomek FX for use in the assay. An automated method was developed, as shown in Figures 3 and 4, to transfer the compounds and reagents from 384-well source plates into 1536-well assay plates in order to perform the screening assay for caspase 3 effectors. On the deck of the Biomek FX Liquid Handling System, compounds from eight 384-well reformatted compound library plates were transferred into two 1536-well plates during the assay process and scheduled as one family. For each family, the associated labware consisted of two 1536-well assay plates, eight 384-well reformatted compound library plates, and four boxes of AP384 30 μ L tips that

were placed by the ORCA® arm on the deck of the Biomek FX Liquid Handling System. Finally, four of the 384-well reformatted compound library plates were linked to one 1536-well assay plate via the use of the Relationship Logger module to enable sample tracking (Figure 3). Using the SAMI™ software, many families can be scheduled for unattended operation in order to generate a large number of 1536-well assay plates.

Given the small well size, pipetting into a 1536-well plate requires accurate positioning of the plate. To ensure accurate positioning for successful pipetting into 1536-well plates, the Biomek FX Liquid Handling System was equipped with a special device called a Positive Positioning ALP that ensures that the 1536-well plates are positioned in the same location to allow accurate and reproducible pipetting into the small wells.

Assembly of the assay occurred in the following order: 3 μ L of buffer, 3 μ L of 0.018 ng/ μ L caspase 3, 1 μ L of the library compound, and 3 μ L of the 50 nm substrate, giving a total volume of 10 μ L/well in the 1536-well plate. The final concentration of the compounds tested was 20 μ M. Tips were washed five times between transfers and tip changes between sources were programmed. A schematic of the method is shown in Figure 4. Each assay plate was assigned a bar code via the “Bar Code Assign” function to enable sample

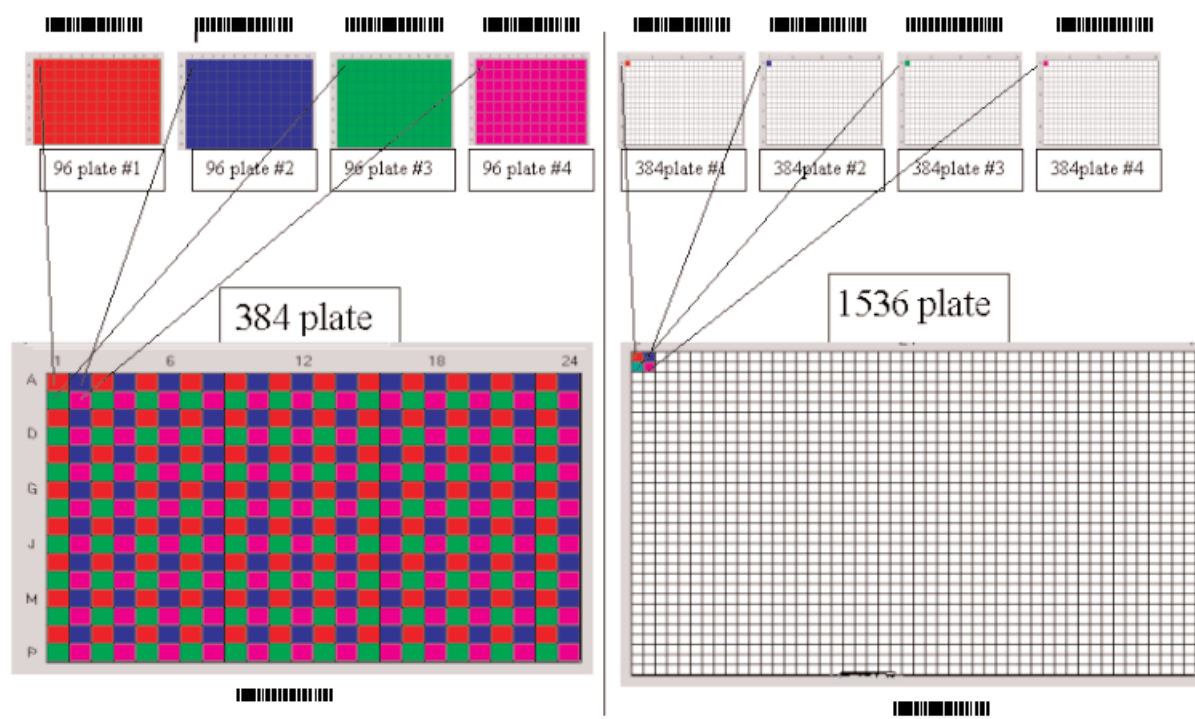


Figure 2. Compound library reformatting scheme. Compounds from four 96-well source plates of the compound library were reformatted into one 384-well plate. For the assay, compounds from four 384-well reformatted compound library plates were transferred into one 1536-well plate. The schematic display of the well assignments follows the tetrad format.

