

# Evaluation of a Novel Automated Machine, the Auto2D, for Two-Dimensional Gel Electrophoresis

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## Abstract

Two-dimensional gel electrophoresis (2D-PAGE) has been a commonly used technique for protein expression studies. Application of a fluorescent dye in 2D-PAGE, namely 2D-DIGE, has improved the performance of 2D-PAGE in terms of sensitivity, reproducibility and throughput. However, 2D-PAGE still requires a degree of skill, and is considerably time-consuming. Recently, a novel automated 2D-PAGE machine, the Auto2D, was developed for use in conventional protein expression studies. Here, we examined the performance of the Auto2D for 2D-DIGE from the viewpoint of proteome coverage, reproducibility, and throughput. We found that a single 2D image of osteosarcoma cells contained 521 protein spots. By running an identical sample, we found that the system reproducibility was high and the intensity of more than 409 protein spots was scattered within a 2-fold range of differences between experiments, with a correlation coefficient of at least 0.60. We also found that a single 2D-PAGE run took 140 min, and that 3 gels could be run per day. We conclude that the Auto2D can be used for conventional protein expression studies. On the other hand, as the number of observed proteins was quite limited, and only three gels can be run per day per device, the Auto2D may not be suitable for a large-scale proteomic study.

**Keywords:** Two-dimensional gel electrophoresis; Automation; Proteome

## Introduction

2D-PAGE has been recognized as one of the most powerful proteomics modalities, having originally been reported almost 40 years ago [1,2]. In 2D-PAGE, intact proteins are separated in a gel matrix according to their isoelectric points and molecular weights, which are all unique to individual proteins. The separated proteins are then detected by colorimetric methods such as silver staining. Depending on the separation area, hundreds to thousands of proteins can be observed as individual spots in a single gel image. Intriguing proteins that may provide clues to the molecular backgrounds of diseases can be selected by integrating biological information from samples and protein expression data. These proteins can then be further examined for potential clinical applications. As a wide spectrum of crude protein samples can be subjected to 2D-PAGE without prior sample purification, 2D-PAGE is applicable to various research fields. One of unique advantages of 2D-PAGE is that it can separate intact proteins. Because of this advantage, 2D-PAGE was used for a pre-fractionation method for mass spectrometry [3] and the activity-based proteomics [4]. 2D-PAGE is still one of the most commonly employed proteomics techniques, despite the advent of more modern proteomics modalities such as gel free proteomics methods.

In the last four decades, innovative tools have been introduced into 2D-PAGE; immobiline gel in the first dimension separation for high reproducibility [5], fluorescent dye for protein labeling prior to electrophoresis for multiplex detection [6], and highly sensitive protein identification by mass spectrometry. These innovations have improved the performance of 2D-PAGE, and solved its inherent problems such as gel-to-gel variation, low sensitivity, and difficulty with protein identification.

In contrast to these advantageous characteristics and substantial innovations, 2D-PAGE has some intrinsic drawbacks; it consists of multiple steps which have to be done manually, and running 2D-PAGE

requires a degree of skill. 2D-DIGE partially solve this problem, as laborious gel staining is substituted by simple laser scanning, and the colorimetric gel staining step can be omitted. However, 2D-PAGE still requires multiple manual procedures, such as sample application to the first-dimension gel, equilibration of the isoelectric focusing gel, and application of the first-dimension gel to the second-dimension one. To bring out the best in 2D-PAGE, detailed protocols and proper instructions are required, which are not always available everywhere. Moreover, as the multiple steps require manual operations, any operator has to devote a lengthy period of time to each 2D-PAGE run. Improvements to these issues would make 2D-PAGE more applicable to conventional experiments.

One remedy for this situation would be automation of 2D-PAGE. In fact, an automated 2D-PAGE machine has been developed and commercialized by Shimadzu Co. (TEP-1, Shimadzu, Kyoto, Japan). The machine employed ampholine isoelectric focusing gel for the first-dimension separation, and vertical 2D-PAGE for the second-dimension separation [7,8]. However, it is now no longer available, probably because it was commercialized before the advent of proteomics. The Auto2D is a novel automated machine for 2D-PAGE, which was developed by Sharp Co. in 2011. It consists of three parts: 1) sample application by rehydration, 2) isoelectric focusing using IPG gel, and 3) horizontal SDS-PAGE. When a sample is applied to the machine,

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it automatically enters the first-dimension gel passively, and the constituent proteins are separated according to their isoelectric points. After equilibration, the first-dimension gel is set on the horizontal SDS-PAGE gel, and the proteins are separated according to molecular weight. As low-fluorescence plastic plates are used in the Auto2D, the technique is compatible with two-dimensional difference gel electrophoresis (2D-DIGE). Thus, the Auto2D combined with 2D-DIGE could be a solution for overcoming the inherent problems of 2D-PAGE. However, although more than 3 years has passed since the introduction of the Auto2D, no report has yet evaluated its performance or discussed its possible applications.

