



## A fully-automated, image-based screen for miRNAs that regulate cancer cell viability and chemosensitivity

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### Abstract

We utilized a screening system to identify miRNA mimics that affect cancer cell viability or sensitivity to chemotherapeutics. The use of live cell imaging enabled data acquisition at multiple time points and provided more hit differentiation than a single endpoint assay. By fully automating library reformatting, cell transfection, compound addition, and image acquisition, we not only reduced the time at the bench and the likelihood of errors, but we enabled the flow of data throughout the screen to ensure the integrity of the screen results.

### Introduction

Screening technologies are frequently used to explore biological questions and to identify potential new treatments for cancer and other diseases. Massive small molecule screens have given way to genome-wide expression or knockdown studies in an effort to better understand the molecular pathways involved in tumorigenesis and cancer metastasis. More recently, microRNAs (miRNAs) have become a focus of inquiry as researchers are trying to determine what role these previously disregarded molecules have in gene regulation and how changes in their expression can promote or inhibit cancer growth.

The large sample throughput typical of screens introduces challenges even when the sample preparation and analysis are relatively straightforward. For cell-based screening, the time needed to manipulate, culture, and analyze the cells can be significant and the increased number of interactions increases the opportunity to introduce errors to the screen. Here we describe a system that can automate all the steps required for the transfection of a miRNA mimic library into a cancer cell line, a subsequent compound treatment, and the acquisition and analysis of images to measure cell toxicity.

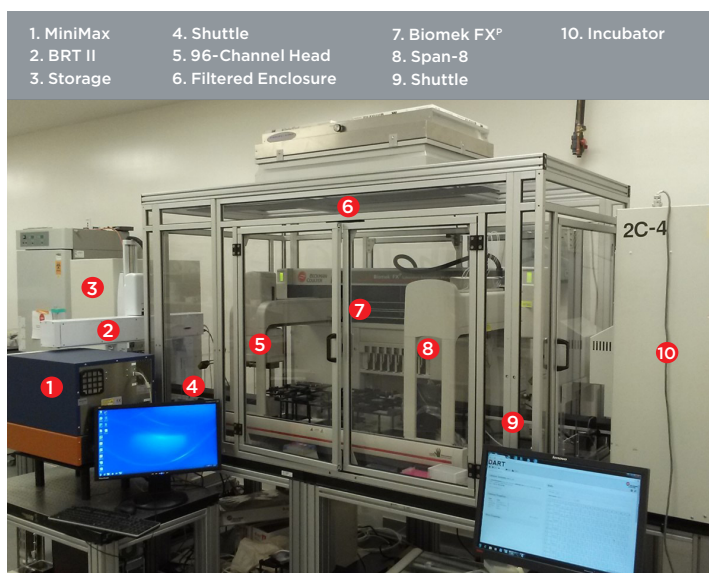
### Materials and Methods

#### Cell Culture and Reagents

HCT 116 colorectal carcinoma cells were cultured in McCoy's 5A Modified Medium supplemented with 10% fetal bovine serum (FBS). All plates were stored within a humidified incubator at 37°C in 5% CO<sub>2</sub>. Transfection complexes were formed from a miRNA mimic library (Sigma Aldrich) and DharmaFECT 1 (GE Life Sciences) in OptiMEM Reduced Serum Medium (Thermo Fisher Scientific). 50 µg/mL 5-fluorouracil (Sigma Aldrich) was used to induce cell death and a 1:2000 dilution of the cell-impermeable nucleic acid stain DRAQ7 (Beckman Coulter) was used to measure cytotoxicity.

#### Automation

The screening system (Figure 1) utilized a Biomek FX<sup>P</sup> Workstation with multichannel and Span-8 pods for all liquid transfer steps. For initial resuspension and reformatting of miRNA mimics into 384-well stock formats, the multichannel pod utilized a 96-channel head with enhanced selective tip pipetting, with which any pattern of tips can be used. The multichannel head was then replaced with a 384-well head for the plating of miRNA mimics, transfection reagent, cells, compound, and staining reagent. The flexible deck configuration of the Biomek FX<sup>P</sup> allowed for customized integration of additional devices including an incubator and storage for tips and plates, with barcode scanners en route to both. The system used an integrated BRT II robotic transport to transfer plates to and from the SpectraMax i3x Multi-Mode Detection Platform with MiniMax 300 Imaging Cytometer (SpectraMax MiniMax) for analysis. A sterile environment for liquid transfers and cell handling was maintained through the use of a HEPA-filtered enclosure.



