High-throughput and automation methods

Appendix

High-Throughput and Automation Methods

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Appendix:

Chapter 1: Robot Operation

Overview

This chapter provides an introduction to successful operation of the Tecan Freedom EVO liquid handling robot. It outlines the system basics, proper power on/off, maintenance, and software usage. This manual should be used in conjunction with the Tecan-provided EVOware Help, a fantastic resource that explains every setting inside the software.

The Tecan Freedom EVO is located in the Beckman Institute in Room 288. The space is owned by the Protein Expression Center, headed by Dr. Jost Vielmetter. The lab benches and pipettes immediately around the robot can be used to prepare your automation experiments. Full size refrigerators at –20°C and 4°C are also on hand to help with experiment preparation. The center cabinet underneath the desk is full of labware and supplies for the Mayo Lab.

The Tecan Freedom EVO 200

The 2-meter robot sits on a reinforced table inside of positive pressure sterile hood and is composed of three unique arms overlooking a precisely manufactured deck (Figure 1). The left-most arm, the 8-tip Liquid Handler (LiHa), has eight liquid-sensing fixed tips that require a washing step after each use. Each tip is individually addressable and ideal for cherry-picking operations. The middle arm, the Multi Channel Arm 96 (MCA96), uses disposable tips to pipet 96 volumes of liquid at once, making it very useful for serial dilutions or buffer additions. The right arm, the Robotic Manipulator (RoMa), can pick up and move any microtiter plate that conforms to the Society for Biomolecular Science (SBS) standard. Different kinds of carriers (holding places for microtiter plates) populate the deck, and although the deck layout was optimized for our applications, not all carriers are accessible to all of the arms.

System Liquid

All fixed tip systems have a tip wash station that requires water from an outside source. In the general case, this is a large water reservoir that is manually refilled as it empties through the system. Luckily, our Freedom EVO has been customized (courtesy of Dr. Vielmetter) with a reservoir that auto-refills from a local house DI water line. The custom reservoir also has a UV filtration unit installed that prevents microbial growth in the standing water. This setup greatly minimizes weekly maintenance of the system. The custom reservoir feeds sterile water through a single tube into the Freedom EVO through the back; this tube is split into 8 channels (as seen by the 8 syringes on the middle top bar of the instrument) that then exit out of the 8 tips of the LiHa.

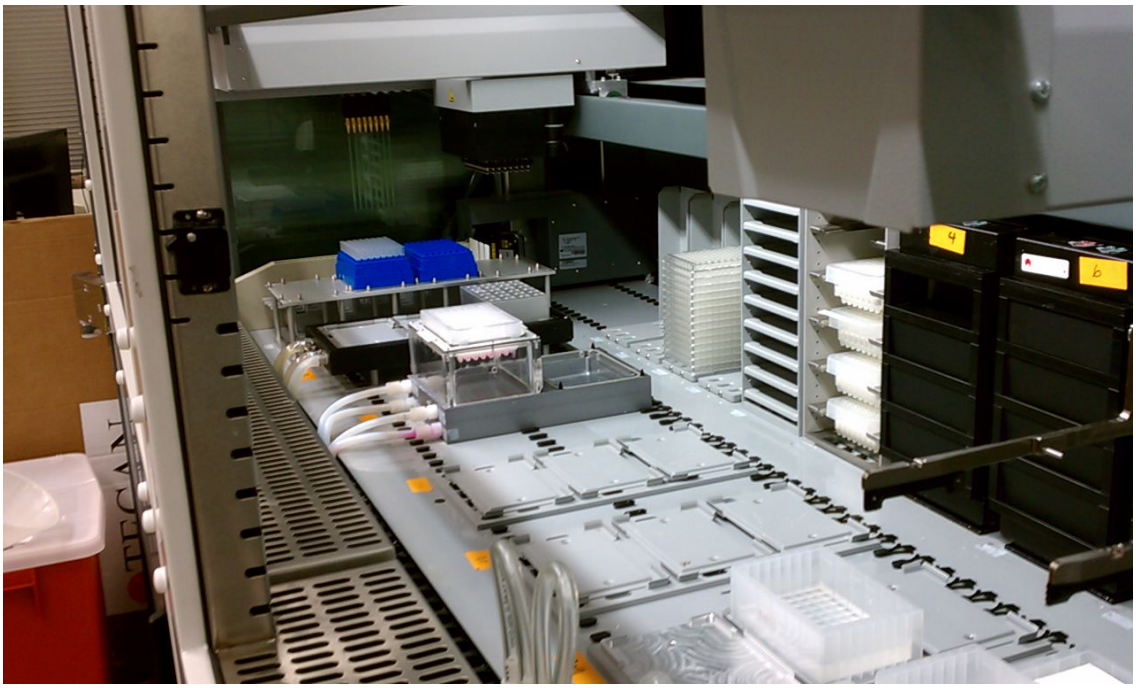


Figure 1. The Tecan Freedom EVO 200, as seen in BI288.

The LiHa wash station drains into the house drainage system on the left side of the instrument. With this modification, the Freedom EVO is essentially a closed loop system: water is pulled from the house line into a sterile reservoir, passed through the robot system, flushed through the LiHa, and finally clears out into the drain.

The LiHa arm in our system is outfitted with the Te-Fill accessory that enables it to dispense large volumes of liquid from reservoirs located on the left side of the instrument. The Te-Fill system can be used to switch between five different buffer sources for a variety of extended automated routines. However, the pump on the Te-Fill is slow and was the motivation for acquiring and integrating the peristaltic pump system (described below).

Deck Layout

The deck layout as seen in the EVOware software (Figure 2) is an accurate portrayal of most of the carriers on the deck that are physically accessible by the liquid handling arms. Two room temperature plate hotels, three controlled temperature plate hotels, and four plate stackers along the back of the instrument are not properly represented in the figure.

Directly below the deck, the robot table houses two peristaltic pumps installed and integrated by Dr. Vielmetter. These devices, in combination with specialized labware also developed by Dr. Vielmetter, are able to provide fast-filling reservoirs of any desirable liquid. One useful arrangement of this subsystem is as a tip wash station for the MCA96 in which the primary reservoir is constantly exchanging water (to wash the tips), and the secondary reservoir is exchanging fresh ethanol (to dry the tips). Outside of the hood and to the left is a variable temperature water recirculator that is connected to a specialized 3 position carrier on the deck. The recirculator can cool the carrier down to 4°C or warm it up to 37°C in 30 minutes.

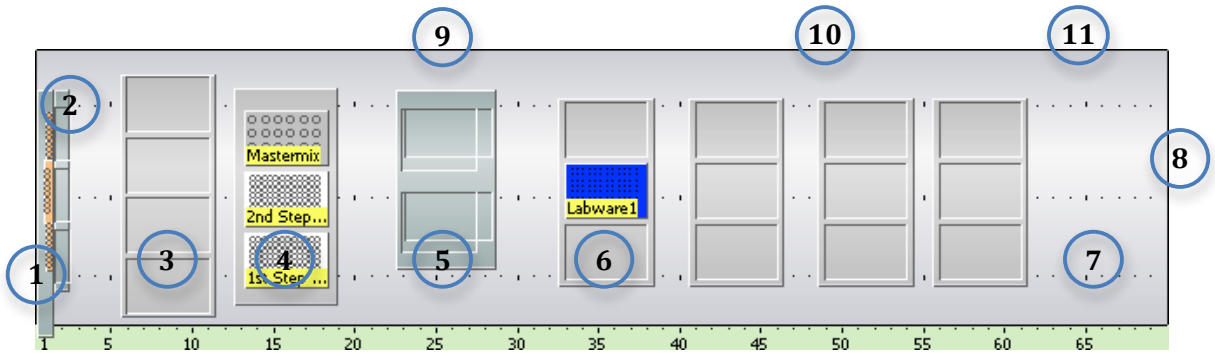


Figure 2. The deck layout, as depicted in the EVOware software. The features on the deck are as follows: (1) LiHa tip wash station, (2) 3 position 100mL trough carrier, (3) 4 position MCA tip box carrier, (4)3 position MP variable temp carrier, (5) 2 position Te-Vac vacuum system, (6) four 3 position MP flat carriers, (7) 2 position Te-Shake MP shaker, (8) attached Tecan Infinite M1000 plate reader, (9) 4 off-deck stackers, 1 SPE hotel, 1MP hotel, (10) 3 off-deck variable temp hotels, (11) BIO-RAD thermalcycler with automated lid. (MP: microtiter plate, SPE: solid phase extraction)

Power ON

The robot platform and accompanying computer can usually be found with the instrument and computer powered on and the computer at the Microsoft Windows login screen. In this instance, simply login to the computer, double-click the EVOware icon, and login to the EVOware software.

After a cold start (the robot was turned off/on), the robot will need to do a long initialization in which the three arms test their range of motion and move to the left-most edge of the deck. It should be noted that when the robot is turned off, the arms are no longer locked in place and can be moved freely by the user. This 5-minute process is not done when the robot is left on. Instead, the robot will move the LiHa and the RoMa to their home positions, which is a much faster process.

Maintenance after Power ON

Immediately after running EVOware, it is very important to run the *Daily_Flush* maintenance routine. This script not only initializes the instrument (prompting the arms to move to their home positions on a warm start) but also flushes and rinses the LiHa with 25mL of water. The act of flushing the LiHa removes bubbles and restores the system liquid trailing air gaps (STAG) in the eight tips. This is important for accurate liquid dispense operations, especially multi-dispense techniques. After flushing, the maintenance routine moves the LiHa over the second microtiter plate carrier and spreads the tips to allow the user to rubdown each tip with an ethanol-soaked kimwipe. This ethanol rubdown step also helps in maintaining the accuracy of the liquid dispensing system. Finally, check that all the twist knobs associated with the LiHa syringes are finger tightened before use. A common reason for inaccurate liquid handling is loose syringes. After this, the robot is ready for use.

Additional maintenance may be needed if your robotic routine calls for the Te-Vac or the thermalcycler. If using the Te-Vac, check that the waste bucket is less than half-full, or preferably empty it if you have a vacuum heavy routine. If using the thermalcycler, the

sealing pad on the automated lid should be washed with a dilute bleach-soaked kimwipe and subsequently washed twice more with a water-soaked kimwipe.

Power OFF

After your robotic routine is completed, clear the deck of your labware, including plates, tubes, and tips. Throw away used tips boxes and eppendorf tubes. Exit the software, double-click the “shutdown EVOware drivers” icon, and log out of the computer. You may leave both the computer and the robot on for the next user in this state.

To leave the instrument in a true OFF state, after the above is completed press the light green arrow button on the robot located on the bottom far right side. Turn off the computer through the Windows Start menu.

Maintenance after Power OFF

Most if not all routines include a tip wash step for the LiHa after use, so any extra wash steps are unnecessary after a script is finished. If the Te-Vac was used, be sure to clean the carrier and the vacuum carrier using a dilute bleach rinse. If the cooling water recirculator was used, remember to turn it off and refill it with DI water if necessary.

Using EVOware 2.1

Upon execution of the EVOware software, the Startup window asks whether you want to run an existing or maintenance script, create a new script, edit an existing one, or configure the system. If you are just starting your session, select “Run maintenance” and then "Daily_Flush" (Figure 4). This opens the Runtime Controller window, where clicking “RUN” sends the script to the robot to be performed. After the process and the appropriate maintenance is completed, the Runtime Controller will alert you of the successful completion of the script. By clicking “Cancel” after a successful finish, the software will return you to the Startup window.

Figure 3. Run the maintenance script "Daily_Flush" from the Startup screen. After clicking "START YOUR SELECTION," click "RUN" at the Runtime Controller window.

After running "Daily_Flush," select "Edit an existing script" to open the list of saved scripts. Select the script of interest (frequently used scripts may be located in the Favorites tab) and open it (Figure 5). Scripts can also be found in separated folders at the bottom of this list. This will open the main functional window of the EVOware software.

Another option is to create a new script and select a template from the Favorites tab. When running one of the methods described later in Chapter 2, this is the preferred option so that any edits made are isolated to that user's script. The majority of users should ignore the other options on the Startup window. Accessing the system configuration is only appropriate for advanced users. Remember, running a script without inspection is decidedly not recommended unless it has been thoroughly debugged and you are an advanced user.

