Implementation of an Automated High-Throughput Plasmid DNA Production Pipeline

Karen Billeci1, Christopher Suh2, Tina Di Ioia1, Lovejit Singh1, Ryan Abraham1, Anne Baldwin1, and Stephen Monteclaro1

Abstract
Biologics sample management facilities are often responsible for a diversity of large-molecule reagent types, such as DNA, RNAi, and protein libraries. Historically, the management of large molecules was dispersed into multiple laboratories. As methodologies to support pathway discovery, antibody discovery, and protein production have become high throughput, the implementation of automation and centralized inventory management tools has become important. To this end, to improve sample tracking, throughput, and accuracy, we have implemented a module-based automation system integrated into inventory management software using multiple platforms (Hamilton, Hudson, Dynamic Devices, and Brooks). Here we describe the implementation of these systems with a focus on high-throughput plasmid DNA production management.

Keywords
compound management, laboratory design, robotics and instrumentation, workflow

Introduction
The high-throughput management of biologics is becoming increasingly important in the drug discovery process. There is therefore a need to develop sample management strategies and infrastructure akin to that developed for compound management and small-molecule drug discovery.1 To this end, we have integrated informatics (registration, inventory management, and request systems) and automation to allow for the request, archive, production, and supply of DNA, RNAi, and proteins to support multiple areas of research, including pathway discovery, antibody discovery, and protein chemistry.

The management of proteins and siRNA from a centralized repository is very similar to compound management workflows established by many institutions, although throughput requirements are generally lower. A typical workflow is that siRNA or protein is synthesized or expressed in large batches or lots. The sample is then aliquoted, diluted, and distributed using standard lab automation equipment. The regeneration of new material is done in a specialized synthesis group outside of the sample management. The management of plasmid-based reagents such as DNA constructs, shRNA, or CRISPR is more complex in that samples can be continuously remade from a source stock such as transformed Escherichia coli (typically stored as glycerol stocks) or as purified plasmid. In a typical molecular biology lab, the manual process of regenerating stocks of purified plasmid for a small number of samples is manageable. However, for large-molecule discovery, such efforts as pathway discovery via expression cloning and antibody discovery have become more miniaturized, and thus throughput has increased dramatically.2 Therefore, the need to automate plasmid production has become important. Here we describe the automation of each step in the plasmid production process from storage/retrieval through basic molecular biology techniques such as transformation, culture media inoculation, colony picking, culture expansion, and plasmid purification. We have taken a modular approach in which each step uses robotics sourced from various vendors that are best suited for the process.

Methods and Materials
All sample management and liquid handling steps were modeled on a standard manual process but optimized to the configuration of the automated platform. SBS format
labware, eight-well agar plates (Teknova, Hollister, CA, L2418), deep-well culture blocks (Costar, Fischer Scientific, Pittsburgh, PA, 3960), and UV plates (Thermo, Thermo Scientific, Waltham, MA, 8404) were used in most experiments and on all platforms unless otherwise noted.

**Plasmid and E. coli Stock Storage and Management**

Plasmids that were used for development and validation of each automation platform are representative of those processed routinely in the lab. Typical plasmids contain inserts with sizes up to 3000 base pair subcloned into the pRK5.smt mammalian expression vector (Genentech, South San Francisco, CA). Plasmids were tracked (location, freeze/thaw, etc.) using Mosaic software (Titan, Westborough, MA), integrated to Brooks automated stores (Brooks Universal Store US-500B and US-450L). Plasmids were stored in REMP tubes (Brooks, Chelmsford, MA) as either purified DNA, at −20 °C, or as glycerol stocks (transformed E. coli suspended in 40% glycerol), at −80 °C. E. coli host cells used in all experiments were generated in-house following a modified manual method. The stability/viability of transformed E. coli stored as glycerol stocks was determined by subjecting sample stored as either 25% or 40% glycerol stocks to multiple freeze/thaw cycles. After zero (control), 5, and 10 freeze/thaw cycles, 2 μL of the glycerol stock was inoculated onto 10 cm agar plates supplemented with antibiotics and incubated overnight at 37 °C. Colonies were then counted and compared to the control.