In the present study, we examined for the first time the performance of the Auto2D for protein expression studies by 2D-DIGE. We assessed the reproducibility, throughput, and comprehensiveness of the Auto2D, and discussed its limitations and possible utilities on the basis of proteome data.

## Materials and Methods

### Protein lysate

Protein lysates of osteosarcoma cell lines were used in this study. The lysates were prepared in the same way as in our previous study [9]. A lysate of HuO9 cells was used to examine the gel images of individual samples, and lysates of HuO9, HOS, MG-63, MNNG-HOS, 143B, HS-Os-1, NOS-1, and NOS-10 cells were used to create the common internal standard sample employed in this study. In brief, the monolayer cells were washed twice with ice-cold PBS, and fixed with 10% trichloroacetic acid for 30 min. The cells were then scraped off, and washed with PBS. They were then treated with protein lysis buffer (2M thiourea, 6M urea, 3% CHAPS, 1% Triton X-100) for 30 min. After centrifugation at 15,000 rpm for 30 min, the supernatant was recovered as a protein lysate.

### 2D-DIGE using the Auto2D

Protein expression profiling was performed using 2D-DIGE and the Auto2D. In brief, 1  $\mu$ g of each HuO9 protein lysate and the internal standard sample were labeled with Cy5 and Cy3 fluorescent dye (CyDy DIGE Fluor saturation dye, GE Biosciences, Uppsala, Sweden), respectively. After stopping the labeling reaction, the labeled samples were mixed, and made up to 10  $\mu$ l with lysis buffer containing 0.5% ampholyte (GE Biosciences) and 20 mM DTT. The sample was then applied to the Auto2D in accordance with the manufacturer's instructions. The running conditions were programmed as follows: sample application by the rehydration method, 35 min; first-dimension separation, 40 min; equilibration, 10 min; and second-dimension separation, 35 min. The equilibration buffer contained 475 mM Tris-HCl (pH6.6), 3.8% SDS, 47.5 mM DTT, 11.875% glycerol, 0.00475% BPB. The first dimension separation was done at 200V constantly for 5 min, gradually increased to 1000V for 5 min, constantly 1000V for 5 min, gradually increased to 7000V for 10 min, and constantly 7000V 15 min. The second dimension separation was done constantly at 400V for 35 min. The temperature was controlled at 20 degree for sample application, IEF and equilibration, and at 8 degree at the second dimension separation, SDS-PAGE. It took 2 hours from the sample application to the end of second dimension separation. The range of isoelectric focusing was between 4 and 7. After gel electrophoresis, the gel sandwiched between plastic plates (70  $\times$  70 mm) was scanned at an appropriate wavelength for Cy3 or Cy5 by a laser scanner (Typhoon Trio, GE Biosciences).

### Image analysis

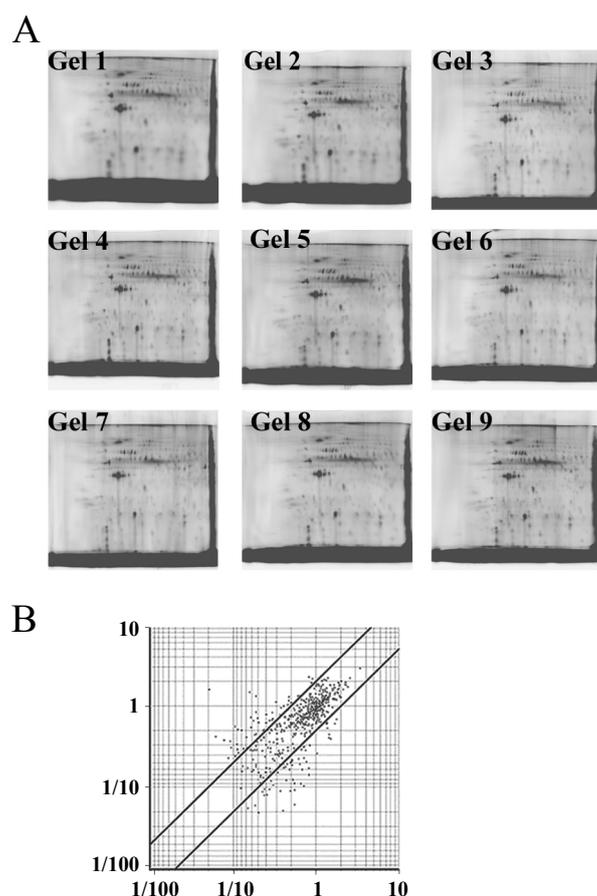
Calculation of protein intensity and standardization of Cy5 intensity with Cy3 intensity for all protein spots were achieved using the ProgenesisSameSpots software package version 3 (Nonlinear Dynamics, Newcastle-upon-Tyne, UK).