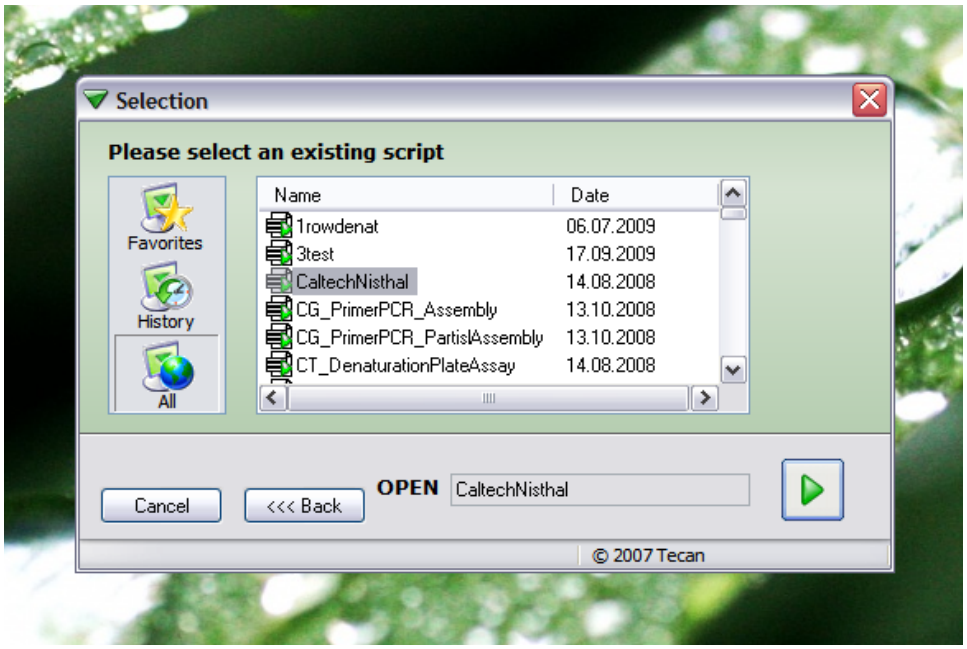


Figure 4. Edit an existing script from the Selection window. Select the script of interest and click the green arrow button.

Line Editing

Along the right side of the screen is the Script Editor window. Here, each line is the equivalent of a line of code that must be performed sequentially. It is extremely important to double check every line of code to make sure the robot is given the correct procedure.

Every available command and popup screen is detailed in the EVOware Help (hit the F1 key or click "Help" along the top of the screen, then "Contents"), so only a brief description of the commonly used "Aspirate" and "Transfer Labware" commands will be provided here.

Many script lines feature information that is useful when scanning a script for possible errors before execution. The "Aspirate" command, for example, shows a cartoon of the tips in use next to a highlighted area of the labware it will pull from (Figure 6). Next to this, the top line shows the amount being aspirated and the liquid class the system will use to do it. The bottom line displays the name of the labware (in quotation marks) followed by the exact positions that will be aspirated (in parentheses). If the command is changed as part of an iterable loop, the number of options that are changing is displayed as well.

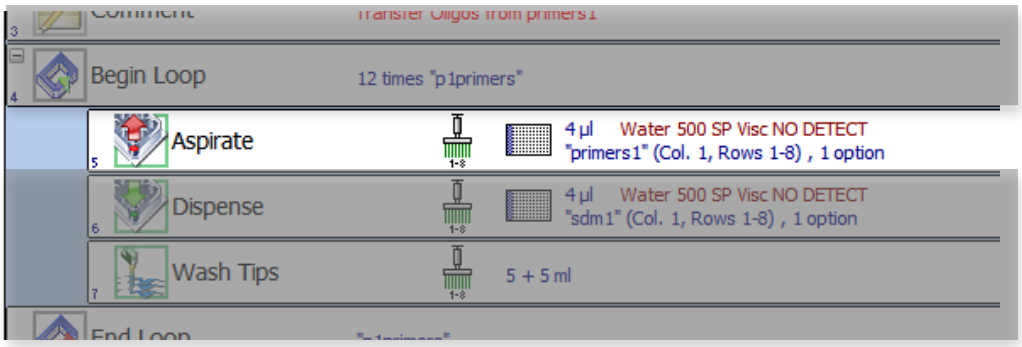


Figure 5. The LiHa "Aspirate" command is highlighted, as seen in the Script Editor window.

The "Transfer Labware" command also contains information that is useful to check before running a particular script. The top line describes a source site using carrier position notation; this is followed by the destination site, which uses the same notation (Figure 7). Finally, the type of grip (narrow or wide) is described. If using a special microtiter plate (such as the PCR plate), a different, user-defined vector is used by the RoMa. All these settings are defined by the user when the script is initially written but can be edited by double-clicking the line of interest.

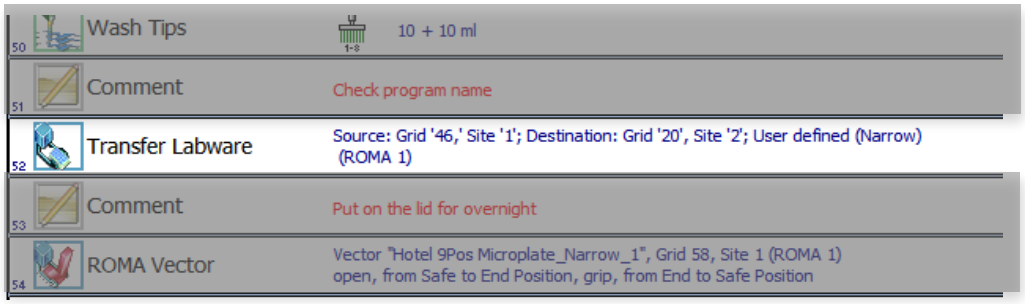


Figure 6. The RoMa "Transfer Labware" command is highlighted, as seen in the Script Editor window.

Worktable Editing

The Worktable Editor window shows a faithful reconstruction of the physical deck configuration of the robot. Through this graphic interface, the user can assign carrier and labware placements and label labware. In general, this window should be used to populate the deck of the robot with the labware needed for a particular script. However, the user should be aware that EVOware creates duplicate instances of labware that is moved by the RoMa. For example, a PCR plate that is labeled "insert-pcr" will show up with the default label, "Labware1" in other sites where it will eventually end up. For this reason, script authors should label all labware before entering any RoMa commands to help in identifying the duplicates.

As noted earlier, carriers that are not accessible by the liquid handling arms (such as the plate stackers and hotels) are pictured without respect to their numerical position on the robot deck (Figure 2). Their physical positions are only indicated when they have been clicked on by the user.

Interface tips:

- ✓ Double-clicking a piece of labware will open the labeling popup.
- ✓ To quickly create a duplicate of a piece of labware, hold down "Control" while dragging the labware.
- ✓ For advanced users: Right-clicking on labware or carriers opens the option to edit them. This is where new labware or where RoMa vectors to carriers can be taught, respectively.

Running a Script

After a script has been inspected and the user is comfortable proceeding, click the "RUN" icon (green arrow) along the top of the screen. If the latest version of the script isn't saved, the program will prompt you to save it before continuing. Now the Runtime Controller window will pop up; click "RUN" to proceed (Figure 8). Near the top of the Runtime Controller window is a check box for running the full script. If unchecked, specific line numbers corresponding to portions of the script can be entered. This is useful in cases in which a script was programmed to handle two microtiter plates, but you want to process one. The Runtime Controller window also has toggle buttons to open extended view and to open the active logfile viewer.

The extended view window is very handy for checking how far along the robot is in processing your script. The top left window prints out comments that are programmed in the script. The bottom left window features loop counters for any loops you may have in the script.

Once the robot is done, the Runtime Controller window will report the elapsed time and allow the user to view the extended view and logfiles of the completed script. Click "Cancel" to return to the main screen of EVOware. Clicking "New" will allow the user to run the same script again right away, which is generally not recommended.

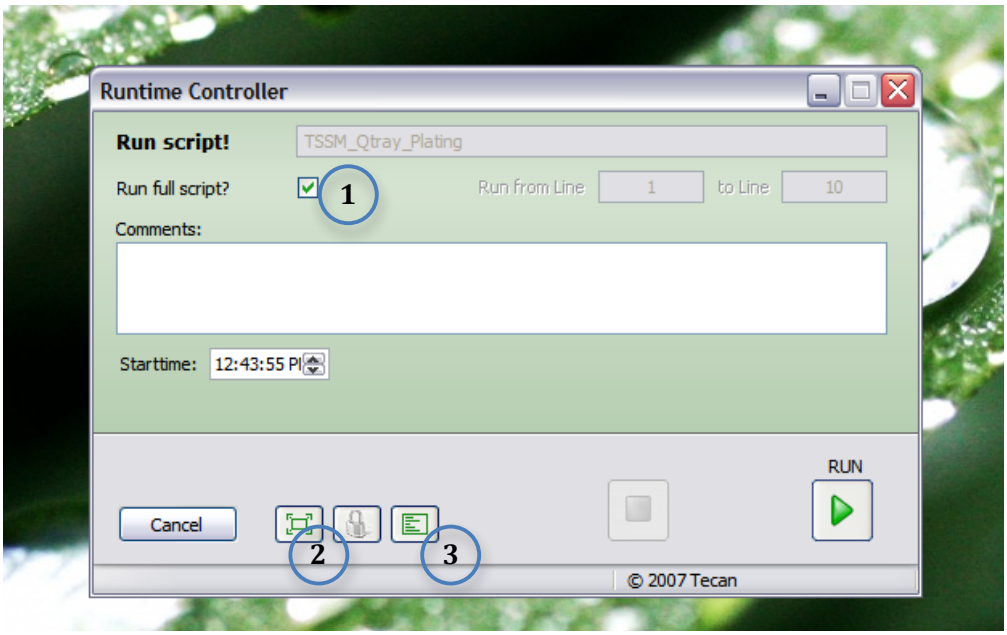


Figure 7. The Runtime Controller window. Important options on this window include: (1) the "Run full script?" toggle, (2) the extended view button, and (3) the logfile viewer button.

Error Messages and Stopping a Script

There are any number of errors that can occur during the execution of a script, but the two most common are hardware crashes and notices of insufficient liquid. Although neither should happen when running verified scripts, they can still creep in if a carrier on the deck has moved or if liquid volumes are adjusted on the fly.

When the software has detected a hardware crash, the script is stopped and the arms lock down into place. EVOware will ask to be placed into virtual mode, where it can no longer communicate with the hardware. In this instance, shut down EVOware and then turn off the instrument. Now the arms should be unlocked and the user can attend to the situation on the deck. Once finished, double-click "Shutdown EVOware drivers," and then turn on the instrument. Open EVOware and login as usual. You may get messages (that you can cancel/ignore) asking you to zip and send the last logfile to Tecan. Opening a script that has caused a crash will open the "Recover" option in the Run menu. Never use the "Recover" feature of EVOware; it is much better to highlight the remainder of the script you wish to execute. The "Run Direct" command simply skips through the runtime controller; there is no need to use it. The script editor will also highlight which line caused the crash.

An insufficient liquid notice is much easier to handle and is only a factor when using liquid detection. A popup box will ask if you wish to detect again, go to z-max, or aspirate nothing.

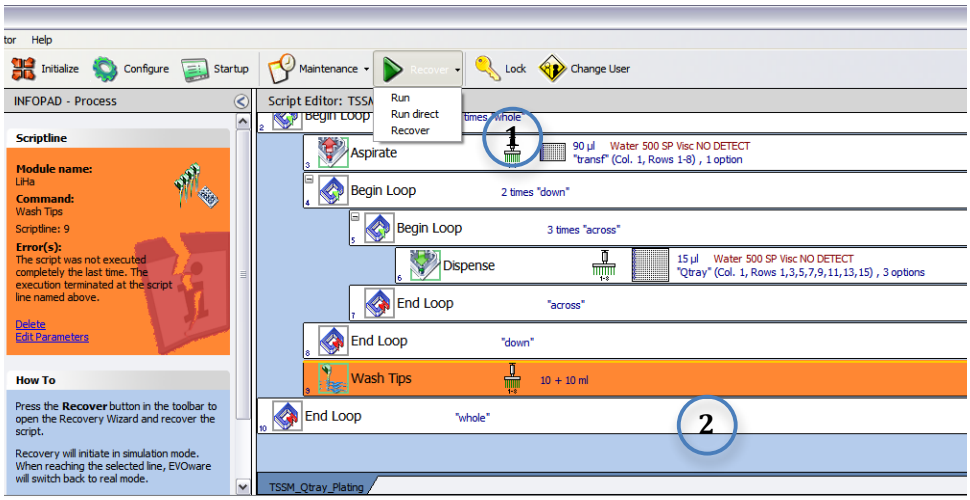


Figure 8. The Run menu in EVOware after an error has occurred. (1) The three options are Run, Run direct, and Recover. (2) The script line that produced the error is also highlighted in orange, and can sometimes be highlighted in red, depending on the severity of the error.