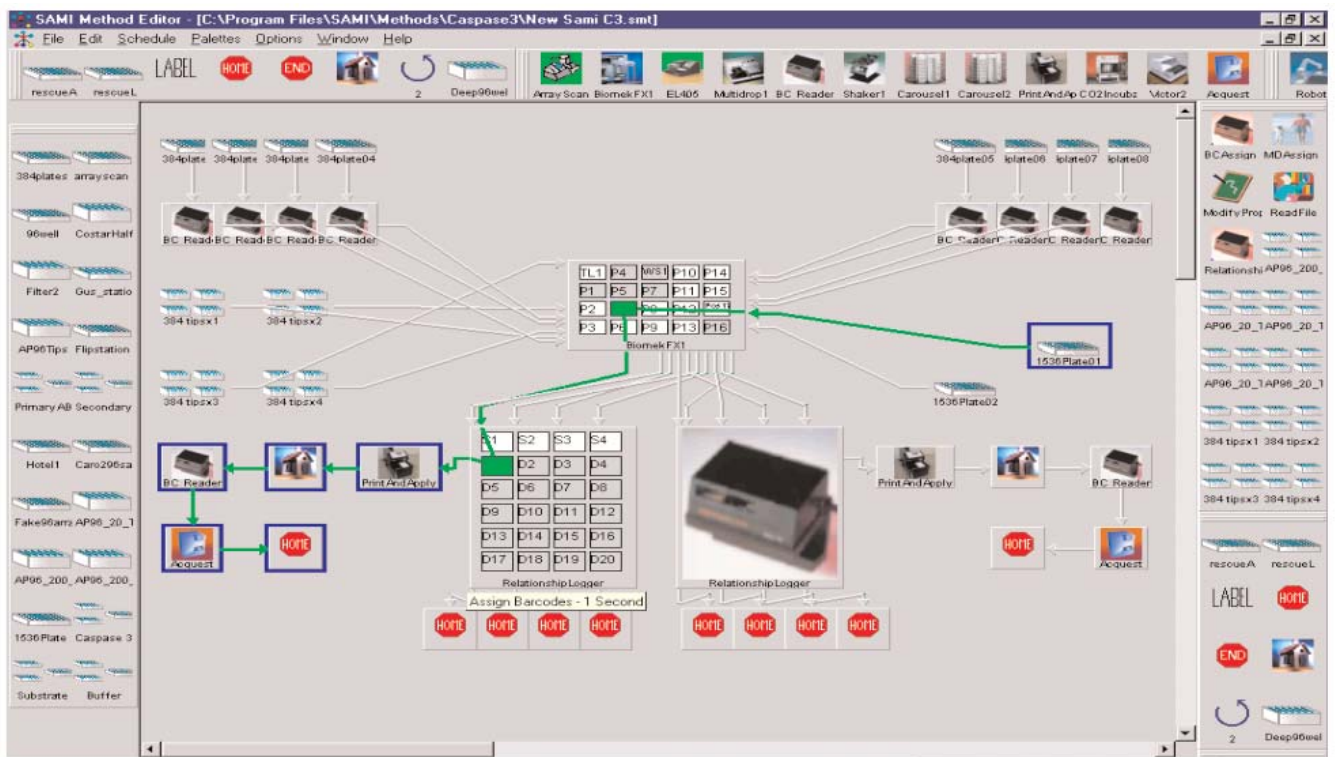


Figure 3. SAMI™ Method Editor showing screening assay method. This figure shows integration of the Biomek® FX Liquid Handling System with resource pools for tips, source plates, 1536-well assay plates, Bar Code Reader, Microplate Print and Apply module for bar coding the plates, Relationship Logger for sample tracking and Acquest reader.