**Figure 1. Screening system.** Plates and tips were loaded into the storage device (Cytomat MPH) and brought onto the deck of the Biomek FX<sup>®</sup> Workstation via an integrated shuttle. The filtered enclosure maintained a sterile environment for the automated addition of media, reagents, and cells. Transfected cells were transported between the incubator (Cytomat 2C) and the SpectraMax MiniMax by the shuttles and the BRT II robotic transport.

three different categories: 1) miRNAs whose overexpression rapidly (<24 hr) initiate cell death, 2) those that either enhance 5FU toxicity or gradually induce cell death (24 hr post-5FU addition), and 3) those that repress 5FU-induced toxicity (24 or 48 hr post-5FU addition).

The library of 1,920 miRNA mimics was originally lyophilized in twenty-four 96-well plates, with the outer columns empty for the addition of controls. To minimize reagent usage and to streamline throughput, we reformatted the libraries into six 384-well plates. After loading 80 tips on the 96-channel head, water was added to the library plates and the mimics were resuspended by shaking each plate for five minutes. The miRNA mimics were then transferred to 384-well stock plates and a portion was diluted to a working stock of 2  $\mu$ M. Two negative control miRNA mimics (Sigma Aldrich) were added to the working plates and water was added to the outer columns to determine any effects of nucleic acids on cell viability. Plates were brought in from storage positions and processed on the Biomek Workstation using SAMI EX software. The entire resuspension, reformatting, and dilution process was completed in 3 hours and 40 minutes. The reformatted library plates were stored at -80°C and the working stock plates were stored at -20°C until used in the screen.