In almost every case, the user should move along and aspirate nothing because moving to z-max may cause a hardware crash and detecting again will give the same error. A note should be made as to why there was not enough volume; sometimes the robot may be attempting to aspirate an unexpectedly viscous liquid or, more likely, a scripting mistake has occurred.

If the user detects a potential upcoming problem in the execution of a script, the Runtime Controller window allows for pausing or stopping a script. After stopping a script, click “Cancel" to return to the main EVOware window. Again, never use the “Recover” feature of EVOware, as it is somewhat unpredictable. Pausing here is identical to pressing the pause button on the robot. Stopping or pausing a script from within the software allows the robot to finish the line it is on before completely stopping and unlocking the shield. If a hardware crash is imminent, it may be necessary to immediately stop the robot by turning it off at the power button.

Email Notification

One of the most useful features in EVOware is the ability to send an email at any point in the script. To do this, you must be entered as a recipient in the EVOware Configuration and then add a “Notification” line to your script.

In the EVOware Configure window, click the “General” tab and then under “Notifications,” click “Recipients” and add the name and email of a new user. Then under “Notifications,” click “Groups” and add the new user’s name as a new group. At the minimum, make the new user and the current automation specialist in the lab members of the newly created group. This configuration allows the automation specialist supervision of new users without flooding other uninvolved users email inboxes. Along the top of the screen, click "Save" and then click "Exit." Restart EVOware to make the changes permanent.

Backing Up/Restoring EVOware

For advanced users only. The home directory for the EVOware installation is "C:\Program Files\TECAN\EVOware."

Complete backups of the EVOware installation can be made by entering the EVOware Configure window and clicking "Backup" at the top of the screen. This will generate a folder with everything needed to restore a broken installation in ".\backup." These backups are also useful for restoring a particular version of a script or for viewing labware/carrier definitions. Every script is saved to a scripts folder in the backup, allowing the user to copy the backup script into the ".\database\scripts" folder in the real EVOware installation directory. Labware and carrier definitions are not saved independently in the backup folder, but instead are saved as one carrier.cfg file that can be copied into the real ".\database" directory.

The software also keeps every saved version of important files so as to immediately recover from a negative change. For example, another user may have edited a carrier definition improperly a few days ago but the last full backup was done two months ago. In that span of time, several positive changes may have been made so that you wish to keep the installation and only fix the carrier file. A user can enter the ".\AuditTrail\configuration" folder and copy the carrier.cfg file from before the negative change was made (minding the dates) and paste it into the actual ".\database" directory.

As in any instance in which a system file is being copied over, remember to create a duplicate of the file in case the older one makes things worse.

Basic Operation Checklist

- ✓ Login to the computer and then into EVOware.
- ✓ Run the maintenance script "Daily_Flush."
- ✓ Check syringe knobs and wipe down the LiHa tips.
- ✓ Create a new script from a template or edit an existing script.
- ✓ Check the script code for possible errors and make adjustments.
- ✓ Populate the physical deck by following the Worktable editor.
- ✓ **Check the script code for possible errors.**
- ✓ Run the script.
- ✓ When finished, clear the deck, close EVOware, and log out of the computer.

Chapter 2: Robot Procedures

Overview

This chapter covers the important procedures that have been developed on the Tecan Freedom EVO 200. Most of these protocols were developed with performing site saturation mutagenesis in mind, but can be run independently.

Each protocol described here features a brief introduction, followed by a materials list (complete with order numbers), and a methods section. In the methods section, the actual robotic script is reprinted from the basic template procedure and important details are highlighted. The corresponding deck image is also included for every full script shown.

All the procedures were saved as separate EVOware templates. To run a protocol, select "Create a new script" from the Startup Menu and select the template of interest. Then save the opened script with your initials as a prefix. This makes for a much faster startup for novice users who wish to run the default protocol. Also, it keeps the core of the program intact, allowing individual users to make any appropriate changes for their particular experimental setup without affecting other users' scripts. Of course, if a user wishes to run the exact same protocol again later, their EVOware script can be found under "Edit an existing script."

IMPORTANT

The robot was upgraded and its deck remodeled in the fall of 2010. As such, the details of many scripts described here are incorrect but the concepts remain the same. A handful of scripts were added after the upgrade, are up-to-date, and are marked appropriately.

Dilute Oligonucleotides (Gene Assembly)

*Current Deck Layout

This script performs the replacement method of library assembly as detailed in chapter 4. The methodology was developed in response to issues with the moderate number of high-ranking sequences found in designed degenerate codon libraries. Explicit consideration of each sequence allows for a more direct translation of protein design results into experimental reactions. The Python script described in chapter 4 produces a robotic worklist, written into the .csv format, which is then translated into the robot-readable .gwl format by the EVOware software.

There are three main specification pages when ordering plate oligos from IDT. For the first page, mark "file upload," "email," "96-well plate," and "column" loading. For the second page, mark "25 nmole DNA," "standard desalting," "no" CE service, "full yield," "V-bottom plate," and shipping "wet." For the third page, mark "150μM," leave the volume blank, and mark "IDTE buffer pH 7.5."

These specifications allow users to start automation experiments much faster because there is no need for resuspension, and they keep the plate in a robot-friendly column-loading scheme. The lower limit for ordering oligos in the plate format is 24. A MS Excel template is available in the lab or from IDT to upload the sequence information.

Materials

96-Well IDT Oligonucleotide Plate(s). The oligo plates from IDT arrive with a rubber capmat, lid, and taped up. Dispose of the tape but keep the capmat and the lid.

96-Well V-Bottom Plate(s). These plates, available through Axygen (P-96-450V-C) were chosen for diluting oligos because of their similarity to the plates from IDT. They are ordered non-sterile and autoclaved before use.

Sterile H_2O in 100mL Disposable trough. This trough, available from Tecan (10613049), fits in the carriers located in grids 2 through 5. Fill with high quality water typically used for diluting the oligos.

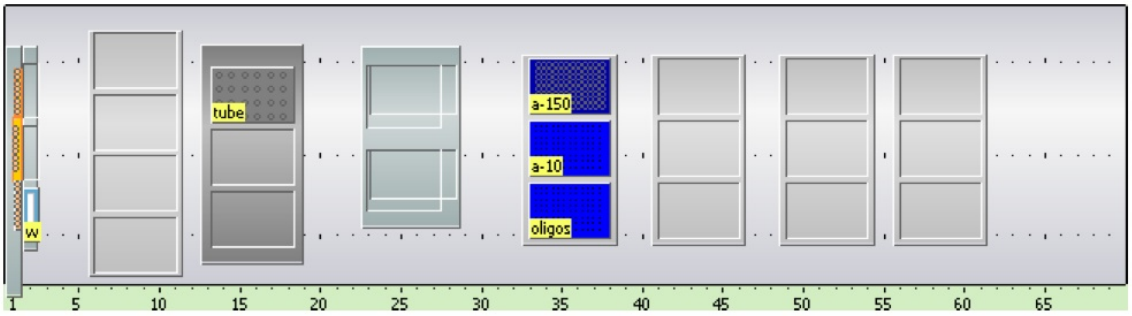
Microtiter Plate Rubber Capmat. The 96-well V-Bottom plates are sealed with an Axygen ImpermaMat (AM-2ML-RD-IMP) to prevent evaporation. Non-frozen sealed plates may need to be centrifuged down prior to opening due to sample adhesion to the seal.

Methods

Setup

The gene assembly oligo dilution script creates 10uM diluted plates from an original 150uM IDT plate. Volumes can be adjusted to dilute to concentrations more applicable to the user's downstream procedures. A robot worklist, "1A53-8ng.csv", then directs the assembly of oligonucleotides into each well of the oligos collection plate.

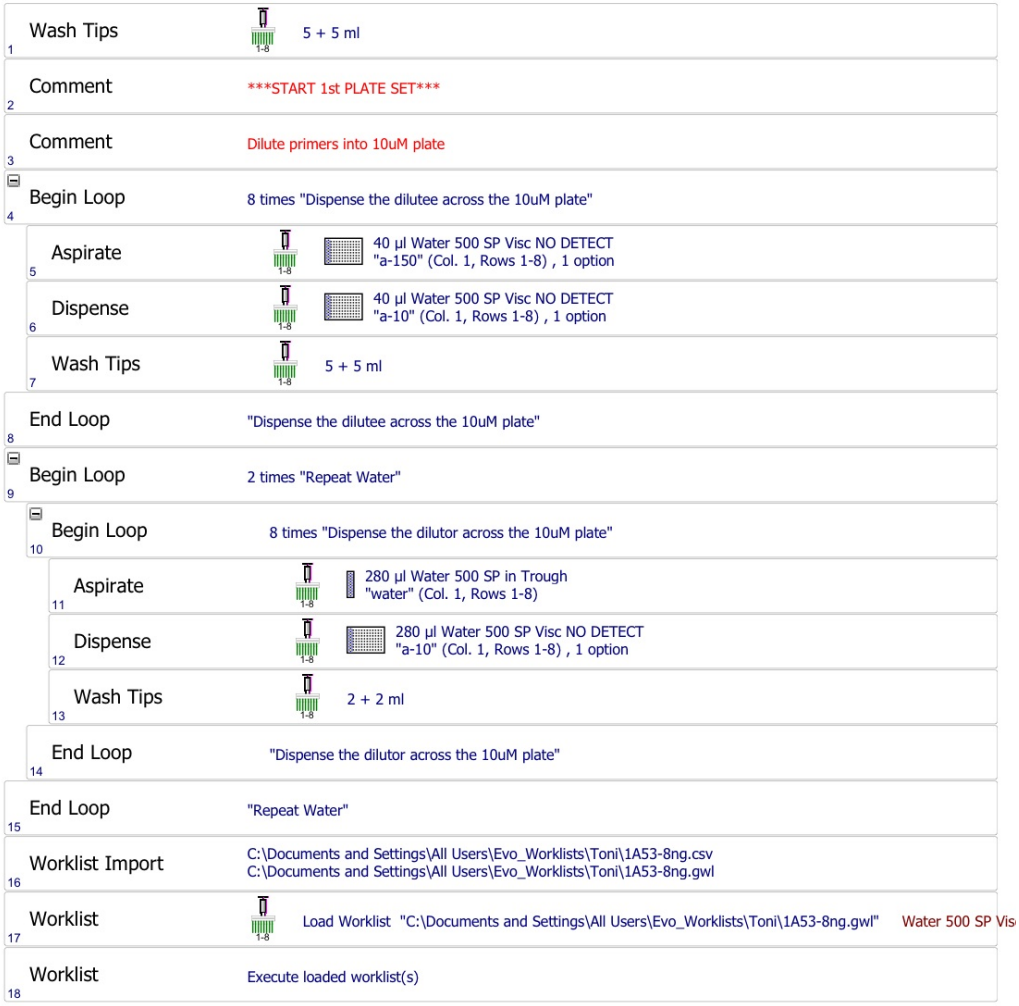
Water: 100ml disposable trough
a-150uM: 96-well IDT oligonucleotide plates
a-10uM, oligos: 96-well V-bottom plates
tube: ignore, not used in the method



Sample data from "1A53-8ng.csv"

source position	source label	dest position	dest label	volume
1	a-10	1	oligos	5
2	a-10	1	oligos	5
3	a-10	1	oligos	5
4	a-10	1	oligos	5
5	a-10	1	oligos	5
6	a-10	1	oligos	5
7	a-10	1	oligos	5
8	a-10	1	oligos	5
9	a-10	1	oligos	5
10	a-10	1	oligos	5
11	a-10	1	oligos	5
12	a-10	1	oligos	5
13	a-10	1	oligos	5
14	a-10	1	oligos	5

Procedure


1 Wash Tips 5 + 5 ml
2 Comment ***START 1st PLATE SET***
3 Comment Dilute primers into 10uM plate
4 Begin Loop 8 times "Dispense the dilutee across the 10uM plate"
5 Aspirate 40 µl Water 500 SP Visc NO DETECT "a-150" (Col. 1, Rows 1-8) , 1 option
6 Dispense 40 µl Water 500 SP Visc NO DETECT "a-10" (Col. 1, Rows 1-8) , 1 option
7 Wash Tips 5 + 5 ml
8 End Loop "Dispense the dilutee across the 10uM plate"
9 Begin Loop 2 times "Repeat Water"
10 Begin Loop 8 times "Dispense the dilutor across the 10uM plate"
11 Aspirate 280 µl Water 500 SP in Trough "water" (Col. 1, Rows 1-8)
12 Dispense 280 µl Water 500 SP Visc NO DETECT "a-10" (Col. 1, Rows 1-8) , 1 option
13 Wash Tips 2 + 2 ml
14 End Loop "Dispense the dilutor across the 10uM plate"
15 End Loop "Repeat Water"
16 Worklist Import C:\Documents and Settings\All Users\Evo_Worklists\Toni\1A53-8ng.csv C:\Documents and Settings\All Users\Evo_Worklists\Toni\1A53-8ng.gwl
17 Worklist Load Worklist "C:\Documents and Settings\All Users\Evo_Worklists\Toni\1A53-8ng.gwl" Water 500 SP Vis
18 Worklist Execute loaded worklist(s)

IPIPE and CPEC PCR (Gene Assembly)

*Current Deck Layout

The gene assembly scripts described here bring the PIPE/CPEC methods established on the benchtop to the robot. Chapter 4 features a full description of the methodology. Since the IPIPE and CPEC methods are very similar, they are grouped into this one entry.

