Original Article

Utilization of an Automated Pipetting System in the Cell Line-Based Screening of the Activity of a DNA-Damaging Anti-Tumour Drug

(cell line screen / drug treatment / liquid handling station / epMotion®)

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Abstract. The principles of large screening strategies, which are developed by industrial companies, have been recently adopted by researchers in the fields of molecular biology and oncology as invaluable tools for translational medicine. The declining costs of laboratory robotic machines have allowed high-throughput screening to become more available to academic centres with limited resources. Here, we describe how a robotic conventional liquid handling system could be used on a daily basis in laboratories to obtain consistent and reproducible results. Our approach allowed us to quickly screen a panel of more than 20 tumorigenic and non-tumorigenic cell lines for their responses to hydroxyurea, which is a DNA-damaging anticancer therapeutic drug. The format of 384-well microplates was used for manual cell seeding, and the effect of hydroxyurea was screened at multiple concentrations. The fluorescence- based CyQuant assay was employed as the readout method to analyse the cellular DNA content. The effectiveness of our approach was demonstrated in the experimental results.

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Abbreviations: CI – confidence interval, DMEM – Dulbecco's modified Eagle's medium, EDTA – ethylenediamine tetraacetic acid, FBS – foetal bovine serum, HU – hydroxyurea, PBS – phosphate-buffered saline, SD – standard deviation, SE – relative variability.

Introduction

Laboratory robotic machines were first introduced in the early 1980s in clinical centres. Since then, the utilization of automation in laboratory practices has continuously increased. Growing efforts to minimize errors, enhance reproducibility, reduce reagents, and test multiple amounts of compounds, along with the introduction of high-throughput screening, have led to the development of robotic systems that can fully automate liquid handling (Ferreira et al., 2011).

Automated stations can now be used in smaller academic centres, thanks to increases in technical progress and decreases in equipment costs. Despite limited resources, academic researchers can use these machines to investigate broader questions, such as basic molecular mechanisms, or screen a larger variety of targets. This eliminates the need to always weigh the costs of the final product against its potential on the market, as is often required in research in industry and pharmacological companies (Baker, 2010).

Systematic screening of gene products (Bürckstümmer et al., 2013), investigation of the response of cancer cells to extensive libraries of drugs (Muellner et al., 2011) and screening of short hairpin RNA collections for stable loss-of-function phenotypes (Silva et al., 2008) are only a few examples of great tools that have been developed for many applications in molecular biology and translational medicine. Thus, various types of medium- to high-throughput screens are nowadays performed in many academic laboratories. However, all screens require precise laboratory procedures, and any step that involves the handling of liquid reagents in very small volumes can suffer from a lack of reproducibility within large amounts of data, which need to be accurately interpreted. Robotic machines can provide an invaluable tool to increase the reproducibility of research work.

Here, we describe the successful usage of an automated liquid handling station to obtain dose-response curves for an anticancer drug in a large panel of cell lines from different types of cancer or healthy tissue.

Material and Methods

Cell culture

The MDCK, MiaPaCa, and PANC-1 cell lines were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Prague, Czech Republic), which was supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml) (PAA, GE Healthcare, Uppsala, Sweden), and 10% foetal bovine serum (FBS; PAA). BPH-1 and BPH-1 CAFTDP4 cells were cultivated in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen), which was supplemented with 5% FBS, streptomycin (0.1 mg/ml), and penicillin (100 U/ml). A2780, A2780cis, H441, MDA-MB-231, and DU-145 cells were cultivated in RPMI-1640 medium, which was supplemented with 10% FBS, streptomycin (0.1 mg/ml), and penicillin (100 U/ml). A549 and PC3 cells were cultivated in F-12 medium (Invitrogen), which was supplemented with 10% FBS, streptomycin (0.1 mg/ml), and penicillin (100 U/ml). SKOV-3, CAKI-2 and Sk-Br-3 cells were maintained in McCoy's 5A modified medium containing 1.5 mM L-glutamine (Sigma-Aldrich, Prague, Czech Republic), penicillin (100 U/ml), streptomycin (0.1 mg/ml), sodium bicarbonate (1.5 g/l; Serva, Heidelberg, Germany), and 10% FBS. HCT-116 $p53^{-/-}$, HCT-116 $p53^{+/+}$, HCT-116 $PTEN^{-/-}$, HCT-116 $PTEN^{+/+}$, SW620, SW480, and HT-29 human colon adenocarcinoma cells were maintained in McCoy's 5A modified medium containing 1.5 mM L-glutamine, penicillin (100 U/ml), streptomycin (0.1 mg/ml), sodium bicarbonate (1.5 g/l), and 10% heat-inactivated FBS (PAA).