### Statistical analysis

System reproducibility was assessed using the scattergram method employing a data-mining software package (Expressionist, GeneData, Basel, Switzerland).

## Results and Discussion

We evaluated the performance of Auto2D from the viewpoints of reproducibility, throughput and comprehensiveness. Auto2D is compatible with 2D-DIGE, and we used the samples prepared for 2D-DIGE in this study. As we labeled the samples with highly sensitive fluorescent dye, only 1  $\mu$ g protein was enough to generate gel images. We found that 1  $\mu$ g protein was also enough for a large format 2D gel (data not shown), and the lower limitation of protein sample for Auto2D with 2D-DIGE remained to be investigated. It should be advantageous



**Figure 1:** 2D images and system reproducibility for the Auto2D. A. Cy5 images of HuO9 osteosarcoma cells obtained using the Auto2D. The gel size was 6.5  $\times$  3.9 cm, and the separation area was 3.0  $\times$  3.9 cm. Note that the protein spots were well focused and separated across the entire area. B. A typical scattergram of protein spots in gels 1 and 2. Among 521 protein spots observed, the intensity of 414 was scattered within a 2-fold range of differences. The correlation coefficient was 0.66 for all 521 protein spots, and 0.87 for the 414 protein spots.

if Auto2D can generate 2D images using sub microgram amount of protein samples, such as those obtained by laser microdissection or biopsy samples.

### Reproducibility

2D images of HuO9 osteosarcoma cells are shown in Figure 1A. The protein spots were well separated and focused within the entire area of the 2D gels. We ran an identical protein lysate of HuO9 nine times, compared the intensity between the gels for all protein spots, and assessed the reproducibility by creating scattergrams. Figure 1B shows a typical scattergram of protein spot intensities for gels 1 and 2. We compared all combinations for 9 runs and evaluated the variations among the runs. We observed 521 protein spots on the master gel image of the internal control sample, and the corresponding protein spots in the individual gel images were then evaluated. Among 521 protein spots, we found that the intensities of 409-450 spots, which accounted for 78.5-86.4% of the total, were scattered within a 2-fold range of

differences (Table 1). The relative correlation for all protein spots ranged from 0.53 to 0.83 (Table 2). When we focused on protein spots that showed within a 2-fold difference between two runs, the relative correlation efficiency was between 0.61 and 0.89 (Table 3).

Based on these observations, we concluded that the reproducibility of the Auto2D was quite acceptable for routine experiments. On the other hand, all procedures after sample injection was performed automatically in Auto2D, and we don't have idea to improve reproducibility. It is noteworthy that the reproducibility of 2D-PAGE may not be perfect, even without operator handling. In this study, the reproducibility may be affected by laser scanning, where the spot intensity can be saturated. These issues should be further investigated in the following studies.

### Throughput

It took us 120 min for 2D-DIGE sample preparation, 120 min for 2D-PAGE using the Auto2D, and 30 min for laser scanning to obtain

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8	Gel 9
Gel 1		414 (79.5)	427 (82.0)	415 (79.7)	415 (79.7)	427 (82.0)	430 (82.5)	440 (84.5)	415 (79.7)
Gel 2			417 (80.0)	409 (78.5)	405 (77.7)	422 (81.0)	425 (81.6)	422 (81.0)	450 (86.4)
Gel 3				436 (83.7)	416 (79.8)	432 (82.9)	426 (81.8)	422 (81.0)	412 (79.1)
Gel 4					420 (80.6)	426 (81.89)	426 (81.8)	450 (86.4)	429 (82.3)
Gel 5						441 (84.6)	429 (82.3)	432 (82.9)	425 (81.69)
Gel 6							426 (81.8)	442 (84.8)	419 (80.4)
Gel 7								428 (82.1)	427 (82.0)
Gel 8									445 (85.4)
Gel 9									

Percentage of the number of protein spots in a total number of protein spots observed in this study is described in parenthesis.