The input to these scripts is a 96-well plate with mixed oligos. The IPIPE method will then perform assembly PCR followed by amplification with IPIPE outside primers. After the IPIPE method the PCR products can be combined with VPIPE products and transformed into bacteria. It is recommended, however, to continue with the CPEC method and use the resulting product to transform. Much better rates of transformed colonies have been found when using CPEC products over the PIPE reaction.

Materials

96-well BIO-RAD PCR Plate(s) on 96-well cooled block. This PCR plate, available from BIO-RAD (HSP9601), is preferred for automated operations due to its robot-friendly hard shell and full skirt. All automated thermocycler operations have been developed exclusively with this plate. The cooled block, along with the water recirculator, can cool the samples down to 4°C.

96-well V-Bottom Plate(s). These plates, available through Axygen (P-96-450V-C) were chosen for diluting oligos because of their similarity to the plates from IDT. They are ordered non-sterile and autoclaved before use.

Microtiter Plate Rubber Capmat. The 96-well V-Bottom plates are sealed with an Axygen ImpermaMat (AM-2ML-RD-IMP) to prevent evaporation. Sealed plates should be centrifuged down (500xg for 30 sec) prior to opening due to sample adhesion to the seal.

1.5mL eppendorf tubes in 24-well cooled block. These are the lab standard autoclavable tubes available in any biochemistry lab. The cooled block, along with the water recirculator can cool samples down to 4°C.

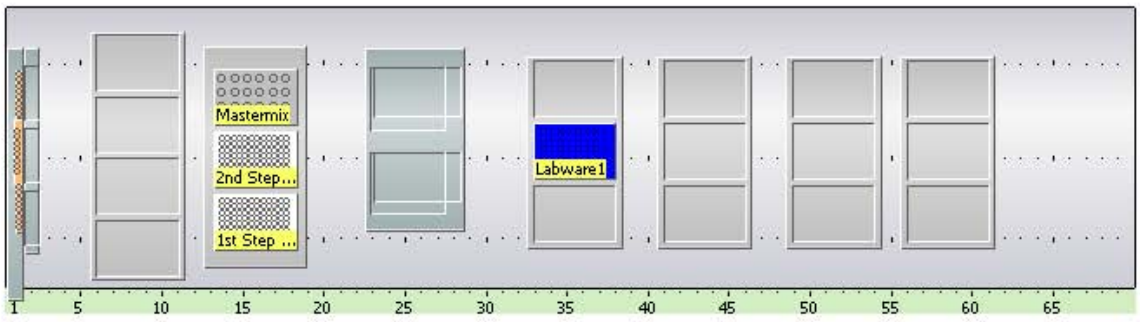
Mastermix Solution. These solutions vary due to their intended use. In general, the assembly mastermix should include DNA polymerase, dNTPs, water and DNA polymerase buffer. The IPIPE amplification mastermix should add the IPIPE primers to the assembly mastermix. The CPEC mastermix should add linearized vector (VPIPE product) to the assembly mastermix.

Methods

Setup – IPIPE

Turn on the water recirculator at least a half-hour before running the procedure to ensure the 24 and 96-well blocks are cool. Organize the deck as shown in the figure below. The script will transfer oligo mixtures from the V-bottom Plate into the 1st Step PCR Plate, and then add assembly mastermix on top of that. The gripper arm will transfer the plate to the thermalcycler, execute the program, and bring it back. A small amount of product is transferred from the 1st Step to the 2nd Step PCR Plate, IPIPE mastermix is added on top of that, and the thermalcycler is engaged again for the insert amplification.

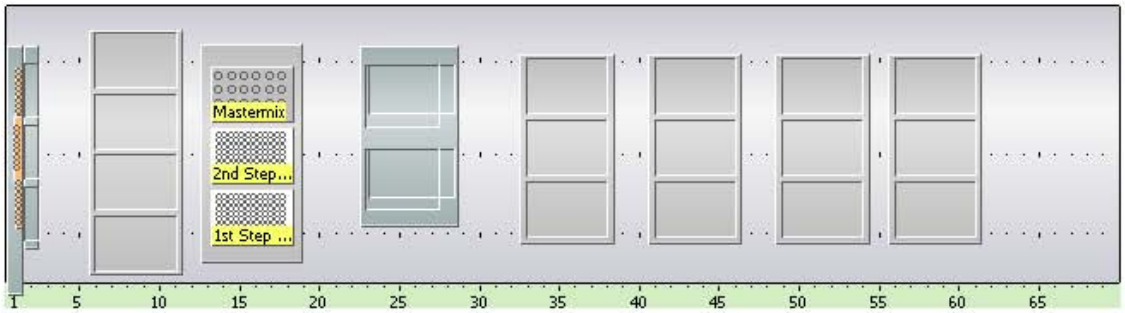
1st Step, 2nd Step PCR Plate: 96-well BIO-RAD PCR Plates
Mastermix: 1.5mL eppendorf tubes in 24-well cooled block (split the assembly mastermix into C1,D1; split the IPIPE amplification mastermix into C2,D2)
Labware1: 96-well V-Bottom Plate



Setup – CPEC

Turn on the water recirculator at least a half-hour before running the procedure to ensure the 24 and 96-well blocks are cool. Organize the deck as shown in the figure below. A small amount of PCR product is transferred from the 1st Step to the 2nd Step PCR Plate and CPEC mastermix is added on top of that. The gripper arm will transfer the 2nd Step PCR Plate to the thermalcycler, execute the program, and bring it back.

1st Step, 2nd Step PCR Plate: 96-well BIO-RAD PCR Plates
Mastermix: 1.5mL eppendorf tubes in the 24-well cooled block (split the CPEC mastermix into C2,D2)



Procedure – IPIPE

1	Comment		Transfer Primers
2	Begin Loop		12 times "Add Primers"
3	Aspirate	1-8	5 µl Water 500 SP Visc NO DETECT "Labware1" (Col. 1, Rows 1-8) , 1 option
4	Dispense	1-8	5 µl Water 500 SP Visc NO DETECT "1st Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
5	Wash Tips	1-8	5 + 5 ml
6	End Loop		"Add Primers"
7	Comment		Add Mastermix
8	Begin Loop		6 times "mastermix"
9	Aspirate	1,2	20 µl Water 500 SP Visc "Mastermix" (Col. 1, Rows 3,4)
10	Aspirate	3,4	20 µl Water 500 SP Visc "Mastermix" (Col. 1, Rows 3,4)
11	Aspirate	5,6	20 µl Water 500 SP Visc "Mastermix" (Col. 1, Rows 3,4)
12	Aspirate	7,8	20 µl Water 500 SP Visc "Mastermix" (Col. 1, Rows 3,4)
13	Dispense	1-8	20 µl Water 500 SP Visc "1st Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
14	Wash Tips	1-8	5 + 5 ml
15	End Loop		"mastermix"
16	Begin Loop		6 times "mastermix"
17	Aspirate	1,2	20 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 1, Rows 3,4)
18	Aspirate	3,4	20 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 1, Rows 3,4)
19	Aspirate	5,6	20 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 1, Rows 3,4)
20	Aspirate	7,8	20 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 1, Rows 3,4)
21	Dispense	1-8	20 µl Water 500 SP Visc "1st Step PCR Plate" (Col. 7, Rows 1-8) , 1 option
22	Wash Tips	1-8	5 + 5 ml

23	End Loop	"mastermix"
24	Comment	Move sdm1 to thermocycler
25	ThermalCycler	OpenLid(1)
26	Transfer Labware	Source: Grid '13,' Site '3'; Destination: Grid '63', Site '1'; Narrow (ROMA 1)
27	ThermalCycler	CloseLid(1,1)
28	Comment	Check program name
29	ThermalCycler	StartProgram(1,TONGA,BLOCK,ON,10)
30	ThermalCycler	WaitForProgram(1)
31	ThermalCycler	OpenLid(1)
32	Notification	Send email now Mayo Lab
33	Comment	Move sdm1 out
34	Transfer Labware	Source: Grid '63,' Site '1'; Destination: Grid '13', Site '3'; Narrow (ROMA 1)
35	Comment	Amplification
36	Begin Loop	12 times "add 1st step product"
37	Aspirate	2 µl Water 500 SP Visc NO DETECT "1st Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
38	Dispense	2 µl Water 500 SP Visc NO DETECT "2nd Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
39	Wash Tips	5 + 5 ml
40	End Loop	"add 1st step product"
41	Comment	Dispense Mastermix Again
42	Begin Loop	6 times "mastermix"
43	Aspirate	23 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
44	Aspirate	23 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)


45 Aspirate 5,8 23 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
46 Aspirate 7,8 23 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
47 Dispense 1-8 23 µl Water 500 SP Visc "2nd Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
48 Wash Tips 1-8 5 + 5 ml
49 End Loop "mastermix"
50 Begin Loop 6 times "mastermix"
51 Aspirate 1,2 23 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
52 Aspirate 3,4 23 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
53 Aspirate 5,8 23 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
54 Aspirate 7,8 23 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
55 Dispense 1-8 23 µl Water 500 SP Visc "2nd Step PCR Plate" (Col. 7, Rows 1-8) , 1 option
56 Wash Tips 1-8 5 + 5 ml
57 End Loop "mastermix"
58 Comment 2nd Step in Thermocycler
59 ThermalCycler OpenLid(1)
60 Transfer Labware Source: Grid '13,' Site '2'; Destination: Grid '63', Site '1'; Narrow (ROMA 1)
61 ThermalCycler CloseLid(1,1)
62 Comment Check program name
63 ThermalCycler StartProgram(1,IPIPE-MM,BLOCK,ON,10)
64 ThermalCycler WaitForProgram(1)
65 ThermalCycler OpenLid(1)
66 Transfer Labware Source: Grid '63,' Site '1'; Destination: Grid '13', Site '2'; Narrow (ROMA 1)
67 Notification Send email now Mayo Lab

Procedure – CPEC

1	Wash Tips	1-8	5 + 5 ml
2	Comment		Amplification
3	Begin Loop		12 times "add 1st step product"
4	Aspirate	1-8	2 µl Water 500 SP Visc NO DETECT "1st Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
5	Dispense	1-8	2 µl Water 500 SP Visc NO DETECT "2nd Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
6	Wash Tips	1-8	5 + 5 ml
7	End Loop		"add 1st step product"
8	Comment		Dispense Mastermix Again
9	Begin Loop		6 times "mastermix"
10	Aspirate	1,2	18 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
11	Aspirate	3,4	18 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
12	Aspirate	5,6	18 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
13	Aspirate	7,8	18 µl Water 500 SP Visc "Mastermix" (Col. 2, Rows 3,4)
14	Dispense	1-8	18 µl Water 500 SP Visc "2nd Step PCR Plate" (Col. 1, Rows 1-8) , 1 option
15	Wash Tips	1-8	5 + 5 ml
16	End Loop		"mastermix"
17	Begin Loop		6 times "mastermix"
18	Aspirate	1,2	18 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
19	Aspirate	3,4	18 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
20	Aspirate	5,6	18 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
21	Aspirate	7,8	18 µl Water 500 SP Visc NO DETECT "Mastermix" (Col. 2, Rows 3,4)
22	Dispense	1-8	18 µl Water 500 SP Visc "2nd Step PCR Plate" (Col. 7, Rows 1-8) , 1 option

23	Wash Tips	 5 + 5 ml
24	End Loop	"mastermix"
25	Comment	2nd Step in Thermocycler
26	ThermalCycler	OpenLid(1)
27	Transfer Labware	Source: Grid '13,' Site '2'; Destination: Grid '63', Site '1'; Narrow (ROMA 1)
28	ThermalCycler	CloseLid(1,1)
29	Comment	Check program name
30	ThermalCycler	StartProgram(1,CPEC,BLOCK,ON,10)
31	ThermalCycler	WaitForProgram(1)
32	ThermalCycler	OpenLid(1)
33	Transfer Labware	Source: Grid '63,' Site '1'; Destination: Grid '13', Site '2'; Narrow (ROMA 1)
34	Notification	Send email now Toni Lee

CPEC Transformation

*Current Deck Layout

Although an automated cell-free expression protocol would obviate the need for bacterial transformation, few assays have the prerequisite sensitivity to deal with the low levels of protein expression from cell-free extracts. In most cases, bacterial transformation and subsequent plating will be a necessary step in automated pipelines.