**Hamilton Microlab STAR Instrument Setup**

A Hamilton Microlab STAR instrument equipped with a 1 mL 96-CORE pipetting head and 12 independent variable span 1 mL channels was set up for automated transformation. The system has a modular deck that is 54 tracks wide, where carriers for all types of labware can be loaded (plates, reservoirs, tips, etc.). Additionally, one Hamilton Heater Shaker and one Hamilton Plate Chiller unit are installed (static) on the deck. Labware can be moved or plates can be lidded/de-lidded with the iSWAP gripper arm or the CORE-grippers. Barcodes can be read by the autoloader when carriers are loaded on the deck or presented to the barcode scanner with the iSWAP gripper arm.

**Automated Transformation**

The basic manual method for transformation is well established. The transformation protocol was modeled on a typical manual method. The automation of transformation was carried out on the Hamilton Microlab STAR instrument described above. All methods were built using Venus Two software. In short, 2–10 μL of plasmid DNA (10–100 ng/μL) was mixed with 10–20 μL of competent cells in a 200 μL 96-well plate and incubated at the chilled position on the deck for 30 min. The competent cell mixture plate was then moved to a heated position and heat shocked at 42 °C for 1 min. Finally, 5 μL of heat shocked/transformed cells was plated onto an eight-well agar dish supplemented with the appropriate antibiotic. Plated agar dish plates were manually moved to a 37 °C incubator and incubated for 18 h until colonies formed.

**Hudson RapidPick Complete System Setup**

The Hudson RapidPick Complete system was equipped with eight labware stackers (maximum capacity of 72 deep-well destination culture blocks), a plate crane for moving labware around the deck, the RapidPick, a plate sealer, and a Macro10× media dispenser. The system can pick ~40 colonies per minute, making a full run of 72 × 96 deep-well destination blocks in just under 3 h. The Hudson RapidPick Complete system was controlled with SoftLinx scheduling software.

**Automated Colony Picking**

Automated colony picking was performed on a Hudson RapidPick Complete system. The input plates (SBS format omni-trays, segmented colony plates, petri dishes, etc.) were moved from the source racks one at a time and imaged by the RapidPick. The colonies were automatically isolated based on defined parameters. Deep-well culture blocks were filled with sterile growth media and then moved to the RapidPick, where colonies were picked with tungsten tips and blocks were inoculated. After each inoculation step, each tip was sterilized and reused. Inoculated deep-well culture blocks were then moved to a 37 °C shaker and incubated for 18 h.

**Dynamic Devices Oasis Instrument Setup**

To obtain the required throughput of 24,000 samples per month or, on average, twelve to fourteen 96-well blocks per day, a custom system was designed using Oasis (LM900, Dynamic Devices) as the core platform. Other off-the-shelf technologies were not evaluated because throughput was not sufficient or resulting plasmids were not suitable for intended downstream applications. The final system is made up of three Oasis platforms that are integrated via a robotic arm (Precise) on a rail. Oasis 1 was used for sample preparation and had 29 positions, including three automatic buffer filling reservoirs. Oasis 2 and 3 each had 29 positions that were used for sample purification. Oasis 2 and 3 each also had two automatic buffer filling reservoirs, two vacuum stations for tip drying, and two positions modified with pins to hold stacked plates. Two pumps located under the integrated platform replenish buffer in the reservoirs equipped with automatic liquid detecting sensors, while two regulators control the vacuum from a house source line. A hotel (Liconic, Woburn, MA) capable of holding UV
Automated Plasmid DNA Purification

DNA purification employed PhyNexus PhyTip (San Jose, CA) column technology as described in detail in Suh et al. In short, 150 µL of resuspension buffer was added to the cells, followed by an 89-cycle mixing step to resuspend the cells. Lysis buffer (180 µL) was added to suspended cell with seven mixing cycles to lyse. Genomic DNA was precipitated by adding 210 µL of precipitation buffer, followed by two cycles of slow air dispense mixing (300 and 200 µL). PhyTip columns were first equilibrated in water with two mix cycles of 450 µL volumes. Plasmid DNA was bound to the silica column by 12 mix cycles, processing 230 µL of lysed, precipitated cells through the column. Bound plasmid DNA was washed by three mix cycles of 450 µL wash buffer twice. The PhyTip columns, with bound plasmid DNA, were dried for 15 min on a drying station to evaporate ethanol carried over from the wash buffer. Following drying of the resin, plasmid DNA was eluted by aspirating 210 µL of elution buffer, pausing 5 min, and dispensing the volume. The final eluted volume ranged from 100 to 120 µL. The proof of concept work was performed using a PhyNexus AutoPlasmid MEA, which can run up to 36 samples per robotic setup.