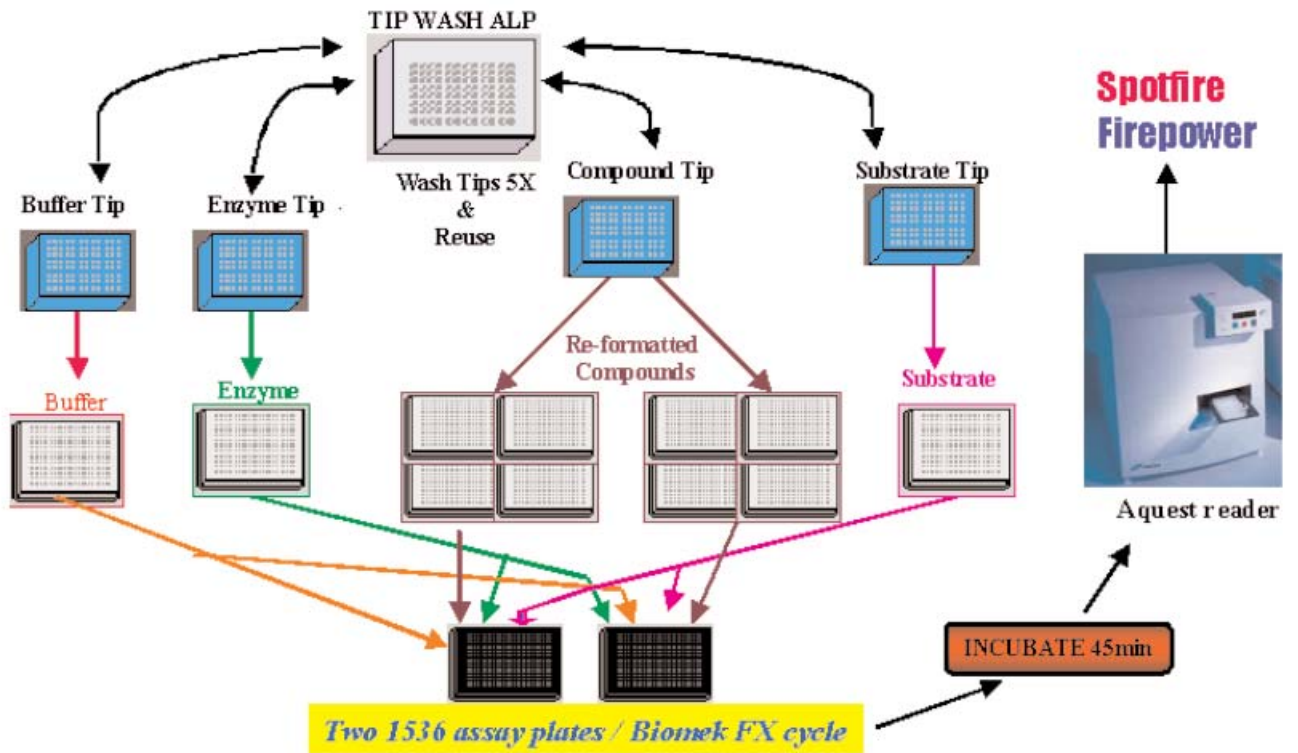


Figure 4. The flow of Biomek FX and SAMI methods for caspase 3 effector screening assay in 1536-well plates. Assembly of the assay occurred as shown above from left to right. Tips were washed 5 times between transfers as well as tip changes between sources were programmed. Two 1536-well assay plates were generated per Biomek FX cycle, incubated for 45 minutes, and read by the Acquest reader. The data stream from the 1536-well plates was captured by the SAMI logger and transferred to Firepower and Spotfire Pro Software.

tracking. Assay plates were incubated for 45 minutes and read by the Aquest reader to measure the fluorescence intensity. Compound library plates were retired immediately after use. The data stream from the 1536-well assay plates was captured by the SAMI™ logger.

Various types of controls were included with each 1536-well plate to monitor the assay and calculate relative fluorescence. The low controls contained no enzyme and show the background produced by the substrate, buffer, and DMSO. The average of the low controls per plate was used as background fluorescence for calculation of relative fluorescence. The high controls were formulated with the caspase 3, substrate buffer, and DMSO (instead of compounds) to show the maximum activity. The average of the high controls per plate was used as the 100% relative fluorescence. To confirm that the observed fluorescence signal is in fact due to caspase 3 activity, the inhibitor, AMC-DEVD-CHO (100 nM to 100 μM), was used. The fluorescent reference standard control, AMC, was diluted from a 1 mM stock and used at a final concentration of 100 nM to monitor the reader performance. There also were controls that contained enzyme only or buffer only.

The relative fluorescence units (RFU) for a well on a given plate were calculated from the average background fluorescence (average of the low controls) and the average of the high controls on that same plate. The fluorescence intensity of a well was first corrected by subtraction of the background fluorescence; then this value was divided by the background corrected fluorescence of the average of the high controls. The resulting quotient was multiplied by 100% to obtain the relative fluorescence for the well. The average of the high controls after correction for background fluorescence is equal to 100% relative fluorescence for that plate. The following formula was used to calculate % relative fluorescence values of the samples which was expressed as % relative activity.

$$\frac{(\text{Fluorescence of Sample}) - (\text{Avg. Fluorescence of Low Controls})}{(\text{Avg. Fluorescence of High Controls}) - (\text{Avg. Fluorescence of Low Controls})} \times 100$$

VI. Software

FirePower Software was used for data computation. FirePower Software allows the user to customize monitoring of assay controls, define any type of assay template desired, and compute the data in any dimension desired. Computed data was fed into Spotfire Pro Software for instant visualization. Heritage information scripts were developed by

Exegetix, Inc., to de-convolute the data from 1536-well plate format to trace the compounds back to the original 96-well compound library plates.