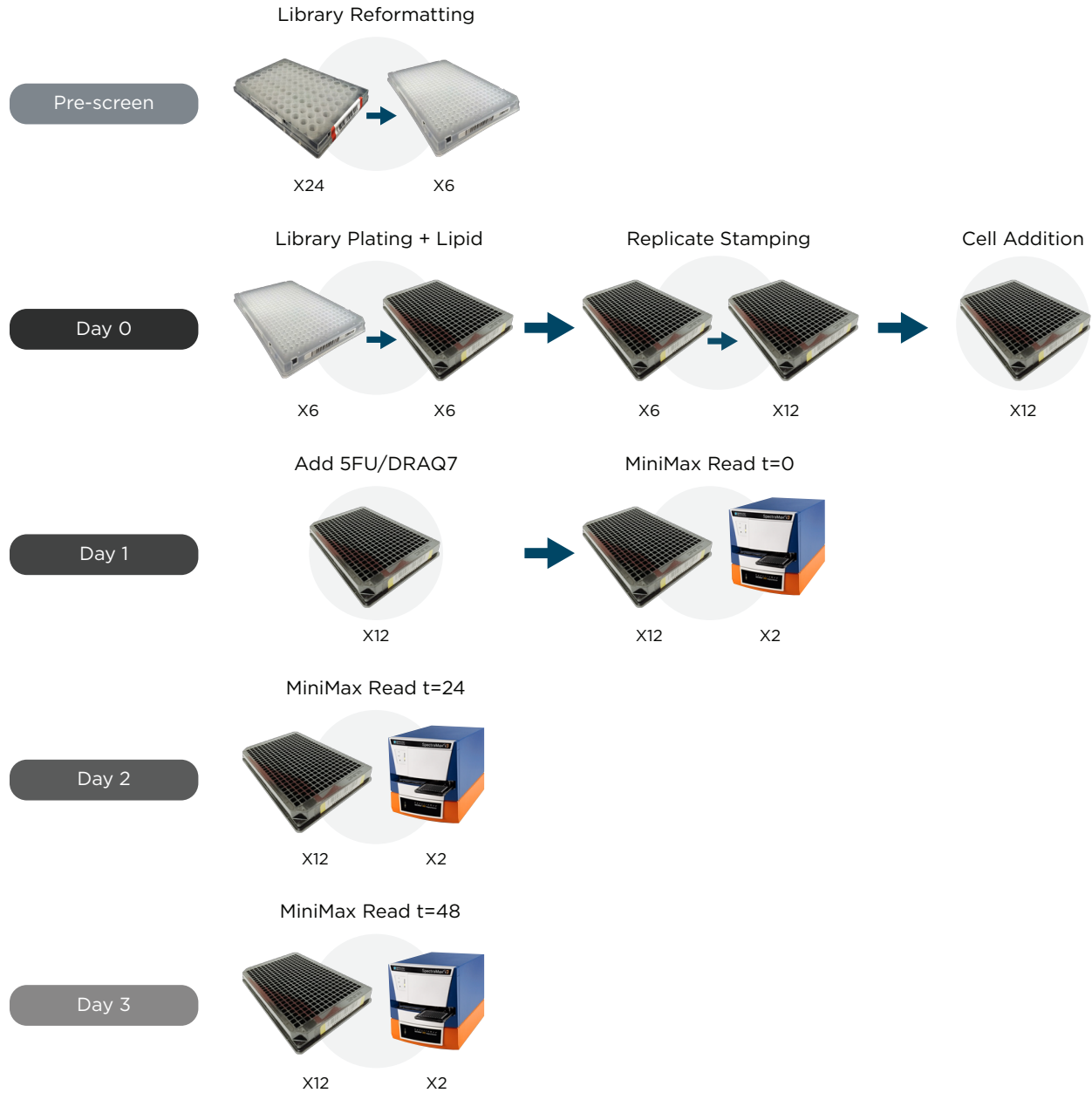
For screening, the 96-channel head was replaced with a 384-well head to automate the transfection and treatment of cells, as shown in Figure 2. Briefly, OptiMEM media was dispensed into clear-bottom black-walled 384-well plates and the working stock miRNA mimics were diluted into the OptiMEM. DharmaFECT 1 was added to the diluted mimics and the plates were incubated to allow transfection complexes to form. After at least 15 minutes, the plates were returned to the deck, and half of the transfection reagents were transferred to replicate plates. Finally, HCT 116 cells in McCoy's Medium + 10% FBS were added to all plates such that 12,000 cells were transfected with 0.4  $\mu$ L DharmaFECT 1 and 40 nM miRNA mimics in 50  $\mu$ L per well. The resulting 12 plates were fully processed in 1 hour and 42 minutes.

## Imaging Cytometry

The SpectraMax MiniMax cytometer was used to image cells at 4x magnification in brightfield and 713 nm emission wavelengths. Images were analyzed using SoftMax Pro 6.5.1 software. The CellsD setting of the SoftMax Pro StainFree Cell Detection Technology was used to count cells in the brightfield images, while DRAQ7-positive nuclei were identified based on size and intensity. DRAQ7-positive nuclei counts were normalized to total cell counts to determine the percent toxicity for each condition.

## Automated Screen

The goal of this work was to demonstrate the automation of a screen that identifies miRNAs that have an effect on cancer cell viability or chemosensitivity. Our model entailed transfecting HCT 116 cells with miRNA mimics and after 24 hours, adding 5-fluorouracil (5FU), a commonly used chemotherapeutic agent for colon cancers. In contrast to endpoint assays, we sought to use cell imaging over time to separate effectors into



**Figure 2. Automated miRNA screen workflow.** Prior to the screen, twenty-four 96-well plates were recombined to six 384-well plates with added controls. For the screen, libraries were diluted and combined with transfection reagents, replicate stamped, and HCT 116 cells were added. 24 hours after transfection, 5-fluorouracil and DRAQ7 were added to the wells and plates were imaged on the SpectraMax MiniMax at two wavelengths (brightfield and 713 nm). Plates were imaged again after 24 and 48 hours of compound treatment.

Plates were incubated for 24 hours and returned to the deck where 5FU and DRAQ7 were added to induce and identify cell death, respectively. Plates were returned to the incubator for 30 minutes before the first plate was scanned on the SpectraMax MiniMax (5FU t=0).