Plasmid DNA Quality Determination

The PhyNexus PhyTip column purification method was validated to ensure that DNA quality was better than or as good as our manual method (Qiagen, Valencia, CA, 27193) using standard criteria such as A280 nm/A260 nm, quality of sequencing (read length and Phred scores), and transfection efficiency. Transfection efficiency was evaluated using a plasmid that expressed green fluorescent protein (GFP). In short, COS7 cells were transfected with plasmid prepared following the PhyNexus PhyTip column protocol or a vacuum plate-based method that served as the control. Three different lipid-based transfection reagents were used according to the manufacturer’s protocol: Fugene 6, Fugene HD, and TransIT LT1. Transfection efficiency was determined via fluorescence by imaging of the cells on an IncuCyte (Essen Bioscience, Ann Arbor, MI) after incubation for either 24 or 48 h.

Plasmid DNA Stability Testing

When validating the high-throughput purification platform, the stability of eluted DNA, cell pellets, and cell lysates was determined using E. coli transformed with test plasmid inoculated into multiple 96-well blocks. After an 18 h incubation, cultures were harvested and the resulting cell pellets were stored at −20 °C. To determine the stability of eluted purified plasmid, a single block was processed. The yield, volume, and purity were determined at two time points: immediately and after sitting on the robotic platform for 7 h. The stability of cell pellets was determined by first leaving one block at room temperature overnight on the robotics platform, while a second block was thawed just prior to processing simultaneously with the first block. Yield and purity were compared between the two blocks. Finally, stability of the cell lysate was determined by preparing a set of samples through the precipitation step and leaving the block on the robotic deck for 7 h prior to processing, while the second set of samples was prepared just prior to purification and processed simultaneously with the first set of samples. Again, yield and purity were compared.

Plasmid DNA Handling and Normalization

Once DNA was purified, it went through further processing, such as concentration normalization, pooling, and preparing assay-ready plates (sequencing or transfection) on the Hamilton Microlab STAR. When normalizing a set of DNA samples (in a 96-well UV plate), the A260 nm/A280 nm absorbance reading was used to calculate the concentration and volume of each well. A target concentration was determined for each plate. A macro then imported the absorbance data file (concentration and volume in each well), calculated the amount of buffer needed to normalize each well to that target concentration, and then exported a .CSV file. The maximum capacity of each UV plate well is 300 µL, and when normalizing a plate of samples to a low target concentration, some wells might exceed this volume if only buffer is added to the starting high concentration material. The macro also calculated a volume of neat material that needs to be removed, so the final well volume is 300 µL at the target concentration. The Hamilton Microlab STAR uses the .CSV file generated by the normalization macro to aspirate from the wells that need starting material to be removed, and then adds buffer to each well to achieve the target concentration across all the wells. Both the macro and the Hamilton Microlab STAR method can process eight 96-well plates at a time. After the plates are normalized, the material is either diluted further by using the 96-CORE head to stamp the plate using a fold dilution or directly stamped into 384-well assay-ready plates.

Results

Viability of E. coli Stocks after Multiple Freeze/Thaw Cycles

When managing large collections of plasmid-based reagents, it is important to determine best practices for storing and handling these reagents. Plasmid DNA is typically
stored as purified material or as transformed *E. coli* suspended in 15%-50% glycerol. Glycerol stocks are routinely used in molecular biology labs as working stocks and are convenient source material in a high-throughput laboratory. Typically, glycerol stocks are stored at −80 °C or in liquid nitrogen. A "stab" of the frozen material is taken to initiate a new culture. It is impractical in a high-throughput setting to ensure that samples remain frozen during processing, and it is difficult to automate stab pipetting. Therefore, we wanted to determine if glycerol stocks could be subjected to multiple freeze/thaw cycles and still retain viability of the transformed *E. coli*. To determine this, an experiment was conducted in which transformed *E. coli* were stored in 25% and 40% glycerol and then subjected to multiple freeze/thaw cycles. As shown in Figure 1, transformed *E. coli* stored in 25% glycerol lost nearly 50% viability after 5 freeze/thaw cycles and retained less than 20% viability after 10 cycles. Transformed *E. coli* stored in 40% glycerol maintained viability after 5 freeze/thaw cycles, with only about a 10% loss in viability after 10 cycles. Thus, we limit freeze/thaw cycles (tracked via Mosaic software) to 5 cycles, and transformed *E. coli* was stored in 40% glycerol for our process.