Results and Discussion

I. Optimization of Caspase 3 Assay

The caspase 3 assay was performed with increasing amounts of caspase 3 enzyme in combination with 75 to 300 μM substrate and the fluorescence intensity measured using the Aquest reader. The combination of 0.054 ng of enzyme and 150 μM of the substrate gave at least 10-fold higher RFUs than the background. This combination also showed a gradual decrease in the caspase 3 activity when used with 100 nM to 100 μM of the caspase 3-specific inhibitor, AMC-DEVD-CHO, which confirms that the observed fluorescence signal was in fact due to caspase 3 activity.

II. Liquid Handling Methods

1. Reformatting

Figure 2 shows the reformatting of the compounds from 96-well plates via 384-well plates into 1536-well plates that are used in the assay. The schematic display of the well assignments follow a “tetrad” format, which is the format for transferring the samples from four 96-well plates into one 384-well plate in alternate wells (Figure 2). All 560 of the 96-well compound library plates were reformatted into a total of 140 384-well plates. Of these 384-well reformatted compound library plates, compounds from 76 plates were transferred into a total of 19 of the 1536-well assay plates during the screening assay for effectors of caspase 3.

2. Performance of the Biomek® FX Liquid Handling System and the Assay in 1536-Well Plates

The 1536-well plates were placed on the positive positioning ALPs to ensure proper positioning of the plate for accurate pipetting. Once the 384-channel pipetting head was calibrated with a 1536-well plate placed on the positive positioning ALP, this ALP clamped the 1536-well plate and kept it in the calibrated position for accurate pipetting.

A 384-channel tip wash test was performed to detect any possible carryover after washing tips by transferring the AMC reference standard from a 384-well source plate into a 1536-well plate and then washing the tips (one to six times) with water between the transfers. The fluorescence intensity of the transferred water after each tip wash was meas-

ured. The results indicated (data not shown) that there is no detectable carryover of reference standard by tips after three tip washes.

The pipetting precision of the 384-tip head was tested by transferring aliquots of the AMC reference standard (5 μM) into all the wells of a 1536-well plate and reading the fluorescence intensity. Six plates were tested. The average fluorescence intensity was calculated and shown for each of the 48 columns and 32 rows from one 1536-well plate in Figure 5. The average fluorescence intensities were approximately 280,000 and only a few columns showed a small degree of variation from this average. Given the miniaturized format, the amount of variation shown here is considered excellent performance.

This data also shows the reader performance. It is evident from the data that there are no edge effects occurring due to the reader. Therefore, the reader is calibrated to read the entire plate.

After testing the pipetting precision and the reader performance, the assay performance was also tested in the 1536-well plate. The caspase 3 assay was performed in a total volume of 10 μL /well in all the wells of a 1536-well plate following the optimized assay protocol. The average fluorescence intensity was calculated and shown for each of the 48 columns and 32 rows from one 1536-well plate in Figure 6. The average fluorescence intensities were approximately 200,000 and only a few columns showed a small degree of variation. Again, these results are indicative of excellent performance for a miniaturized assay.

This data demonstrates that we have successfully pipetted the optimized assay reagents into 1536-well plates using the 384-channel disposable tip pipetting head and the positive-positioning ALPs on the deck of the Biomek[®] FX Liquid Handling System to perform the caspase 3 assay in 10 μL volume. The Z' values calculated using the data from this assay were 0.6 which is further evidence of a robust assay.