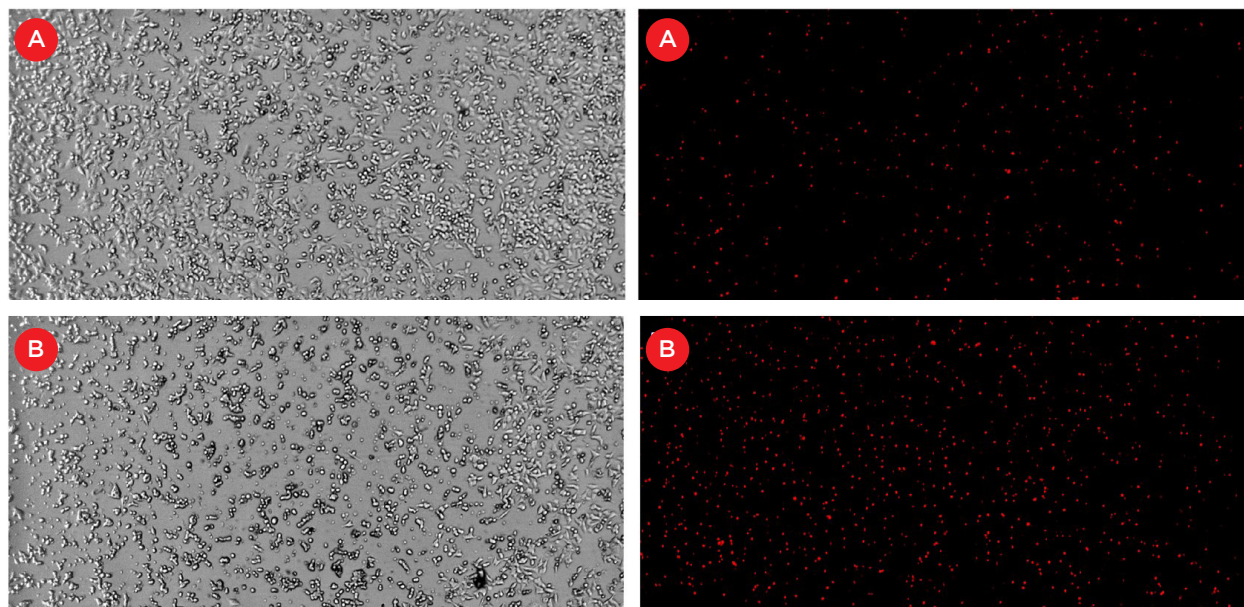
Plates were scanned twice – once to count cells using brightfield microscopy and once to count DRAQ7-positive nuclei using the 713 nm filter. Each plate required approximately 30 minutes to process fully. Prior testing showed that there was no significant effect of 5FU in the first six hours of treatment, so any cell toxicity detected at t=0 should be the direct result of the miRNA mimic overexpression. Cells were returned to the incubator after scanning and the analysis was repeated at 24 hours and 48 hours after 5FU treatment.

All plates were barcoded, allowing library information (e.g., miRNA mimic identity and sequence) to be passed on to the 384-well stock and assay plates during the liquid transfers through the use of the Data Acquisition and Reporting Tool (DART 2.0) software. This ensured that screen results could be directly linked with the miRNA library, thereby removing the potential for errors and ensuring the correct identification of hits. For more information on handling screen data, see “Automated data management for reliable cell-based screening” (AAG-1735APP06.16).