Automated Transformation

The manual transformation is a well-established process consisting of five basic steps:

Step 1: Plasmid DNA is mixed with competent cells and incubated on ice for 30 min.

Step 2: The competent cell mixture is subjected to heat shock at 42 °C for 1 min to allow the cell to take up the plasmid DNA.

Step 3: The cells are allowed to recover, on ice, for 2 min.

Step 4: The cells are diluted with medium and incubated at 37 °C with shaking for 1 h to promote cell growth and division.

Step 5: The cells are then plated onto agar plates supplemented with the appropriate antibiotic to generate single colonies.

To automate the transformation process, a number of steps within the manual method were characterized to make the method more suitable for automation. These variables include pipetting volume, range of plasmid DNA concentration, the requirement of the recovery step after heat shock, and the inoculation volume. Each of these variables was studied in a series of experiments.

For step 1 of the manual method, plasmid DNA concentration, plasmid DNA volume, and competent cell volumes were varied to determine the best condition for step 1 of the transformation. Tables 1 and 2 summarize the effect of DNA concentration on transformations. As shown from Tables 1 and 2, plasmid DNA samples with concentrations between 10 and 100 ng/µL generally yield “ideal” colony numbers and density. However, most DNA constructs in our collection range in concentration from 40 to 500 ng/µL, and thus all samples went through 1:4 dilution prior to transformation. This had the added benefit of increasing the sample volume and in turn minimizing dead volume, thus making automation more robust. We tested three different plasmid DNA volume additions and three different competent cell volumes, as shown in Table 3. We were concerned that the Hamilton Microlab STAR instrument could not consistently pipette 2 µL of DNA. When pipetting small volumes, droplets can form on the pipette tip without releasing into the destination tube/well. In addition, without careful positioning of the pipette head, small-volume droplets would not passively combine with the competent cells; droplets would simply adhere to the side of the tube/well. By increasing the volume to 6 µL, the droplets were heavy enough to consistently fall into the destination well, thus avoiding the need

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<th>Table 1. Effect of DNA Concentration on Transformation Efficiency.</th>
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Three test plasmids were diluted to 10, 100, and 500 ng/µL and transformed manually. The resulting colonies per 10 cm dish were determined.

- Step 1: Plasmid DNA is mixed with competent cells and incubated on ice for 30 min.
- Step 2: The competent cell mixture is subjected to heat shock at 42 °C for 1 min to allow the cell to take up the plasmid DNA.
- Step 3: The cells are allowed to recover, on ice, for 2 min.
- Step 4: The cells are diluted with medium and incubated at 37 °C with shaking for 1 h to promote cell growth and division.
- Step 5: The cells are then plated onto agar plates supplemented with the appropriate antibiotic to generate single colonies.
to manually manipulate the destination labware. We also needed to increase the competent cell volume to 20 µL to ensure that sufficient dead volume was available to automate pipetting. As shown in Table 3, the best results are achieved using 6 µL of plasmid DNA with 20 µL of competent cells, as described above.

Step 2, the heat shock, was readily automatable using heated deck positions on the liquid handling robotic platform. However, steps 3–5 would require significant modification in order to automate. In manual method steps 3 and 4, the heat shocked sample was recovered on ice and then diluted with medium, followed by 1 h of incubation at 37 °C. For ease of automating the entire transformation process, we tested to see if skipping transformation steps 3 and 4 was possible. We found that skipping these steps did not significantly affect the colony size or density (data not shown). Finally, in step 5, heat shocked cells were plated on eight-well dishes and then incubated. We determined that

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Forty test plasmids ranging in concentration from 9 to 684 ng/µL were transformed manually and evaluated for density of colony formation. Highly dense = no individual distinct colonies; dense = some distinct colonies; ideal = distinct colonies; poor = <20 colonies.
increasing the inoculation volume from 2 µL to 5 µL made
the automation method more reproducible (data not shown).