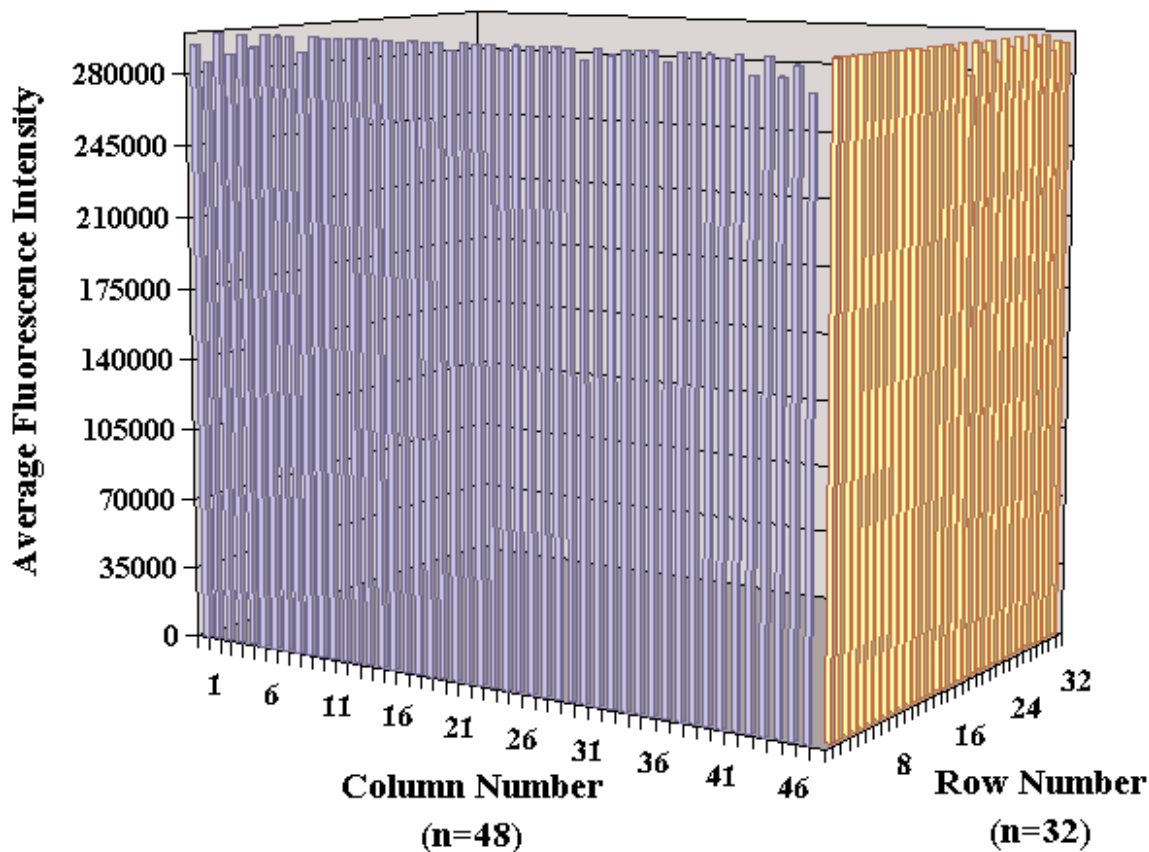


Figure 5. Pipetting reproducibility of the Biomek FX 384-channel disposable tip pipetting head into a 1536-well plate. A method was developed to pipette 3 μL 5 μM AMC reference standard and 7 μL buffer added in aliquots corresponding to the other assay components from 384-well source plates into three 1536-well plates. The average fluorescence intensity was calculated and shown for each of the 48 columns and 32 rows from one 1536-well plate. The average fluorescence intensity was approximately 280,000 and only few columns showed a small degree variation from this average.