## Results

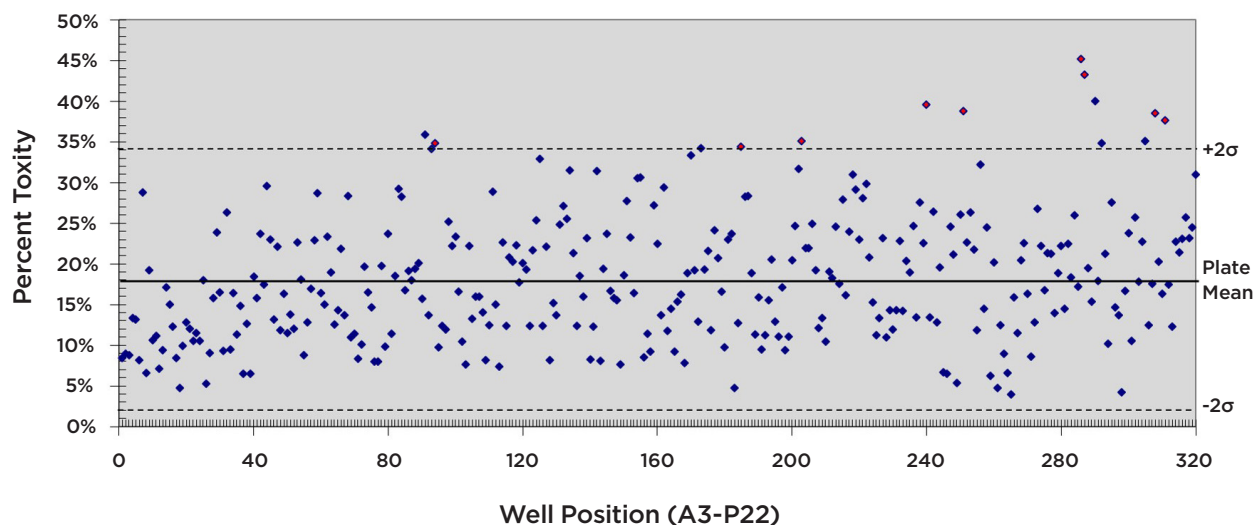
Replicate screen wells were assayed for total cell counts and DRAQ7-positive nuclei to determine the relative level of cytotoxicity. Screen hits were identified at each time point to identify miRNA mimics that: 1) rapidly induce cytotoxicity in HCT 116 cells (5FU t=0 hr), 2) slowly induce cytotoxicity/enhance 5FU toxicity (t =24 hr) or 3) repress 5FU toxicity (t=24, 48 hr).

Primary hits were identified as those miRNAs generating cytotoxicity measurements more than two standard deviations from the plate mean on both replicate plates. Figure 3 shows one such comparison with brightfield and 713 nm images from an average responding well 24 hours after transfection (5FU t=0) (A), and a screen hit that induced roughly 3-fold more toxicity than the plate mean (B). The plate means were used for comparison because one of the two negative control miRNA mimics induced significant toxicity in the HCT 116 cells. This intra-plate comparison to the mean was also useful in normalizing any effects from the six hour lag between the first and last plate read for each assay time point.



**Figure 3. Well images.** A SpectraMax MiniMax was used to acquire brightfield images (left) to count cells and 713 nm images (right) to count DRAQ7-positive nuclei. The upper images (A) correspond to a well that shows the plate mean level of toxicity 24 hours after transfection (5FU t=0) while the lower images (B) show a well on the same plate where the miRNA mimic expression induced 3-fold more toxicity.

Figure 4 shows the results of a typical plate at the 5FU 24-hour time point. Nine of the fourteen wells with cytotoxicity greater than two standard deviations from the plate mean were also hits in the replicate plate. The overall hit rate for the screen was 2.6% (50 of 1,920), with 52% of the hits inducing toxicity independent of 5FU (Table 1). The remaining 48% also increased cell toxicity but were either slower-acting or enhanced the effects of 5FU. A number of wells showed reduced toxicity that approached the two standard deviation threshold, but none of these surpassed the threshold on both replicate plates. This was likely due in part to using the broader standard deviation expected from the library plate mean rather than the tighter standard deviation expected from negative controls.



**Figure 4. Single plate toxicity scatter plot.** Percent toxicity was calculated as the number of DRAQ7-positive nuclei divided by the number of cells per well. The scatter plot represents the percent toxicity for each well of one plate 24 hours after 5FU treatment (48 hours post-transfection). The dashed lines indicate the threshold for hits at  $>2$  standard deviations from plate mean. Only wells that were also above the threshold in the replicate plate were considered true hits and are colored red.

**Table 1.** Unique screen hits and the time points at which they were identified.

	24hr miRNA	48hr miRNA + 24hr 5FU	72hr miRNA + 48hr 5FU
<b>Hits (<math>+2\sigma</math> toxicity)</b>	26	16	8

23 of the 50 hits had published information suggesting a potential role in cancer and the breakdown of these hits is shown in Table 2. 21 of the 23 cancer-related hits had data supporting a tumor suppressor role such that overexpression of these miRNAs would be predicted to contribute to cell toxicity. The two miRNAs with a predicted oncogene role were found to increase cytotoxicity in the presence of 5FU, suggesting that these miRNAs might have enhanced cell growth in the presence of the thymidylate synthase inhibitor and driven the cells to further damage. Given that 90-100% of hits with known functions give the expected phenotypes, these results lend significant support to the validity of the screen. This support also indicates that the remaining 27 hits with little to no published information regarding a role in cancer might be novel potential therapeutic targets.