As the automated method was implemented, final consider-
ations were to ensure that there was no cross-contamination of
the agar plates. As shown in the “preoptimization” panel of
Figure 2, contamination was observed in the negative con-
trol wells. To eliminate cross-contamination, combined liq-
uid class definitions, pipetting heights, robot movement
speeds, and aspiration times were varied to eliminate cross-
contamination. In addition, the pipetting strategy was con-
figured to be as rapid as possible with successful inoculation.
The results of the optimization are shown in the “after opti-
mization” panel of Figure 2. No colony growth appeared in
negative control wells.

Following the implementation of the final transforma-
tion protocol, 480 transformations were performed over
several weeks. The transformation efficiency was success-
ful at a rate of 94%. Of the 27 failures, 20 were successful
when the transformation was repeated. For the final seven
failures, the transformation failures were determined to be
inherent to the plasmid and not a failure of our process (data
not shown).

Automated E. coli Culture for Plasmid DNA
Generation

Once the transformation process was complete, to prepare
sufficient quantities of plasmid DNA for downstream pro-
cesses, a single colony was picked and inoculated into fresh
medium. The manual process of colony picking uses a
wooden toothpick to pick a specific colony from an agar
media plate. The toothpick is then dropped into the well of
a 96-well culture block. The process is repeated up to 95
times from a single or multiple agar wells to complete inoc-
ulation of the deep-well block. The block is sealed with a
semipermeable filter and placed in an incubated shaker at
37 °C for 16–20 h.

The manual process is time-consuming, physically dif-
cult, and error-prone, especially as sample throughput
increases. An automated colony picker was configured as
an integrated, enclosed automated platform. To ensure pick-
ing efficiency (99% success rate), optimization of both the
transformation efficiency and imaging is required. Transfor-
mation efficiency must be such that colonies are dense, but
individually distinguishable. Imaging parameters must also
be optimized such that the majority of colonies are in focus
regardless of slight differences in agar height, colony diam-
eter, and color. Optimal colony density was optimized on
the Hamilton Microlab STAR. The imaging parameter was
optimized by trial and error until consistently focused
images were achieved over multiple transformation experi-
ments. Unfortunately, the colony picker needs to be opti-
mized from the default setting by trial and error by varying
the parameters mentioned. Once optimized, the colony
picker does not need to be optimized again. Without going
through the optimization process, only 10% of the picks
were successful as measured by successful bacterial growth.

With optimization, the colony picker efficiency improved to
99%. Figure 3 shows an image of an agar plate for which
the colony density and imaging parameter are ideal. Even
though the top right and bottom right quadrants are slightly
denser, the colony picker was able to successfully pick from
these quadrants without any issues. Finally, it should be
noted that additional optimization of culture time (increased
by 2 h) was required with implementation of automated
colony picking.

Automated Plasmid DNA Purification

The ability to produce high-quality DNA in a high-throughput
manner has become an important step in the large-molecule
drug discovery process. Automation of this process in high-
throughput platforms can dramatically increase throughput
and accuracy and reduce ergonomic stress. Automation of
DNA production is challenging because of the diversity of
yield requirements. We decided to focus on miniscale purifi-
cation, as the 96-well format for many methodologies is
adaptable to many lab automation platforms.

Historically, DNA production at the miniscale was based
on a 96-well format semiautomated vacuum-driven chromo-
matography method using silica membranes in a plate for-
mate. As can be seen in Figure 4, the variation of the sample
pellet size made it difficult to transfer the supernatant con-
taining plasmid DNA from the processing wells to the
membrane. Also, due to varying amounts of particulate
matter, liquids would not process through the plate in a pre-
dictable manner (Fig. 4). Manual monitoring and frequent
intervention to attempt to “rescue” samples were necessary.
Finally, the vacuum-driven process of the plates appeared to
increase cross-contamination, presumably via aerosols. In
order for the technology to be adapted to our process, the
following criteria had to be met: the resulting purified DNA
(1) was sequencing and transfection grade, (2) had to be
compatible with standard lab automation liquid handling
equipment, (3) had to be capable of starting from frozen and
then be thawed or freshly harvested on a cell pellet, (4) had

Table 3. Determination of Plasmid DNA and Competent Cell
Volume.