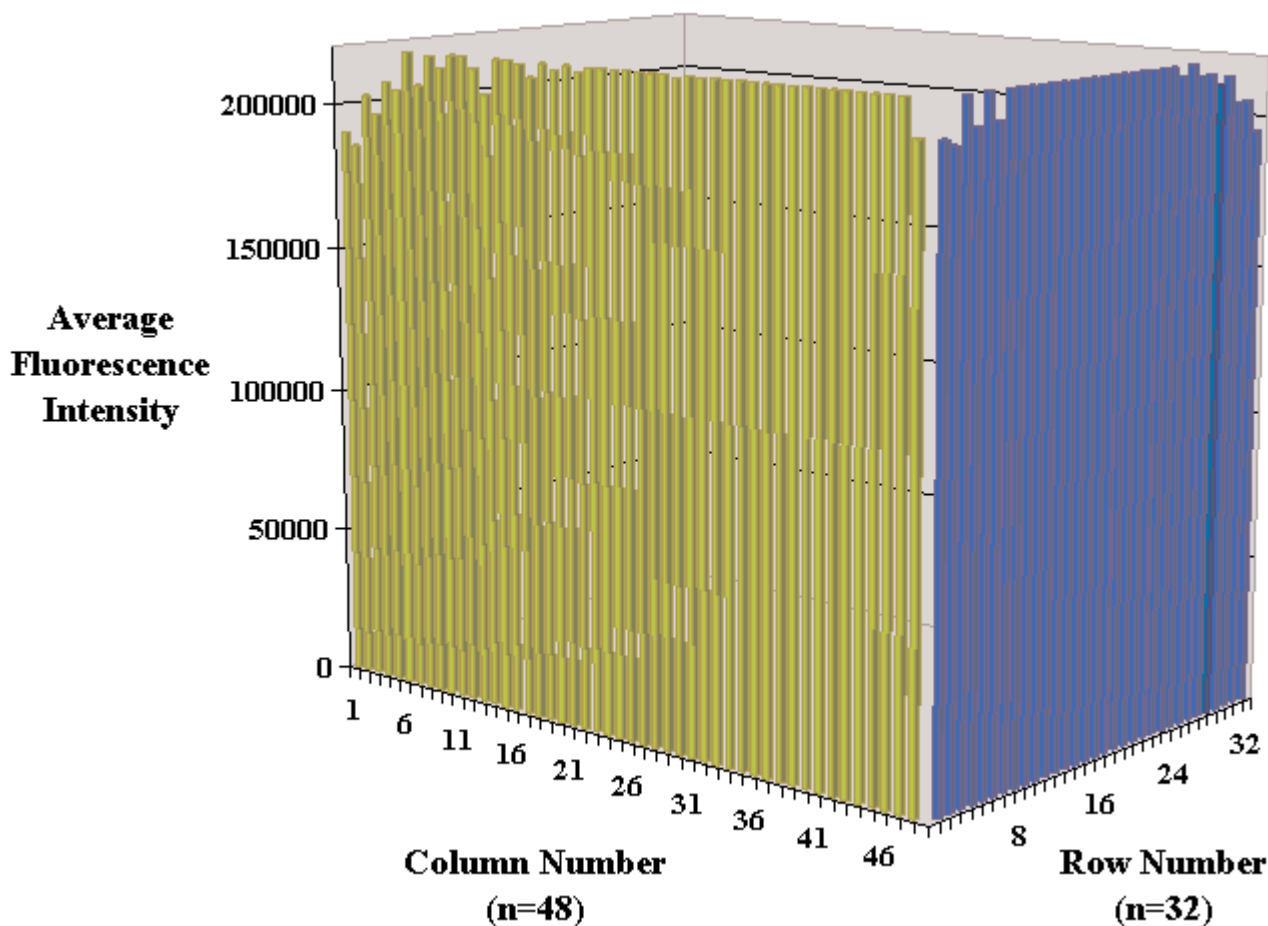


Figure 6. Reproducibility of the miniaturized caspase 3 assay in a 1536-well plate: The caspase 3 assay was performed in a total volume of 10 μL /well in all the wells of a 1536-well plate following the optimized assay protocol. The average fluorescence intensity was calculated and plotted for each of the 48 columns and 32 rows from one 1536-well plate. The average fluorescence intensity was approximately 200,000 and only few columns showed a small degree of variation from this average.

III. Efficiency of Robotic Handling of the Screening Process

A fully automated, miniaturized caspase 3 assay was adapted to the 1536-well plate format on Beckman Coulter's SAGIAN™ Core system. Several advanced features of the Core System allowed for enhanced process flow: resource pooling, bar coding, and sample tracking using the relationship logger.

Core system processing times were very efficient with the Biomek® FX Liquid Handling System as the central liquid handler. This efficiency was attributable to the 384-channel pipetting capability and large-capacity deck space of the Biomek FX Liquid Handling System. Actual processing time for the screening assay from 384-well to 1536-well plate was ~6 hours for 24,320 compounds plus controls. The process-flow was such that two 1536-well assay plates were generated per Biomek FX Liquid Handling System cycle (Figure 4). Furthermore,

there is no discernible error introduced by the washing and re-use of tips within the design parameters.

IV. Data Analysis

FirePower software and Spotfire Pro software were used for data computation and data viewing, respectively. SAMI™ collects data in a single line format which is not compatible with Spotfire Pro software for direct visualization. Therefore, FirePower software was used as a “plug-in” for the Spotfire Pro software to compute and visualize the results. Another very important reason for using FirePower software in the present experimental design is that it acts as a tool to associate heritage information of the hits from the original library compounds in the 96-well plate format. With the help of the FirePower software, we were able to compute the raw data along with the QC controls for the data analysis using a customized assay template.

A total of 24,320 compounds were screened with the miniaturized caspase 3 assay. The computed relative fluorescence data was fed into Spotfire Pro software for instant visualization as shown in Figure 7. Relative activity values below 30% were scored as inhibitor hits. There were ~0.2% (62 out of 24,320 compounds) of the compounds identified as inhibitor hits.

This screening assay also showed activators of caspase 3 among the compounds screened. Compounds with relative activity values greater than 150% were scored as activators. There were ~0.2% (62 out of ~24,320 data points) of the compounds identified as activator hits.

After the identification of the hits in the Spotfire Pro software, with the help the scripts generated by Exegetix, Inc., the heritage information of the hits was derived. This was done by de-convoluting the hits back to the original compound ID in the 96-well plates of the compound library. The hits were picked from the original 96-well compound library plates and placed in a 384-well plate. For the confirmation of the hits, the caspase 3 assay was again performed in the 1536-well plates using the same protocol that was used for the primary com-

pound library screening. Each potential inhibitor and activator hit and eight neutral compounds with no effect in the initial screening were tested in quadruplicate assays. Of the original hits, 12 of the inhibitor hits and 14 of the activator hits were confirmed. The results of the confirmed hits are shown in Figure 8. The neutral compounds showed little effect on caspase 3 with relative activities ranging from 70 to 112%. Of the confirmed hits, the inhibitors ranged from 21 to 50% relative activity, which corresponds to 50 to 79% inhibition of caspase 3. The confirmed activators had relative activities ranging from 202% to 1293%, which correspond to two- to over six-fold activation of caspase 3.