**Table 2.** Hit correlation with published data.

	24hr miRNA	48hr miRNA + 24hr 5FU	72hr miRNA + 48hr 5FU
<b>Known Tumor Suppressors</b>	11	9	2
<b>Known Oncogenes</b>	0	2	0
<b>Unknown</b>	15	5	6

## Discussion

Cancer treatments are widely variable in their effects. miRNAs may explain some of the different responses seen in different cancers or even in different cells within a single tumor. Identifying miRNAs that play a role in cancer viability or that enhance resistance or susceptibility to given chemotherapeutics could lead to novel treatments or identify potential combination therapies. Here we have identified 50 candidate miRNAs whose overexpression may induce apoptosis in HCT 116 colon carcinoma cells or enhance their killing by 5-fluorouracil. This work benefitted from many advantages of a fully-automated image-based screening system.



One of the most important aspects of automation is the ability to reduce errors in a manual workflow. This includes physical errors, such as placing a plate in the wrong orientation or in the wrong position, to pipetting errors during sample preparation. By using barcodes to ensure all plates are processed in the proper way and facilitating data continuity throughout the process, one can be assured that screen hits are reliable.

By automating what would otherwise be 18 hours of resource-consuming data acquisition on the SpectraMax MiniMax we were able to analyze the screen over a time course. This time course increased our chances of successfully identifying hits since 5FU toxicity could vary between experiments and if one is looking at a single time point, there is a high likelihood that optimal conditions will not be captured. The time course also gave a greater detail of data than would be provided by an endpoint assay. For example, by being able to distinguish the 26 miRNAs that induced toxicity prior to the addition of 5FU, the number of hits taken into secondary screens would be reduced if we were particularly interested in miRNAs that affect 5FU sensitivity. Utilizing imaging also provides the ability to eliminate false positives, such as the presence of an air bubble resulting in low cell counts, which would be difficult to discern in lysis-based endpoint assays. The more hits that can be triaged before moving candidates forward to secondary screens, the less resources will be required to identify the most significant effectors.

A major motivation for the miniaturization of the screen to a 384-well format was limiting resources. While 384-well plates can be challenging to work with manually, a majority of a well of a 384-well plate can be assayed with a single 4X image from the SpectraMax MiniMax. To acquire a similar percentage of a 96-well plate, one would need to acquire four images. This would significantly increase processing time and create the need to manipulate four times as many plates. By moving to smaller volume wells, we also extended the life of the library while reducing the usage of costly reagents such as transfection lipids.

Despite these reductions in reagent volumes per well, the dead volumes of large reservoirs (>20 mL) is still a significant cost. For lower throughput applications, the Biomek Workstation can dispense reagents from tubes or smaller reservoirs using either Span-8 or enhanced selective tip capabilities, as the increased processing time is likely balanced by decreasing reagent waste.

Another advantage of having automated the process is that the optimized system makes it simple to change factors such as compound, cell type, or transfection reagent. Changing these factors could show which miRNAs have broad applicability across cell lines or which hits are specific to 5FU toxicity. Determining 5FU-specificity would provide significant information toward potential targets of the miRNAs in addition to identifying miRNAs that may be specific to a second compound.

It is worth noting that the screening system utilized here is just an example of what is possible when integrating other instruments to the Biomek Workstation. The ability to configure the Biomek deck allows the addition of on-deck instrumentation or shuttling of transport plates to and from other instruments while the Biomek and SAMI EX software can drive the processing and analysis of plates and even use assay information to drive liquid transfers. The Biomek Workstation has been integrated with over 300 different instruments that cover a wide array of uses and new integrations are added regularly so that nearly any workflow can be automated for higher throughput. By enabling such screens as the one described here, automation can accelerate biological innovations and discoveries of new treatments.

## References

1. <http://www.cancer.org/cancer/colonandrectumcancer/detailedguide/colorectal-cancer-treating-chemotherapy>



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