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<td>&lt;5</td>
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</tr>
<tr>
<td>40</td>
<td>8</td>
<td>&gt;100</td>
<td>76</td>
<td>40</td>
</tr>
</tbody>
</table>

Three different plasmid DNA volumes were tested with three different
volumes of competent cells.
to employ absolutely no hands-on processing, and (5) had to be scalable to our lab throughput of 24,000 samples per month.

The solution for completely walkaway automation was changing the purification process from vacuum-driven silica membrane filters to pipette tip columns. The pipette tip columns used for the purification are shown in Figure 5. The PhyNexus Lysate Direct technology using PhyTip columns was chosen for several reasons. The PhyTip technology had been successfully implemented for protein purification in multiple groups at Genentech. The tip concentrating effect of the column resulted in good recoveries even from limited sample. The column style is very compatible with automated robotic liquid handlers. The plasmid purification is based on chaotropic silica chemistry with modifications to ensure transfection quality plasmid. Further details of the technology are described elsewhere.6

Initial development of the pipette tip column for plasmid purification work used a 200 µL pipette tip column body on a Dynamic Devices Oasis system with a 96-channel 200 µL head. With the eventual availability of a 96-channel head capable of processing 1 mL volumes, the pipette tip column was further developed and configured based on the 1 mL

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**Figure 2.** Effect of transformation. Eight-well agar plates were used to monitor colony formation when optimizing liquid handling operations and movements. Alternating wells were plated with 5 µL of a transformation mixture using automation. Top plate: The preoptimization wells indicate cross-contamination in the negative control wells. Bottom plate: No colony growth was detected in negative control wells after optimization.

**Figure 3.** Optimization of colony picking. The colorized image shows ideal colony density and the setting of imaging parameters after optimization.
pipette tip. Initially, characterization work for the 1 mL column was performed on the MEA. The PhyNexus method was characterized for yield, purity, and suitability in downstream application such as transient transfection and sequencing.

As shown in Figure 6, plasmid DNA purified from the pipette tip columns using this automation was comparable to that of current manual methods. Yields met the requirement of ≥5 µg. For cultures grown in the 96-well format, the yield is routinely in the range of 5–10 µg. DNA quality was assessed by the ratio of absorbance at A260 nm/A280 nm. This is an indication of purity of DNA; a value of ≥1.8 is acceptable. DNA purity was assessed by the ratio of absorbance at A260 nm/A230 nm. This is an indication of buffer constituent contamination, and a value of ≥1.6 is acceptable. From these data, the 1 mL pipette column body with an 80 µL bed volume PhyTip column (Lysate Direct column) was chosen for final development, as experiments suggested that the liquid dynamics in the larger column improved the purity of the recovered plasmid.

Although standard culture conditions had been implemented, we still wanted to ensure that the purification method could handle pellets from cultures that were overgrown or pellets that were highly compact. Plasmid DNA was purified using a manual vacuum-driven method and the automated Lysate Direct PhyTip columns. The manual minipreps were chosen as a control, as this was the established protocol in the group. The A260 nm/A280 nm and A260 nm/A230 nm ratios observed for all conditions were between 1.8 and 2.0. As shown in Figure 6, the plasmid DNA yield and purity compared well to the manual process, and all results were within an acceptable range.
yields > 5 µg, A260 nm/A280 nm and A260 nm/A230 nm ratios > 1.8. However, the automated process did not yield as much DNA from overgrown cultures (Fig. 7). As the method began to more fully implement the automated purification method, we frequently saw variability in the A260 nm/A230 nm ratio. We wanted to determine how critical this ratio was in downstream applications.

**Plasmid DNA Quality, Sequencing, and Transient Transfection**

Although yield and purity are good indicators of plasmid DNA quality, it is also important to evaluate the DNA in downstream processes such as sequencing and transient transfection. Sequence quality was indicated by the read length and Phred scores. The maximum base pair read using

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**Figure 5.** Pipette tip column for automated plasmid purification. (A) A pipette tip column. A thin frit screen at the bottom of the pipette tip column holds the resin in place. (B) Columns are configured to be used with a robotic liquid handler equipped with a 96-channel head.