SW480, SW620, HT-29, DU-145, and H441 cell lines were obtained from American Type Culture Collection (LGC Standards, Warsaw, Poland).

All cells were harvested after a brief incubation in 0.05% ethylenediamine tetraacetic acid (EDTA) in phosphate-buffered saline (PBS), followed by trypsinization (0.25% w/v trypsin/0.53 mM EDTA in PBS). They were then counted by using a CASY TT automatic cell counter (Roche Diagnostics, Prague, Czech Republic), diluted in the appropriate volume, and seeded in 384-well plates (80 µl of cell suspension/well).

Drug treatment

Cells were seeded at a density of 20,000 or 30,000 cells/cm^2. Hydroxyurea (Sigma Aldrich) was dissolved in PBS and diluted to the appropriate concentrations (0.037 mM to 27 mM). Treatments with 5 µl of the diluted drug were performed with the epMotion® 5075 (Eppendorf, Prague, Czech Republic), which is a liquid handling workstation. The time scheme is depicted in Figure 1.

The epMotion® 5075 procedures

In this study, the worktable of the epMotion 5075 was equipped with the following labware: 50/300/1000-µl filters, sterile tips (epT.I.P.S.® Motion), racks with 1.5-ml Safe-Lock tubes that were used to store stock solutions of drugs, and 96-well plates (TPP, Trasadingen, Schaffhausen, Switzerland) for the dilution of the drug for direct treatment.

Cell proliferation assay

The CyQUANT cell proliferation assay (Invitrogen) was performed according to the manufacturer's recommendations, and the results were analysed using a Fluostar Galaxy reader (BMG Labtech, Ortenberg, Germany).

Statistical analysis

Data were standardized as a percentage of control and further summarized as means ± standard errors (SE) for each concentration of hydroxyurea. The variability of the experimental data of different cell lines was assessed as the mean relative SE. Non-linear regression was applied to generate curves that came closest to the data. A four-parameter dose-response model with a sigmoidal shape was used: Y = Bottom + (Top-Bottom)/(1+10^five[(LogIC50-X)*HillSlope]), where IC50 denoted the concentration of the agonist that gave a response

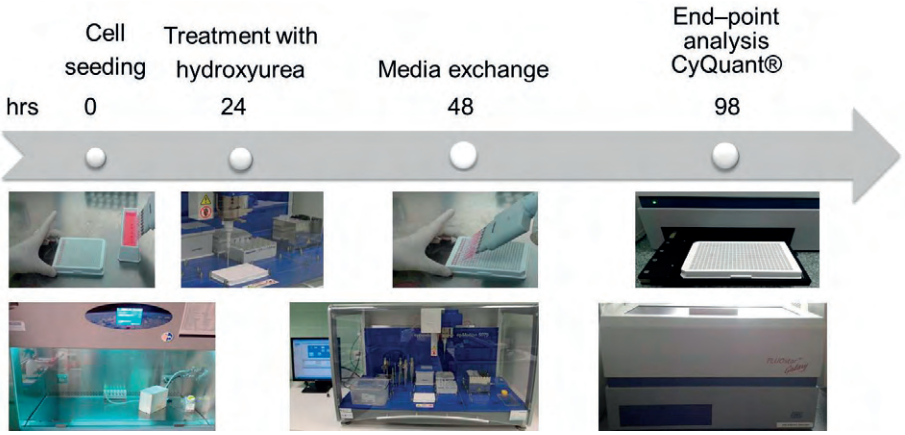

Fig. 1. Experimental design for cell seeding, drug treatment, and analysis

that was halfway between Bottom and Top. HillSlope described the steepness of the curve, and the Top and Bottom denoted plateaus in the units of the Y-axis.

Results

We screened a panel of 23 mammalian cell lines to assess their responses to hydroxyurea, a potent anti-tumour drug, and to obtain dose-response curves. Hydroxyurea has been used for decades, and it is still valuable for the treatment of some types of cancer (Saban and Bujak, 2009). The origins and histology of the cell lines are summarized in Table 1. In research laboratories, the 96-well format of assay plates is usually used to screen the cytotoxicity of compounds, and it is also included in many commercially available viability kits. This format enables one to easily and manually perform all experimental steps, including the seeding of the cells, the addition of the drugs, and the final measurement of viability and cytotoxicity, within one plate. However, due to the accessibility of the automated laboratory station and the requirement to generate large amounts of data in a short time, we decided to use 384-well cultivation plates. These plates allowed us to design layouts that covered control wells and a broad range of drug concentrations, which were performed in quadruplicate.