Conclusions

A fluorescence-based caspase 3 assay was optimized and miniaturized in 1536-well plates in a total volume of 10 μ L/well. Excellent pipetting was achieved into 1536-well plates by the Biomek[®] FX 384-channel disposable tip pipetting head and handling of the 1536-well plates by the positive positioning ALP on the deck of the Biomek FX Liquid Handling System.

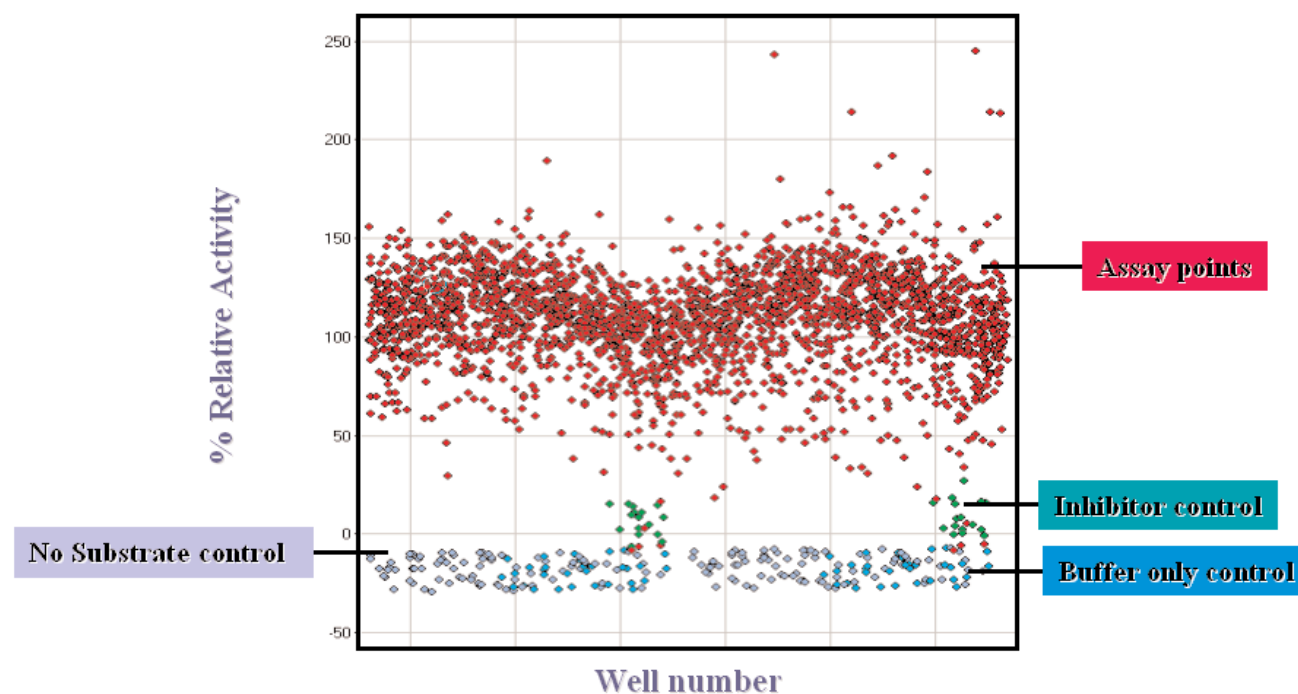


Figure 7. 1536-well plate caspase 3 assay data overview using Spotfire Pro software. A total of 24,320 compounds were screened using the caspase 3 assay. The fluorescence intensity of each data point relative to the average of the 100% relative activity of the high controls in each plate was calculated and expressed as % relative activity. The average of the no enzyme control wells per plate was zero % relative activity. The computed data was visualized by Spotfire. The above data is for two 1536-well assay plates and showed a Z' value of 0.6. Assay points below 30% relative activity were considered inhibitor hits and assay points greater than 150% of relative activity were identified as activator hits. Of the 24,320 compounds, 62 (~0.2%) were identified as inhibitor hits and 62 were identified as activator hits.