**Figure 6.** Comparison of plasmid purification technologies. Identical pellets were purified using the manual vacuum-based method and two prototype automated pipette tip columns (200 µL and 1 mL). Yields were measured using a UV plate reader.
Sanger sequencing was between 800 and 1000 bp, so a read length within this range is considered good. Phred scores greater than 50 suggest an accurate call of each base of 99.999%. DNA purified using our automated method resulted in an average read length of greater than 800 bases of the purified DNA for 96 different DNA constructs, and the Phred scores were 55.

Transient transfection efficiency was evaluated using a plasmid expressing GFP, following a forward or reverse transfection approach with three transfection lipid-based kits: Fugene 6, Fugene HD, and TransIT LT1. Transfection efficiency was measured by imaging transfected COS7 cells and counting the number of green cells per condition. The results shown in Figure 8 are representative of multiple transfection experiments in which we compared the transfection efficiency of plasmid DNA purified using the manual and automated methods. In general, the transfection efficiency was comparable between the two methods.

As the automated PhyTip column method was further optimized, some variability in the A260 nm/A230 nm ratio was observed. As it was quite challenging to optimize the conditions to ensure this ratio was above 1.8, we wanted to determine how ratios below the ideal specification would affect transfection efficiency. Plasmid DNA prepared on multiple days with A260 nm/A230 nm ratios of <1.0 to >2.0 was transfected into COS7 using Fugene HD following a forward transfection protocol. The transfected cells were imaged at 24 and 48 h, and the number of transfected cells were counted for each condition. The results from this experiment are shown in Figure 9; transfection efficiency drops off at A260 nm/A230 nm ratios of 1.5/1.6. We therefore concluded that special attention should be paid to this ratio when optimizing the final method on the custom system.

![Figure 7. Plasmid prep robustness testing. To simulate the variety of cell pellets received by the Biologics Resource Management facility, 96-well blocks were inoculated and harvested under three conditions: optimal conditions (ideal), overgrown (overgrown), or such that pellets were highly compact and dense (compact). Samples were then purified via the manual vacuum-based method or using the automated system employing Lysate Direct PhyTip columns. Yields and purity were determined using a UV plate reader.](image1)

![Figure 8. Transient transfection. Plasmids cloned with GFP were purified by the manual vacuum-driven method and, alternatively, the Lysate Direct PhyTip columns. The purified plasmid was then transfected into COS7 via forward or reverse transfection using either Fugene 6, Fugene HD, or TransIT LT1. After 24 h, the number of green cells were counted.](image2)
Design and Implementation of Automated Workflow

The design of the final Dynamic Devices platform is shown in Figure 10. One limitation of the PhyNexus method is the robotic head’s inability to multitask while it is occupied with tips. This made scheduling difficult. Initially, consideration was made to configure the three platforms as independent systems such that sample prep and purifications would be performed independently on each platform. Analysis of the throughput using independent platforms versus specialized platforms showed that the specialized platform was more efficient. Scheduling efficiency was gained by allowing the sample preparation platform to multitask sample prep while purification was conducted on platforms 2 and 3. Additional efficiency was gained by initiating a second purification on each platform during the drying phase of the first purification, in which the pipetting head could be disengaged. Capacity per setup was maximized by loading the first 4 blocks per setup directly on the deck, while the remaining 12 were in the hotel. The purification columns are also loaded directly on the deck. The final design yielded a system with a throughput of sixteen 96-well plates in 7 h.

Typically, when purifying plasmid DNA manually, there is very little delay between each step in the process. On an automation platform, samples may sit on the deck or hotel throughout the robotic run. Therefore, we wanted to determine the stability of purified plasmid, the cell pellets, and the sample lysate at room temperature for 7–16 h, the maximum time a sample may sit on the platform before the sample is processed or removed from the platform. Table 4 summarizes the results from these experiments. In all three experiments, purity was not affected by delayed purification or yield determination. Some differences in yield were observed when pellets or lysate was allowed to sit for 7–16 h on the platform prior to purification. Interestingly and unexpectedly, yield and concentration tended to improve by as much as 40%. Buffer evaporation was observed for the eluted purified plasmid plates after 16 h, but yield was unaffected.

Once DNA is purified, it either is used as is or goes through further processing, such as concentration normalization and preparation of assay-ready plates using the Hamilton Microlab STAR. Given that support is needed for the diversity of downstream assays that require different concentrations and plate formats, Hamilton methods are written with logic that allows the user to select only certain steps in the protocol, volumes, and the number of destination plates. Figure 11 is an example of the logic used to program a method.