Competent cell manipulation was kept at a minimum to avoid disturbing the cells. The proper setting for the outgrowth step (2 hr shaking at room temperature) was determined empirically.

This method is updated from the older Bacterial Transformation script. Instead of shaking on the robot at room temperature, this method calls for off-robot shaking at 37°C that improves the number of colony forming units.

Materials

CPEC Product. This is typically carried over from the "CPEC PCR (Gene Assembly)" procedure in a 96-well BIO-RAD PCR plate.

1.5mL Eppendorf Tubes in 24-well Cooled Block. These are the lab standard autoclavable tubes available in any biochemistry lab. The cooled block, along with the water recirculator can cool samples down to 4°C.

BL21 Gold DE3 Competent Cells. These homemade chemically competent cells are very effective in transforming plasmid and nicked DNA and not very sensitive to variations in heatshock protocols. For one 96-well plate, thaw 2.4mL of competent cells from the -80°C freezer. Their preparation protocol is included in the appendix of this manual.

96-well BIO-RAD PCR Plate(s) on 96-Well Cooled Block. This PCR plate, available from BIO-RAD (HSP9601), is preferred for automated operations due to its robot-friendly hard shell and full skirt. All automated thermocycler operations have been developed exclusively with this plate. The cooled block, along with the water recirculator, can cool the samples down to 4°C.

96-well Costar Sterile Round Bottom Plate(s). This plate, available from Costar (3788), is preferred for automated operations due to its robot-friendly hard shell.

200uL Robot Tips. These automation tips are available from USA Scientific (TipONE #1188-1700). They are stacked with yellow inserts.

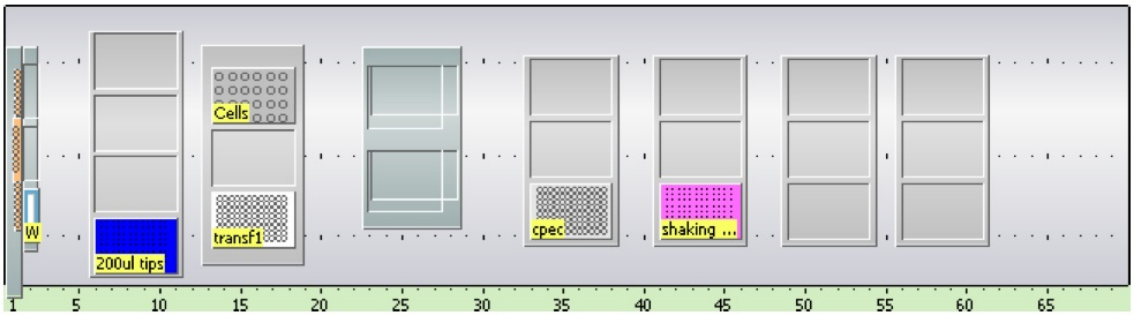
LB in 100mL Trough. These troughs, available from Tecan (10 613 049), only fit in the carrier located at grid 2. Fill with at least 15 mL of lab standard LB liquid media for the transformation of one 96-well plate. Keep warm before use for better results.

Methods

Setup

Turn on the water recirculator at least a half-hour before running the procedure to ensure the 24 and 96-well blocks are cool. Organize the deck as shown in the figure below. The script will transfer a small amount of CPEC product to the cooled PCR plate. Competent cells are aliquoted on top of this product, incubated for 15 min, and then the plate is heatshocked in the thermalcycler. Once the plate is returned, LB media is added to the cells as recovery media. The MCA96 arm then transfers the cells to a sterile 96-well plate for off-robot shaking at 37°C.

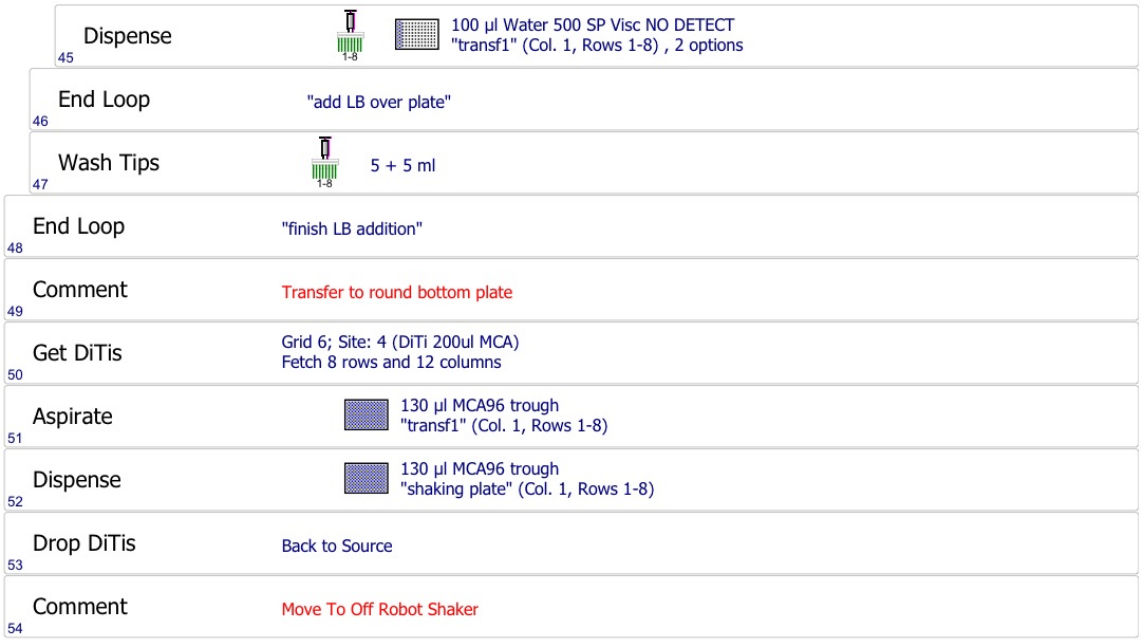
Cells: Competent cells split into 2 tubes in C1, D1 of the 24-well cooled block
cpec: CPEC PCR product in 96-well BIO-RAD PCR Plate
transf1: 96-well BIO-RAD PCR plate on 96-well cooled block
shaking plate: 96-well Costar sterile round bottom plate
200uL tips: 200uL disposable tips for the MCA96
Warm LB: LB in 100mL trough



Procedure

1	Comment	Check PCR Plate types
2	Wash Tips	1-8 5 + 5 ml
3	Comment	Add dpn1 Product
4	Begin Loop	12 times "add cpec product"
5	Aspirate	1-8 2 µl Water 500 SP Visc NO DETECT "cpec" (Col. 1, Rows 1-8) , 1 option
6	Dispense	1-8 2 µl Water 500 SP Visc NO DETECT "transf1" (Col. 1, Rows 1-8) , 1 option
7	Wash Tips	1-8 5 + 5 ml
8	End Loop	"add cpec product"
9	Comment	Add Cells
10	Begin Loop	6 times "add cells"
11	Aspirate	1,2 18 µl Water 500 SP Visc "Cells" (Col. 1, Rows 3,4)
12	Aspirate	3,4 18 µl Water 500 SP Visc "Cells" (Col. 1, Rows 3,4)
13	Aspirate	5,6 18 µl Water 500 SP Visc "Cells" (Col. 1, Rows 3,4)
14	Aspirate	7,8 18 µl Water 500 SP Visc "Cells" (Col. 1, Rows 3,4)
15	Dispense	1-8 18 µl Water 500 SP Visc "transf1" (Col. 1, Rows 1-8) , 1 option
16	Wash Tips	1-8 5 + 5 ml
17	End Loop	"add cells"
18	Begin Loop	6 times "add cells"
19	Aspirate	1,2 18 µl Water 500 SP Visc NO DETECT "Cells" (Col. 1, Rows 3,4)
20	Aspirate	3,4 18 µl Water 500 SP Visc NO DETECT "Cells" (Col. 1, Rows 3,4)
21	Aspirate	5,6 18 µl Water 500 SP Visc NO DETECT "Cells" (Col. 1, Rows 3,4)
22	Aspirate	7,8 18 µl Water 500 SP Visc NO DETECT "Cells" (Col. 1, Rows 3,4)

23	Dispense	1-8	18 µl Water 500 SP Visc NO DETECT "transf1" (Col. 7, Rows 1-8) , 1 option
24	Wash Tips	1-8	5 + 5 ml
25	End Loop		"add cells"
26	Comment		Wait 15 minutes for cells and product to incubate
27	Start Timer		1
28	Wait for Timer		Timer 1 : 900 sec
29	Comment		Heat Shock
30	ThermalCycler		OpenLid(1)
31	Transfer Labware		Source: Grid '13,' Site '3'; Destination: Grid '63', Site '1'; Narrow (ROMA 1)
32	ThermalCycler		CloseLid(1,1)
33	ThermalCycler		StartProgram(1,AN-HS,BLOCK,ON,10)
34	ThermalCycler		WaitForProgram(1)
35	ThermalCycler		OpenLid(1)
36	Comment		Move Plate Out
37	Transfer Labware		Source: Grid '63,' Site '1'; Destination: Grid '13', Site '3'; Narrow (ROMA 1)
38	Comment		Wait 1 minute to return back to 4 degrees
39	Start Timer		4
40	Wait for Timer		Timer 4 : 60 sec
41	Comment		Add warm LB
42	Begin Loop		3 times "finish LB addition"
43	Aspirate	1-8	400 µl Water 500 SP Visc in Trough "Warm LB" (Col. 1, Rows 1-8)
44	Begin Loop		4 times "add LB over plate"


45
Dispense
1-8
100 µl Water 500 SP Visc NO DETECT
"transf1" (Col. 1, Rows 1-8) , 2 options
46
End Loop
"add LB over plate"
47
Wash Tips
1-8
5 + 5 ml
48
End Loop
"finish LB addition"
49
Comment
Transfer to round bottom plate
50
Get DiTis
Grid 6; Site: 4 (DiTi 200ul MCA)
Fetch 8 rows and 12 columns
51
Aspirate
130 µl MCA96 trough
"transf1" (Col. 1, Rows 1-8)
52
Dispense
130 µl MCA96 trough
"shaking plate" (Col. 1, Rows 1-8)
53
Drop DiTis
Back to Source
54
Comment
Move To Off Robot Shaker

Qtray Plating w/Sterile Beads

*Current Deck Layout

Directly after the bacterial transformation step is completed, the outgrowth cultures are plated onto 48-segmented Qtrays. This protocol is the most improvised procedure in the entire chapter because the Qtray is not a standard microtiter plate. Load the prepared Qtray (see appendix) with >4 sterile beads in each segment. Follow the placing diagram on the following page.

Materials

Transformed bacterial culture. This is carried over from the "CPEC Transformation" procedure as a 96-well Costar round bottom plate.

96-well Costar Sterile Round Bottom Plate(s). This plate, available from Costar (3788), is preferred for automated operations due to its robot-friendly hard shell.