**Table 1:** The number of protein spots whose intensity ranged within 2 fold differences in two 2D-PAGE gels.

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8	Gel 9
Gel 1		0.6623	0.7085	0.6260	0.6008	0.6542	0.6337	0.7111	0.6504
Gel 2			0.6353	0.6093	0.6000	0.5918	0.6951	0.6857	0.7834
Gel 3				0.6103	0.5580	0.6363	0.5780	0.6417	0.6060
Gel 4					0.5273	0.5394	0.6079	0.6772	0.6206
Gel 5						0.5976	0.5937	0.5768	0.6213
Gel 6							0.8361	0.6155	0.6118
Gel 7								0.6560	0.6579
Gel 8									0.7033
Gel 9									

**Table 2:** The relative coefficient value of the intensity of 521 protein spots in two 2D-PAGE gels.

	Gel 1	Gel 2	Gel 3	Gel 4	Gel 5	Gel 6	Gel 7	Gel 8	Gel 9
Gel 1		0.8690	0.8891	0.8671	0.8503	0.8627	0.8714	0.8680	0.8563
Gel 2			0.8760	0.6093	0.8312	0.8375	0.8718	0.8509	0.8973
Gel 3				0.8538	0.8441	0.8538	0.8392	0.8443	0.8524
Gel 4					0.8112	0.8242	0.8405	0.8517	0.8299
Gel 5						0.8286	0.8576	0.8371	0.8123
Gel 6							0.8361	0.8333	0.8326
Gel 7								0.8491	0.8459
Gel 8									0.8407
Gel 9									

**Table 3:** The relative coefficient value of the intensity of protein spots whose intensity was scattered within 2 fold differences in two 2D-PAGE gels.

2 images. While the most of times are latency time, it took us 270 min to obtain 2D gel images. When multiple gels are used to examine multiple samples, the Auto2D may not have such a high throughput. By overlapping the experiments, we ran 2D gels 3 times per day in the working time available, and it took us 3 days to run 9 gels. Parallel use of multiple Auto2D devices may improve the throughput. However, because of its high price, this idea is not practical. In contrast, the price of conventional devices for 2D-PAGE is relatively low, and it is more realistic to run multiple samples using multiple low-cost classical 2D-PAGE devices.

We conclude that the Auto2D may not be applicable to studies in which large numbers of samples need to be examined. The development of automated 2D-PAGE device for simultaneous running multiple samples is desired to improve the throughput.

### Comprehensiveness

In this study, we observed 521 protein spots by Auto2D. Although the number of protein spots may vary depending on the type of protein sample, it may not exceed 1,000 according to the 2D images (Figure 1A). Thus, the Auto2D may not be used for serious global protein expression studies. This limitation is a stark contrast to the performance of 2D-PAGE with a large-format gel apparatus. When we used the same protein lysate of HuO9 cells in our original large-format electrophoresis device, which has a 24 x 32-cm separation area [10], we observed 4762 protein spots [9]. This may be because the number of protein spots is proportional to the separation area of the 2D gel.

To increase the number of protein spots, we may be able to attempt the combined use of IPG gels with different pI ranges [11,12]. There are several types of narrow-range IPG gel for the Auto2D, and these have different pI ranges, covering pH 4-5.5, 5-6.5, 6-10, and 7-10, and thus it is worth applying those gels to obtain a greater number of protein spots. Alternatively, protein fractionation prior to gel electrophoresis may be worth trying to increase the number of protein spots. The number of protein spots observed using multiple narrow-range IPG gels and pre-fractionation should be evaluated using real samples. It should be noted that the combined use of multiple pI gel formats and pre-fractionation of protein samples will increase the number of 2D gels required for analysis, and thus more time will be required in order to complete a series of experiments.

### Conclusions: Possible Utility of the Auto2D

Automation of 2D-PAGE procedures would seem attractive for researchers who do not have experience of running 2D-PAGE. The system reproducibility of the Auto2D with 2D-DIGE is quite high, and as it does not require previous 2D-PAGE experience, it will be applicable for routine experiments. It is also noteworthy that the operation time for the Auto2D is considerably short, i.e. about 2 hour per run. As long as the target proteins are included in the 2D image,

the Auto2D can be used for expression studies. On the other hand, as the number of protein spots is obviously small, the Auto2D may not be used for global protein expression studies. One possible application of the Auto2D may be protein fractionation prior to mass spectrometry, two-dimensional separation for Western blotting, quality examination of protein products, and development of methods for protein pre-fractionation and gel staining. These possibilities should be examined in further studies.

### Conflict of Interest

This study was performed through collaboration between the National Cancer Center and Sharp Co., and the Auto2D device and reagents were provided by Sharp.

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