Table 1. Panel of mammalian tumorigenic or non-tumorigenic cell lines that were screened for responses to hydroxyurea treatment

Cell line	Tissue	Histological Type
A2780	Ovary	Adenocarcinoma
A2780cis	Ovary	Adenocarcinoma
A549	Lung	NSC carcinoma
BPH-1	Prostate	Benign hyperplasia
BPH-1 CAFTD04	Prostate	Tumour, clone of BPH
CAKI-2	Kidney	Renal cell carcinoma
DU145	Prostate	Carcinoma
H441	Lung	Adenocarcinoma
HCT-116 $p53^{-/-}$	Large intestine	Carcinoma
HCT-116 $p53^{+/+}$	Large intestine	Carcinoma
HCT-116 $PTEN^{-/-}$	Large intestine	Carcinoma
HCT-116 $PTEN^{+/+}$	Large intestine	Carcinoma
HT-29	Large intestine	Adenocarcinoma
MCF10A	Breast	Non-tumorigenic
MDA-MB-231	Breast	Carcinoma
MDCK	Kidney	Non-tumorigenic
MiaPaCa2	Pancreas	Ductal carcinoma
PANC-1	Pancreas	Ductal carcinoma
PC3	Prostate	Adenocarcinoma
Sk-Br-3	Breast	Carcinoma
SKOV-3	Ovary	Adenocarcinoma
SW480	Large intestine	Adenocarcinoma
SW620	Large intestine	Adenocarcinoma

The liquid handling station that was used in this study did not provide a sterile environment for cell culture processing. Thus, the first step involved using a multichannel pipette to manually seed cells in microplates in a standard laminar flow cabinet. For drug treatments, we developed a programme for the Eppendorf liquid handling station that allowed all cells to be treated precisely with the drug.

The experimental design and procedure are summarized in Figure 1. On day 0, we used a manual pipette to seed cells at the appropriate densities. One plate was used for each cell line, and the dimensions of our liquid handling system allowed us to screen seven plates per run of the experiment. On the second day, we manually prepared a stock solution of hydroxyurea and placed it into the liquid handling system, which dispensed the solution into the first column of a 96-deep-well plate. Dilutions were consequently performed using a multichannel pipetting head to obtain the concentration range. According to the sensitivity of each cell line (based on a literature search or our previous measurements; data not shown), we chose to use 1 : 5 or 1 : 4 serial dilution of the drug to obtain an adequate rank of tested concentrations. For the automated treatments, the multichannel head distributed 5 µl of the drug solution from the 96-well plate to the desired positions of the 384-well plate that contained the cells. After an additional 24 h, we manually refreshed the media, and the measurement of cell proliferation, which was the end-point analysis, was performed on day 4.

Cell proliferation was evaluated by using the fluorescence-based CyQUANT® assay, which measures the DNA content in the wells of the microplate (Jones et al., 2001). Raw data that were obtained by the fluorescence reader were processed and statistically evaluated, according to the procedures that are outlined in Material and Methods.

For all measured cell lines, regardless of the tissue of their origin, we were able to evaluate variability with relative SE and generate proper dose-response curves with intervals of confidence. The relative deviations of the means of experimental values varied from 2.39 % to 9.25 %. The lowest numbers were obtained for the HCT-116 $p53^{+/+}$ (2.9 %), HT29 (3.16 %), and MDCK (3.01 %) cell lines. On the other hand, higher values were obtained for the MCF10A (9.25 %), CAKI-2 (8.01 %), and PC-3 (6.64 %) cell lines. The r^2 values for six of the investigated cell lines (CAKI-2, H441, MDA-MB-23, PANC-1, PC-3, and Sk-Br-3) were less than 0.9. The r^2 values for the remaining cell lines were higher, and the models were able to fit the data well.

The dose-response curves with calculated intervals of confidence are shown in Figure 2. Table 2 depicts a summary of the results of statistical analyses of both experimental data and fitted dose-response curves. The relative variability (SE) of experimental data and the goodness of fit of nonlinear regression, as described by the r^2 value, were compared. The outliers are highlighted in grey in Table 2.