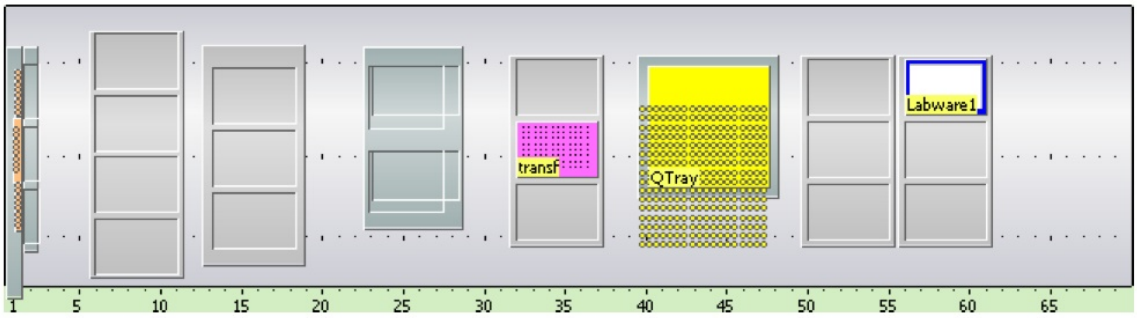
Qtray(s). This specialty petri plate, available from Genetix (x6029), has 48 subdivisions upon which different bacterial cultures can be plated. The plate was chosen as it is the only input to the Genetix Qbot, a colony picking robot located on the 2nd floor of the Beckman Institute.

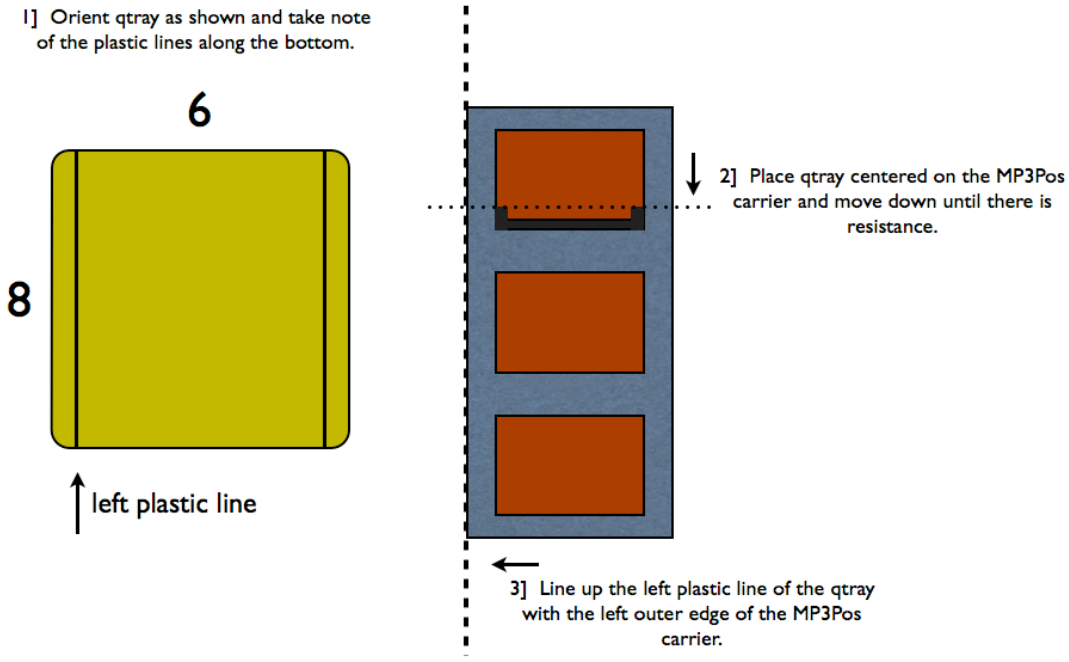
Methods

Setup

The script takes less than 5min to complete. Load a Qtray with sterile beads precisely as shown on the following page and organize the deck as seen in the figure below. After the procedure is finished cover the Qtray and shake it. Remove the beads and let the solutions air-dry under a flame. Replace the cover and incubate overnight.

Qtray: 48-segment Qtray
transf: Transformed culture in a 96-well Costar round bottom plate
Labware1: Placeholder reference for arm movements, ignore





QTray Loading

Procedure

1	Comment	Move the MP 3Pos flat carriers that normally reside at 33 and 41 to 32 and 40
2	Group	Move Roma and MCA out of the way
3	ROMA Vector	Vector "AN Move RoMa Safe-Right", Grid 56, Site 1 (ROMA 1) open, from Safe to End Position
4	Move	Positioning with global Z-Travel "Labware1" (Col. 1, Rows 1-8)
5	Group End	Move Roma and MCA out of the way
6	Comment	Plate (First 6 Columns)
7	Wash Tips	5 + 5 ml
8	Begin Loop	6 times "whole"
9	Aspirate	72 µl Water 500 SP Visc NO DETECT (Asp High) "transf" (Col. 1, Rows 1-8) , 1 option
10	Begin Loop	2 times "down"
11	Begin Loop	3 times "across"
12	Dispense	12 µl Water 500 SP Visc NO DETECT "QTray" (Col. 1, Rows 1,3,5,7,9,11,13,15) , 3 options
13	End Loop	"across"
14	End Loop	"down"
15	Wash Tips	10 + 10 ml
16	End Loop	"whole"
17	Comment	Plate (Last 6 Columns)
18	User Prompt	"Switch the Qtray" sound : once
19	Begin Loop	6 times "whole"
20	Aspirate	72 µl Water 500 SP Visc NO DETECT (Asp High) "transf" (Col. 7, Rows 1-8) , 1 option
21	Begin Loop	2 times "down"
22	Begin Loop	3 times "across"


Dispense
1-8
12 µl Water 500 SP Visc NO DETECT
"QTray" (Col. 1, Rows 1,3,5,7,9,11,13,15) , 3 options
23
End Loop
"across"
24
End Loop
"down"
25
Wash Tips
1-8
10 + 10 ml
26
End Loop
"whole"
27

Dilute IDT Oligonucleotides

Most automation projects start with ordering oligos through Integrated DNA Technologies (IDT) to either generate site-directed mutants or assemble library variants. Luckily, oligos are much cheaper when specified to arrive on a 96-well plate. Of course, certain options such as PAGE purification are unavailable in the plate format, but that feature is prohibitively expensive when ordering the amount of oligos used in automation experiments anyway.

There are three main specification pages when ordering plate oligos from IDT. For the first page, mark "file upload," "email," "96-well plate," and "column" loading. For the second page, mark "25 nmole DNA," "standard desalting," "no" CE service, "full yield," "V-bottom plate," and shipping "wet." For the third page, mark "150μM," leave the volume blank, and mark "IDTE buffer pH 7.5."

These specifications allow users to start automation experiments much faster because there is no need for resuspension, and they keep the plate in a robot-friendly column-loading scheme. The lower limit for ordering oligos in the plate format is 24. A MS Excel template is available in the lab or from IDT to upload the sequence information.

Materials

96-Well IDT Oligonucleotide Plate(s). The oligo plates from IDT arrive with a rubber capmat and lid, and taped up. Dispose of the tape but keep the capmat and the lid.

96-Well V-Bottom Plate(s). These plates, available from Nunc (249944), were chosen for diluting oligos because of their similarity to the plates from IDT. They are ordered non-sterile and are autoclaved before use.

Sterile H_2O in 100mL Disposable Trough. This trough, available from Tecan (10 613 049), only fits in the carrier located at grid 2. Fill with standard molecular biology water for diluting the oligos.

Microtiter Plate Rubber Capmat. The 96-well V-bottom plates are sealed with an Axygen ImpermaMat (AM-2ML-RD-IMP) to prevent evaporation. Non-frozen sealed plates may need to be centrifuged down prior to opening due to sample adhesion to the seal.

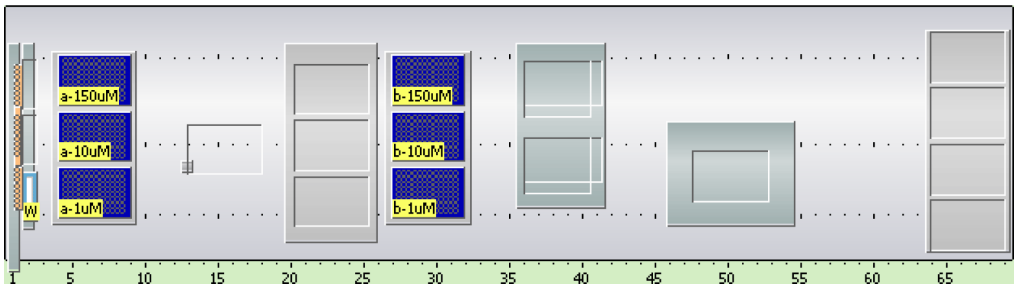
Methods

Setup

The template script is named "AN_Dilute_IDT_Oligos" and takes roughly 48 min to complete. Fill the water trough to at least 100mL. Organize the deck as shown in the figure below.

This template file creates 10µM and 1µM diluted plates from two original 150µM IDT plates. Volumes can be adjusted to dilute to concentrations more applicable to the user's downstream procedures.

Water: 100ml disposable trough
a/b-150µM: 96-well IDT oligonucleotide plates
a/b-10µM, a/b-1µM: 96-well V-bottom plates



Procedure

1	Wash Tips		5 + 5 ml
2	Comment		***START 1st PLATE SET***
3	Comment		Dilute primers into 10uM plate
4	Begin Loop		12 times "Dispense the dilutee across the 10uM plate'
5	Mix		3 x 80 µl LiHa Tip Mixing AN "a-150uM" (Col. 1, Rows 1-8) , 1 option
6	Aspirate		18 µl Water 500 SP Visc NO DETECT "a-150uM" (Col. 1, Rows 1-8) , 1 option
7	Dispense		18 µl Water 500 SP Visc NO DETECT "a-10uM" (Col. 1, Rows 1-8) , 1 option
8	Wash Tips		5 + 5 ml
9	End Loop		"Dispense the dilutee across the 10uM plate'
10	Begin Loop		12 times "Dispense the dilutor across the 10uM plate"
11	Aspirate		252 µl Water 500 SP in Trough "Water" (Col. 1, Rows 1-8)
12	Dispense		252 µl Water 500 SP "a-10uM" (Col. 1, Rows 1-8) , 1 option
13	Wash Tips		2 + 2 ml
14	End Loop		"Dispense the dilutor across the 10uM plate"
15	Comment		Dilute primers into 1uM plate
16	Begin Loop		12 times "Dispense the dilutee across the 1uM plate'
17	Aspirate		25 µl Water 500 SP Visc NO DETECT "a-10uM" (Col. 1, Rows 1-8) , 1 option
18	Dispense		25 µl Water 500 SP Visc NO DETECT "a-1uM" (Col. 1, Rows 1-8) , 1 option
19	Wash Tips		5 + 5 ml
20	End Loop		"Dispense the dilutee across the 1uM plate'
21	Begin Loop		12 times "Dispense the dilutor across the 1uM plate"
22	Aspirate		225 µl Water 500 SP in Trough "Water" (Col. 1, Rows 1-8)
23	Dispense		225 µl Water 500 SP "a-1uM" (Col. 1, Rows 1-8) , 1 option
24	Wash Tips		2 + 2 ml
25	End Loop		"Dispense the dilutor across the 1uM plate"

See Note 1

See Note 2

See Note 3

26	Comment	***START 2nd PLATE SET***	
27	Comment	Dilute primers into 10uM plate	
28	Begin Loop	12 times "Dispense the dilutee across the 10uM plate'	
29	Mix	3 x 80 µl LiHa Tip Mixing AN "b-150uM" (Col. 1, Rows 1-8) , 1 option	
30	Aspirate	18 µl Water 500 SP Visc NO DETECT "b-150uM" (Col. 1, Rows 1-8) , 1 option	
31	Dispense	18 µl Water 500 SP Visc NO DETECT "b-10uM" (Col. 1, Rows 1-8) , 1 option	
32	Wash Tips	5 + 5 ml	
33	End Loop	"Dispense the dilutee across the 10uM plate'	
34	Begin Loop	12 times "Dispense the dilutor across the 10uM plate"	
35	Aspirate	252 µl Water 500 SP in Trough "Water" (Col. 1, Rows 1-8)	
36	Dispense	252 µl Water 500 SP "b-10uM" (Col. 1, Rows 1-8) , 1 option	
37	Wash Tips	2 + 2 ml	
38	End Loop	"Dispense the dilutor across the 10uM plate"	
39	Comment	Dilute primers into 1uM plate	
40	Begin Loop	12 times "Dispense the dilutee across the 1uM plate'	
41	Aspirate	25 µl Water 500 SP Visc NO DETECT "b-10uM" (Col. 1, Rows 1-8) , 1 option	
42	Dispense	25 µl Water 500 SP Visc NO DETECT "b-1uM" (Col. 1, Rows 1-8) , 1 option	
43	Wash Tips	5 + 5 ml	
44	End Loop	"Dispense the dilutee across the 1uM plate'	
45	Begin Loop	12 times "Dispense the dilutor across the 1uM plate"	
46	Aspirate	225 µl Water 500 SP in Trough "Water" (Col. 1, Rows 1-8)	
47	Dispense	225 µl Water 500 SP "b-1uM" (Col. 1, Rows 1-8) , 1 option	
48	Wash Tips	2 + 2 ml	
49	End Loop	"Dispense the dilutor across the 1uM plate"	
50	Notification	Send email now Mayo Lab	

See Note 4

Notes

1. All mixing steps in the scripts are based on there being at least 150µL in each well. Adjust the volume if this is not the case.
2. All "1 option" loop tags instruct the LiHa to move over 1 column in every cycle of the loop.
3. If only 1 plate needs to be diluted, highlight script lines 1 to 25. Alternatively, delete all script lines after line 25.
4. At the end of the dilution script there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

Site-Directed Mutagenesis

The procedure outlined here is based on the article, "A novel megaprimed and ligase-free, PCR-based, site-directed mutagenesis method" by Tseng *et al.*, Anal. Biochem, 2008. This method was chosen and optimized as an automated protocol for the total site-saturation mutagenesis of the β1 domain of protein G. More details on the background of the method can be found in Chapter 3.