Conclusion

The large-molecule drug discovery process is increasingly becoming higher throughput. As was accomplished in...
small-molecule drug discovery and compound management, automation plays a key role in the success of managing large numbers of samples. The management of plasmid-based reagents is particularly challenging and unique. Unlike compound management where the samples are managed via dilution and liquid transfers of bulk synthesized materials, plasmid-based reagents are regenerated/expanded as requested within the sample management facility. Here we have demonstrated the successful implementation of modular automation platforms to support transformation, colony picking, and purification.

### Table 4. Robustness of Robotics Platform: Sample Stability

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Immediate</th>
<th>16 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (µg)</td>
<td>6.5 ± 1.2</td>
<td>5.6 ± 1.2</td>
</tr>
<tr>
<td>Concentration (ng/µL)</td>
<td>55 ± 11</td>
<td>72 ± 14.2</td>
</tr>
<tr>
<td>Volume (µL)</td>
<td>118 ± 8.0</td>
<td>78 ± 7.6</td>
</tr>
<tr>
<td>A260/A280</td>
<td>1.8 ± 0.1</td>
<td>1.8 ± 0.1</td>
</tr>
<tr>
<td>A260/A230</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>Cell pellet</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (µg)</td>
<td>6.4 ± 1</td>
<td>7.4 ± 1</td>
</tr>
<tr>
<td>Concentration (ng/µL)</td>
<td>50 ± 8.1</td>
<td>59 ± 7.8</td>
</tr>
<tr>
<td>A260/A280</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>A260/A230</td>
<td>2.0 ± 0.1</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (µg)</td>
<td>6.2 ± 1</td>
<td>8.7 ± 1.2</td>
</tr>
<tr>
<td>Concentration (ng/µL)</td>
<td>51 ± 7.2</td>
<td>70 ± 8.9</td>
</tr>
<tr>
<td>A260/A280</td>
<td>1.9 ± 0.1</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>A260/A230</td>
<td>1.9 ± 0.1</td>
<td>2.23 ± 0.1</td>
</tr>
</tbody>
</table>

The stability of cell pellets, lysed cells, and purified plasmid DNA was tested at two different time points (immediately and after 16 h) and compared for yield, volume, and purity.
Billeci et al.

DNA purification, and preparation of assay-ready plates for sequencing or transfection.

Implementation of an automated high-throughput plasmid DNA production pipeline was achieved through optimization of several modules. Storage of plasmid DNA is most stable stored as purified DNA. However, it is more practical to initiate a large number of cultures from transformed *E. coli* stored as glycerol stocks. To manage glycerol stocks via automation, the ability to subject the sample to multiple freeze/thaw cycles is required. We have found that storage of transformed *E. coli* requires at least 40% glycerol to maintain viability through five freeze/thaw cycles.

Transformations when viewed for automation suitability are a simple process in that small volumes of sample are combined and transferred to various destination labware. There are, however, various steps that are challenging to automate. The transformations were simplified by eliminating the recovery step that required an incubation on ice and a second incubation with shaking at 37 °C. In addition,
pipetting volumes were increased to improve accuracy and characterized the range of concentration that would yield ideal colony density. Finally, liquid handling was optimized by carefully defining liquid classes and robotic movements minimizing contamination.

Optimization of colony picking was performed through trial and error until imaging parameters were set to accommodate different agar heights, colony sizes, and color.

The automation of plasmid purification at the miniscale was implemented on a customized system that allows the purification of up to sixteen 96-well blocks per setup. The method was validated to ensure that the resulting purified material was suitable in downstream assays, such as sequencing, or methods that require transient transfection. Interestingly, lysates, cell pellets, and eluted DNA were very stable at room temperature; thus, we could confidently set up robotic runs up to 16 h.

Finally, automation was put in place to support the generation of assay-ready plates. The methods developed were optimized and streamlined to allow flexibility and rapid adaptability to changing downstream assay requirements.

Implementation of automation to support large-molecule drug discovery was successful. Automation has allowed us to manage 24,000 plasmid purifications per month, while freeing up resources to provide more in-depth project support and improved sample tracking and storage.

Declaration of Conflicting Interests
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References