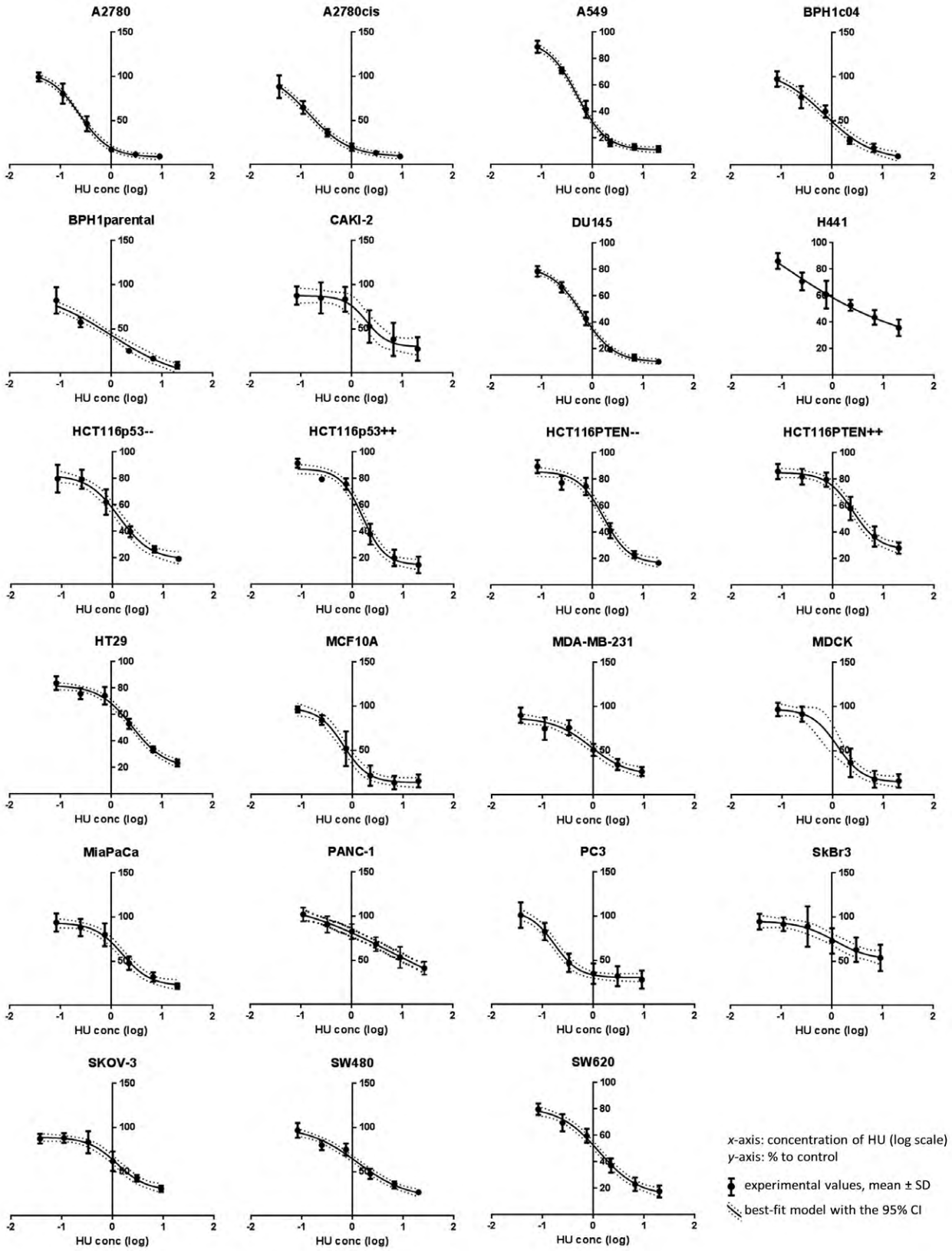

Fig. 2. Statistical evaluation of dose-response curves for hydroxyurea in mammalian cell lines
SD – standard deviation, CI – confidence interval, HU – hydroxyurea, conc – concentration

Discussion

Many sophisticated automated platforms have been developed in the rapidly growing laboratory robotic field. In particular, robotic stations with fully automated tissue culture handling systems have been designed for drug screenings that require long-term sterile cultivation. These machines, which are mainly used in the

Table 2. Analysis of relative deviations of the experimental data and the goodness of fit of nonlinear regression