Although this site-directed mutagenesis (SDM) protocol was initially developed using New England Biolab (NEB) Hot Start Phusion DNA polymerase, the user can easily change the mastermix and appropriate dispense volumes. The thermalcycler program must be entered directly using the thermalcycler number pad.

Materials

Diluted Oligonucleotide Plate(s). Typically, the diluted mutagenic oligos are in 96-well V-bottom plates at a concentration of 1μM. These are prepared by running the "Dilute IDT Oligonucleotides" protocol.

96-Well BIO-RAD PCR Plate(s) on 96-Well Cooled Block. This PCR plate, available from BIO-RAD (HSP9601), is preferred for automated operations due to its robot-friendly hard shell and full skirt. All automated thermocycler operations have been developed exclusively with this plate. The cooled block, along with the water recirculator, can cool the samples down to 4°C.

1.5mL Eppendorf Tubes in 24-Well Cooled Block. These are the lab standard autoclavable tubes available in any biochemistry lab. The cooled block, along with the water recirculator, can cool samples down to 4°C.

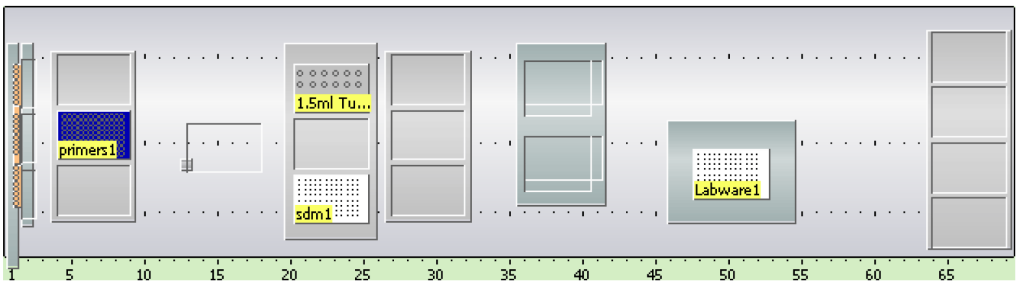
Mastermix Solution. For one 96-well plate, prepare (1) 520μL of 5× Phusion HF buffer, (2) 52μL of 10μM reverse primer, (3) 52μL of 10mM each dNTPs, (4) 13μL of 50mM $MgSO_4$, (5) 13μL of 160ng/μL plasmid template, (6) 16.25μL of Phusion Hot Start DNA Polymerase, and (7) 1430μL of sterile H_2O. This is a 130× mix (total volume: 2.1mL) of a 16μL recipe that is combined with 4μL of 1μM forward primer to obtain a final volume of 20μL in each well.

Methods

Setup

The template script is named "AN_SDM_1PLATE" and takes roughly 2.5 hr to complete, including 2 hr and 12 min for the thermalcycler protocol. Turn on the water recirculator at least 30 min before running the procedure to ensure the 24- and 96-well blocks are cool. Evenly split the 2.1mL mastermix into two 1.5mL eppendorf tubes. Organize the deck as shown in the figure below. You can ignore the labware with generic names (Labware1, Labware2, etc.) as they are only placeholders for the software.

1.5mL tubes: SDM mix split into two tubes in C1, D1 of the 24-well block
sdm1: 96-well BIO-RAD PCR plate
primers1: 96-well V-bottom plate



Troubleshooting

Even after optimizing the melting temperature (T_m) of an oligo, transformable plasmid may not have been generated after the SDM procedure. In that case, several variables can be adjusted. One could vary the 1st step annealing temperature and increase the concentration of template, $MgSO_4$, or enzyme. Of course, there may be negative consequences to changing some of these parameters (see Table 2-1 on the next page).

The mastermix components and thermalcycler program for a 96-well plate SDM are listed below. The user may use these values to calculate the appropriate amount of mastermix to prepare, depending on the number of columns and plates to be processed.

130×	1×	Mastermix Component	AN-SDM1
520	4	5× HF Phusion buffer	1. 98°C, 30 sec
52	0.4	reverse primer (10µM)	2. 98°C, 6 sec
52	0.4	dNTPs (10mM each)	3. 64°C, 15 sec
13	0.1	template (160ng/µL)	4. 72°C, 20 sec (go to step 2 for 10×)
13	0.1	$MgSO_4$ (50mM)	5. 98°C, 6 sec
16.25	0.125	HS Phusion enzyme	6. 72°C, 3 min (go to step 5 for 25×)
1430	11	water	7. 72°C, 10 min

Table 2-1. SDM Troubleshooting.

Condition	Pro	Con
1st step anneal (°C)	A temperature gradient may reveal a condition that improves the generation of 1st step product	None
Increase template	Generates more 2nd step product (transformable plasmid)	Increases wild-type recovery during sequence verification
Increase $MgSO_4$	Generates more 1st and 2nd step product	May lead to non-specific banding and/or incorrect products
Increase enzyme	Generates more 1st and 2nd step product	May lead to non-specific banding and/or incorrect products

Procedure

1	Wash Tips		5 + 5 ml
2	Comment		***SETUP PLATE 1***
3	Comment		Transfer SDM (2 tubes of 1.05mL each = 1 96 well plate)
4	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
5	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
6	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
7	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
8	Begin Loop		6 times "mastermix1-6"
9	Dispense		16 µl Water 500 SP Visc "sdm1" (Col. 1, Rows 1-8) , 1 option
10	End Loop		"mastermix1-6"
11	Wash Tips		5 + 5 ml
12	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
13	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
14	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
15	Aspirate		96 µl Water 500 SP Visc "1.5ml Tubes" (Col. 1, Rows 3,4)
16	Begin Loop		6 times "mastermix7-12"
17	Dispense		16 µl Water 500 SP Visc "sdm1" (Col. 7, Rows 1-8) , 1 option
18	End Loop		"mastermix7-12"
19	Wash Tips		5 + 5 ml
20	Comment		Transfer Oligos from primers1
21	Begin Loop		12 times "p1primers"
22	Aspirate		4 µl Water 500 SP Visc NO DETECT "primers1" (Col. 1, Rows 1-8) , 1 option
23	Dispense		4 µl Water 500 SP Visc NO DETECT "sdm1" (Col. 1, Rows 1-8) , 1 option
24	Mix		2 x 12 µl LiHa Tip Mixing AN "sdm1" (Col. 1, Rows 1-8) , 1 option
25	Wash Tips		5 + 5 ml
26	End Loop		"p1primers"
27	Comment		Move sdm1 to thermocycler
28	ThermalCycler		OpenLid(1)

See Note 1

See Note 2

See Note 3

See Note 4

29	Transfer Labware	Source: Grid '20,' Site '3'; Destination: Grid '46', Site '1'; User defined (Narrow (ROMA 1)	
30	ThermalCycler	CloseLid(1,1)	See Note 5
31	Comment	Check program name	
32	ThermalCycler	StartProgram(1,AN-SDM1,BLOCK,ON,10)	
33	Comment	program will run for ~2hr	See Note 6
34	Group	Put lid on primers1	
35	Comment	Put on the lid for overnight	See Note 7
36	ROMA Vector	Vector "Hotel 9Pos Microplate_Narrow_1", Grid 58, Site 1 (ROMA 1) open, from Safe to End Position, grip, from End to Safe Position	
37	ROMA Vector	Vector "MP 3Pos Flat: Lid Grip", Grid 4, Site 2 (ROMA 1) move from Safe to End Position, open, from End to Safe Positior	
38	Group End	Put lid on primers1	
39	ThermalCycler	WaitForProgram(1)	
40	Comment	Move sdm1 out	
41	ThermalCycler	OpenLid(1)	
42	Transfer Labware	Source: Grid '46,' Site '1'; Destination: Grid '20', Site '3'; User defined (Narrow (ROMA 1)	
43	Group	Put lid on sdm1	
44	Comment	Put on the lid for overnight	
45	ROMA Vector	Vector "Hotel 9Pos Microplate_Narrow_1", Grid 58, Site 2 (ROMA 1) open, from Safe to End Position, grip, from End to Safe Position	
46	ROMA Vector	Vector "MP 3Pos Cooled-Lid Grip: PCR plate", Grid 20, Site 3 (ROMA 1) move from Safe to End Position, open, from End to Safe Positior	
47	Group End	Put lid on sdm1	
48	Notification	Send email now Mayo Lab	See Note 8

Notes

1. The amount of mastermix placed in each tube is key to the script moving forward because the aspirate commands use liquid detection. If it measures an insufficient volume, the script will stop and ask for input from the user. If this happens, click "Go to Z-max," which will move the tip to the bottom of the tube to aspirate.
2. Script lines 8 and 16 control how many columns of the sdm plate the robot will dispense into. For example, if you wish to only dispense into 9 columns and not 12, change script line 16 to 3 loops. Don't forget to change the aspiration volumes of lines 12–15 to reflect the smaller amount of mastermix needed. Continuing the example, each aspiration volume would be changed to 48μL.
3. Script line 21 controls how many columns of the sdm plate to fill and mix with oligos from the oligo plate. For example, if you only had 9 columns of oligos, change the number in line 21 to 9 loops.
4. The liquid class "LiHa Tip Mixing AN" does a satisfactory job in mixing the oligos into the mastermix. The volume to mix is based on a 20μL total volume; adjust this if the script is being changed to accommodate larger total reaction volumes.
5. The Transfer Labware commands used in lines 29 and 42 employ custom made vectors that handle the tricky BIO-RAD PCR plates. The plates are difficult for the RoMa to work with primarily because of the restricted space around the thermalcycler loading area.
6. The actual thermalcycler program call occurs here on line 32. Adjust the program name as necessary.
7. Grouped commands on lines 34–38 and 43–47 pick up a manufacturer agnostic lid (it can fit plates from Greiner, Costar, Nunc, etc.) from sites 1 and 2 of the 9pos microplate hotel and place them on the oligo plate and sdm plate. These lines are useful if this script is going to be run overnight.
8. At the end of the mutagenesis script, there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

Dpn1 Digestion

The Dpn1 digestion step is necessary in the site-directed mutagenesis automated pipeline to remove the wild-type template plasmid. A more efficient digestion step will directly lead to decreased wild-type contamination in the sequencing results and improved recovery of the mutant of interest.

A traditional bench PCR digestion step maintains the Dpn1 enzyme concentration at 2–4% of the reaction mixture. The Dpn1 mix described in the Materials below keeps the enzyme concentration at 8% to digest as much methylated and hemi-methylated wild-type DNA as possible.

The default thermalcycler protocol is 37°C for 2 hr. This can probably be adjusted to 30 min, or even decreased to 5 min if the manufacturer's (NEB) advertising is true.

Materials

Site-Directed Mutagenesis DNA Product on 96-Well Cooled Block. This is typically carried over from the "Site-Directed Mutagenesis" procedure as a 96-well BIO-RAD PCR plate. The cooled block, along with the water recirculator, can cool the samples down to 4°C.

1.5mL Eppendorf Tubes in 24-Well Cooled Block. These are the lab standard autoclavable tubes available in any biochemistry lab. The cooled block, along with the water recirculator, can cool samples down to 4°C.

Dpn1 Mix. For site-directed mutagenesis product in 10µL aliquots, prepare (1) 175µL of 10× buffer 4, (2) 140µL of Dpn1 enzyme, and (3) 185µL of Diluent B for one 96-well plate. When 4µL of this mix is added to 10µL PCR product, buffer 4 and the Dpn1 enzyme attain effective concentrations of 1× and 8%, respectively.