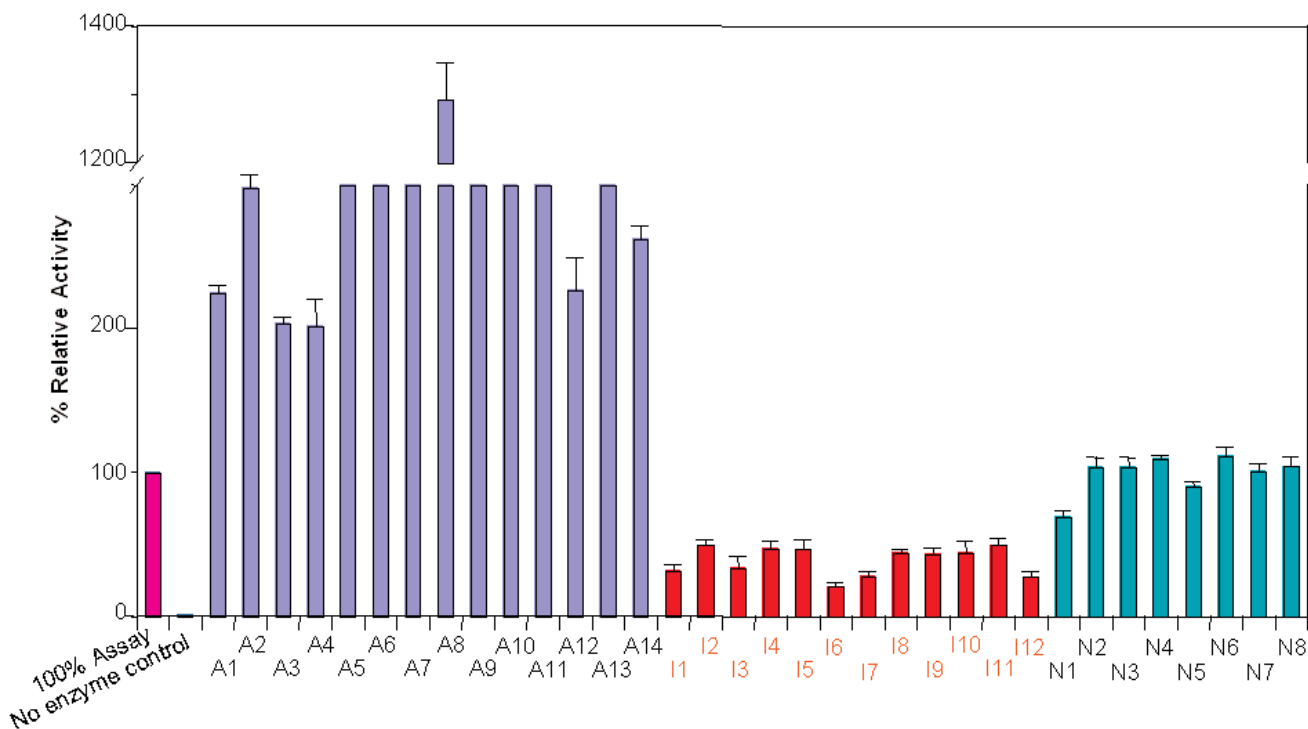


Figure 8. Confirmed activators and effectors of the caspase 3 assay. Approximately 24,320 compounds were screened with the miniaturized caspase 3 assay. Out of these compounds, 62 activator (0.2%) and 62 inhibitor hits were identified. In order to confirm the hits, each identified hit compound was assayed for caspase 3 in quadruplicate 1536-wells along with eight compounds that had no or neutral effect in the screening assay. Shown are the average \pm SEM ($n=4$) of the relative activity for the confirmed hits. Of the original hits, 14 activators (A1-A14) and 12 inhibitors (I1-I12) were confirmed. The non-effect of the neutral compounds (N1-N8) was confirmed.

By performing the assay in 1536-well plates, one uses at least ten times less substrate, enzyme, and buffer as compared to a 96-well plate assay, yet similar answers are obtained as with the 96-well plate. The use of 1536-well format also decreases the plate handling time. The 1536-well plate holds up to 16 of the 96-well plates or four of the 384-well plates along with a number of internal controls.

A total of 24,320 compounds were screened with the miniaturized caspase 3 assay and identified 0.2% inhibitor hits and 0.2% activator hits. Of these, 12 inhibitor hits and 14 activator hits were confirmed. The fully automated caspase 3 assay in a 1536-well plate format has been adapted on the SAGIAN™ Core System from Beckman Coulter. Using the present experimental design on the SAGIAN Core System, screening assays can be performed in 1536-well plate format on 100,000 compounds within a day.

Acknowledgements

The authors would like to thank Alan Main, Ph.D., Dave Bolton, Ph.D., and Hartmuth Kold, Ph.D., from Lexicon Pharmaceuticals; Dan Root, Ph.D., and Lora Heiner from Exegetix, Inc; Lena Sripitisawad, Alice Tanibata, Larry Weiss, and Bryan Shannon from Beckman Coulter, Inc.

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