Cell line	Relative deviation (SE) [%]	r^2 value
A2780	6.19	0.972
A2780cis	6.45	0.957
A549	5.60	0.985
BPH1c04	5.48	0.944
BPH1parental	5.24	0.936
CAKI-2	8.01	0.722
DU145	3.74	0.986
H441	5.55	0.876
HCT-116p53$^{-/-}$	4.64	0.932
HCT-116p53$^{+/+}$	2.39	0.964
HCT-116PTEN$^{-/-}$	3.74	0.962
HCT-116PTEN$^{+/+}$	3.46	0.931
HT29	3.16	0.961
MCF10A	9.25	0.918
MDA-MB-231	4.49	0.873
MDCK	3.01	0.930
MiaPaCa	4.78	0.917
PANC-1	5.69	0.861
PC3	6.64	0.864
SkBr3	3.74	0.548
SKOV-3	5.39	0.902
SW480	4.06	0.939
SW620	4.28	0.953

pharmacological field for high-throughput screens, can also provide the integrated reader with desired signals that can be used to automate the whole procedure (Kempner, 2002).

In our screen, cell seeding was performed by manual pipetting using a multichannel pipette. Because the seeding process is not demanding, the utilization of manual pipetting tools, which can be used for all types of plate formats, can easily provide accuracy for cell density. However, the process of manually adding drugs to dense grids, especially to those of 384-well plates, can be time-consuming and highly inaccurate. Additionally, larger volumes of cell suspension are typically used, compared with the few microliters of expensive and/or toxic drugs that are dispensed for the treatment. Therefore, the automated machine was used to dispense drug volumes for the cell treatments. The last step of the experiment was performed using the manual multichannel pipette, as it included only the disposal of the media and the addition of the diluted fluorescent dye.

The liquid handling station that was used in our screen is designed to automate a broad spectrum of biological and biochemical methods in a semi-sterile environment in a medium-throughput manner. It guarantees accuracy for pipetting 1-µl volumes (Grzeskowiak and Oltmanns, 2004; Wehrhahn, 2007). Nevertheless, we decided to seed cells in media volumes of 80 µl, and volumes of 5 µl were used for the drug treatments. This proportion allowed the pipetting to be performed with great precision, and it protected cells from any additional effects that may be caused by the drug solvent.

The r^2 value, which was used to compare the quality of the obtained data, quantifies the goodness of fit of the regression model and is a value between 0 and 1. The higher values indicate a better fit. The analysis of our r^2 values shows that the dose-response curves for 18 of the 23 investigated cell lines were very good. However, the calculated r^2 values for several cell lines (CAKI-2, MDCK, and Sk-Br-3) were lower than those for other cell lines. Although pipetting errors may account for these differences in values, the variations may be more likely due to the differences in the adherence of nearly confluent cells to the cultivation surface at the time of the end-point analysis. This phenomenon was cell line dependent and could be partly resolved by specifying seeding density, if needed.

The relative errors of the measured values varied from 2.39 % to 9.25 %. For most of the cell lines, these numbers were satisfactory. For comparison, results from a similar experiment in which all steps, including drug dilutions and treatments, were performed using manual pipetting revealed relative deviation values that ranged from 3.2 % to 3.8 % (data not shown). This indicates that our medium-throughput type of screen can provide results that are comparable to very careful manual pipetting. Additionally, the layout for automated treatments was designed to obtain data for three dose-response curves that consisted of six different concentrations of the drug from one 384-well plate, where each data point was measured in quadruplicate in each assay. The multichannel pipetting head was able to distribute the stock solution of the drug in seven 384-well plates within 20 min. It is difficult to manually generate this amount of data within such a short period of time.

Based on our experience in working with the liquid handling station, we can propose advantages and disadvantages to help other laboratories assess the usefulness of this system in their research. The main benefits of using automated stations included the ability to develop programs that can be routinely used to screen a large panel of cell lines. However, every laboratory has to consider the time that must be invested to develop the reliable program. In addition, the price of the robotic machine and its pipetting tips for routine usage can still be relatively high for smaller research teams. Furthermore, it has to be taken into account that the consumption of drugs for treatment can be higher, due to the extra volume that is needed for automated pipetting.

In summary, by preparing a simple and user-friendly program that was used by the automated station for diluting drugs and treating cells, we overcame the main obstacles, such as the accurate addition of reagents and time consumption, to large-scale screening in smaller research laboratories. Furthermore, we were able to obtain consistent dose-response curves for hydroxyurea in a panel of 23 tumorigenic or non-tumorigenic cell lines.

These data allowed us to quickly identify the most promising candidates that can be used to further investigate the biological mechanisms of response to drug treatments.

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