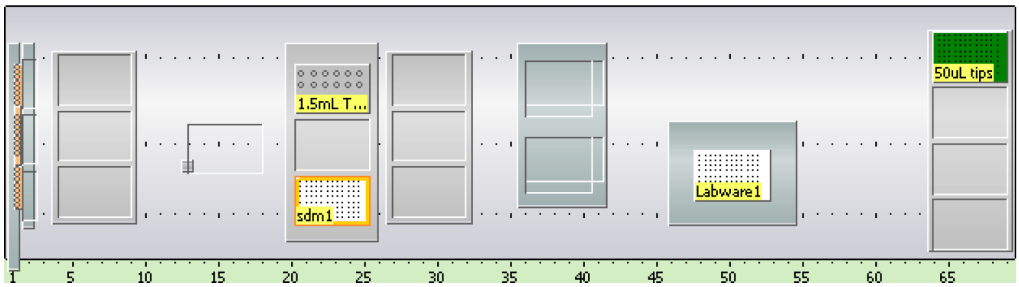
50µL Robot Filter Tips. These automation tips are the ART BioRobotix tips available from Molecular BioProducts (#906-021). They are individually wrapped in green boxes.

Methods

Setup

The template script is named "AN_Dpn1_1PLATE" and takes roughly 2.5 hr to complete, including the 2 hr digestion time. Turn on the water recirculator at least 30 min before running the procedure to ensure the 24- and 96-well blocks are cool. Organize the deck as shown in the figure below. You can ignore the labware with generic names (Labware1, Labware2, etc.), as they are only placeholders for the software.

1.5mL tubes: Dpn1 mix split into two tubes in C1, D1 of the 24-well block
sdm1: 96-well BIO-RAD PCR plate
50µL tips: 50µL robot filter tips (black box)



Advanced Technique:

The Dpn1 digest protocol can be tagged onto a trusted SDM procedure to save time. In addition, a few different techniques can be used to get the SDM samples down to 10µL for efficient digestion. First, 10µL of each sample is needed to run the HT DNA Agarose Gel Electrophoresis procedure. This puts the surplus volume to good use. Second, a more complicated yet time efficient procedure is to aspirate 10µL with the MCA96, dispense that volume into a waste trough, use LiHA multi-pipetting to deliver the Dpn1 mix, and finally mix the samples with the same 50µL robot filter tips used in the first aspirate. This will decrease the procedure's time by about 25 min.

Procedure

1	Wash Tips	5 + 5 ml	
2	Comment	Pipet out sdm product, leaving only 10uL	See Note 1
3	Group	MCA96 commands	
4	Group	Move_MCA_Safe_to_Right_Back_Corner	See Note 2
5	Command	"W1PAA,,2362," wait	
6	Command	"W1PAA,10,," wait	
7	Move Vector	Vector "Safe-RightBackCorner", Grid 64, Site 1(MCA 1) move from Safe to End Positior	
8	Group End	Move_MCA_Safe_to_Right_Back_Corner	
9	Get DiTis	Grid 64; Site: 1 (DiTi 50ul Filter MCA) Fetch 8 rows and 12 columns	
10	Move	Positioning with global Z-Trave "1.5mL Tubes" (Col. 1, Rows 1-4)	See Note 3
11	Aspirate	10 µl MCA96 trough "sdm1" (Col. 1, Rows 1-8)	
12	Move	Positioning with global Z-Trave "1.5mL Tubes" (Col. 1, Rows 1-4)	
13	Drop DiTis	Back to Source	
14	Group End	MCA96 commands	
15	Comment	***Dpn1 DIGEST PLATE 1***	
16	Comment	2 tubes of .25ml = 1 96 well plate	
17	Begin Loop	12 times "dpn1addmix"	
18	Aspirate	4 µl Water 500 SP Visc "1.5mL Tubes" (Col. 2, Rows 3,4)	
19	Aspirate	4 µl Water 500 SP Visc "1.5mL Tubes" (Col. 2, Rows 3,4)	
20	Aspirate	4 µl Water 500 SP Visc "1.5mL Tubes" (Col. 2, Rows 3,4)	
21	Aspirate	4 µl Water 500 SP Visc "1.5mL Tubes" (Col. 2, Rows 3,4)	
22	Dispense	4 µl Water 500 SP Visc NO DETECT "sdm1" (Col. 1, Rows 1-8) , 1 option	

See Note 4

23	Mix	 	3 x 6 µl LiHa Tip Mixing AN "sdm1" (Col. 1, Rows 1-8) , 1 option
24	Wash Tips		10 + 10 ml
25	End Loop		"dpn1addmix"
26	Comment		Move sdm1 out for incubatior
27	ThermalCycler		OpenLid(1)
28	Transfer Labware		Source: Grid '20,' Site '3'; Destination: Grid '46', Site '1'; User defined (Narrow (ROMA 1)
29	ThermalCycler		CloseLid(1,1)
30	Comment		Check program name
31	ThermalCycler		StartProgram(1,AN-37INC,BLOCK,ON,10)
32	ThermalCycler		WaitForProgram(1)
33	ThermalCycler		OpenLid(1)
34	Transfer Labware		Source: Grid '46,' Site '1'; Destination: Grid '20', Site '3'; User defined (Narrow (ROMA 1)
35	Comment		Put on the lid for overnight
36	ROMA Vector		Vector "Hotel 9Pos Microplate_Narrow_1", Grid 58, Site 1 (ROMA 1) open, from Safe to End Position, grip, from End to Safe Position
37	ROMA Vector		Vector "MP 3Pos Cooled-Lid Grip: PCR plate", Grid 20, Site 3 (ROMA 1) move from Safe to End Position, open, from End to Safe Positior
38	Notification		Send email now Mayo Lab

See Note 5

See Note 6

See Note 7

See Note 8

Notes

1. The Group “MCA96 commands,” encompassing lines 3–14, instruct the robot to pick up tips, aspirate 10µL from the “sdm1” plate, and drop off the tips (along with the volume) back into the tip box. There is no need to dispense the 10µL volume because the tips (and tip box) will not be used for anything else in this script.
2. The Group “Move_MCA_Safe_to_Right_Back_Corner” is a set of commands that orient the MCA96 into a safe position starting from anywhere on the deck. This helps to avoid crashes when executing the “Get DiTis” command.
3. Lines 10 and 12 instruct the MCA96 to move to a far site so that it won’t collide with the thermalcycler as it moves between the MP3Pos carriers and its DiTi carrier.
4. The liquid class “LiHa Tip Mixing AN” does a satisfactory job in mixing the samples with the Dpn1 enzyme. The volume to mix is based on a 14µL total volume. Adjust this if the script is being changed to accommodate larger total volumes of product and Dpn1 enzyme.
5. The Transfer Labware commands used in lines 28 and 34 employ custom made vectors that handle the tricky BIO-RAD PCR plates. The plates are difficult for the RoMa to work with primarily because of the restricted space around the thermalcycler loading area.
6. The actual thermalcycler program call occurs here on line 31. Adjust the program name as necessary.
7. Lines 36 and 37 pick up a manufacturer agnostic lid (it can fit plates from Greiner, Costar, Nunc, etc.) from site 1 of the 9pos microplate hotel and place it on the newly Dpn1 digested SDM product plate. These lines are useful if this script is going to be run overnight.
8. At the end of the digestion script, there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

Bacterial Transformation

Although an automated cell-free expression protocol would obviate the need for bacterial transformation, few assays have the prerequisite sensitivity to deal with the low levels of protein expression from cell-free extracts. In most cases, bacterial transformation and subsequent plating will be a necessary step in automated pipelines.

Competent cell manipulation was kept at a minimum to avoid disturbing the cells. The proper setting for the outgrowth step (2 hr shaking at room temperature) was determined empirically.

Currently (3/24/10), the RoMA is having difficulty loading the PCR plate onto the Te-Shake. We therefore recommend running the script to line 47, manually loading the PCR plate, and then running the remainder of the script after line 49.

Materials

Dpn1 Digested Product. This is typically carried over from the "Dpn1 Digestion" procedure as a 96-well BIO-RAD PCR plate.

1.5mL Eppendorf Tubes in 24-Well Cooled Block. These are the lab standard autoclavable tubes available in any biochemistry lab. The cooled block, along with the water recirculator, can cool samples down to 4°C.

BL21 Gold DE3 Competent Cells. These homemade chemically competent cells are very effective in transforming plasmid and nicked DNA and are not very sensitive to variations in heatshock protocols. For one 96-well plate, thaw 2.2mL of competent cells from the –80°C freezer. Their preparation protocol is included in the appendix of this manual.

96-Well BIO-RAD PCR Plate(s) on 96-Well Cooled Block. This PCR plate, available from BIO-RAD (HSP9601), is preferred for automated operations due to its robot-friendly hard shell and full skirt. All automated thermocycler operations have been developed exclusively with this plate. The cooled block, along with the water recirculator, can cool the samples down to 4°C.

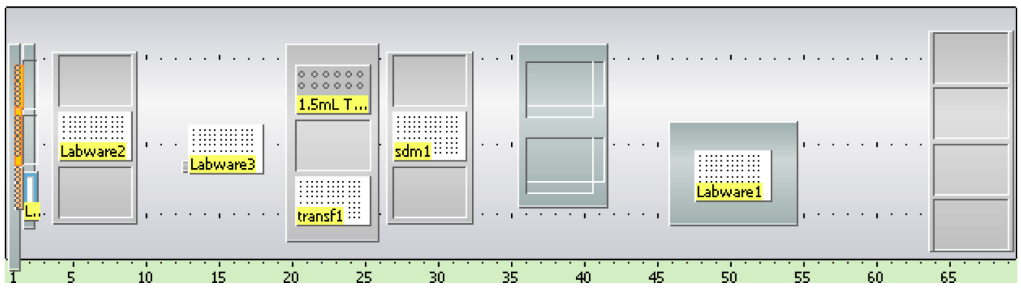
LB in 100mL Trough. These troughs, available from Tecan (10 613 049), only fit in the carrier located at grid 2. Fill with at least 15mL of lab standard LB liquid media for the transformation of one 96-well plate. Keep warm before use for better results.

Methods

Setup

The template script is named "AN_Transformation_1Plate" and takes roughly 2.5 hr to run, including the 2 hr shaking outgrowth period. Turn on the water recirculator at least 30 min before running the procedure to ensure the 24- and 96-well blocks are cool. Organize the deck as shown in the figure below. You can ignore the labware with generic names (Labware1, Labware2, etc.), as they are only placeholders for the software.

1.5mL tubes: Competent cells split into 2 tubes in C1, D1 of the 24-well block
sdm1: Dpn1 digested product
transf1: 96-well BIO-RAD PCR plate on 96-well cooled block
LB: LB in 100mL trough



Remember, as of 3/24/10, run the script to line 47, manually load the PCR plate onto the Te-Shake, and then run the rest of the script starting from line 49.

Procedure

See Note 1

See Note 2

See Note 3

1	Wash Tips		5 + 5 ml
2	Comment		***Add Dpn1 product to chilled plate***
3	Begin Loop		12 times "add dpn1 product"
4	Aspirate	 	2 µl Water 500 SP Visc NO DETECT (Asp High) "sdm1" (Col. 1, Rows 1-8) , 1 option
5	Dispense	 	2 µl Water 500 SP Visc NO DETECT "transf1" (Col. 1, Rows 1-8) , 1 option
6	Wash Tips		5 + 5 ml
7	End Loop		"add dpn1 product"
8	Comment		***Add competent cells to plate 1***
9	Comment		Use 2 tubes of 1.1 ml of cells each per 96 well plate
10	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
11	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
12	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
13	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
14	Begin Loop		6 times "add cells 1-6"
15	Dispense	 	18 µl Water 500 SP Visc "transf1" (Col. 1, Rows 1-8) , 1 option
16	End Loop		"add cells 1-6"
17	Wash Tips		5 + 5 ml
18	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
19	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
20	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
21	Aspirate	 	108 µl Water 500 SP Visc "1.5mL Tubes" (Col. 1, Rows 3,4)
22	Begin Loop		6 times "add cells 7-12"
23	Dispense	 	18 µl Water 500 SP Visc "transf1" (Col. 7, Rows 1-8) , 1 option
24	End Loop		"add cells 7-12"
25	Wash Tips		5 + 5 ml
26	Comment		Wait 15 minutes for cells and product to incubate
27	Start Timer		1
28	Wait for Timer		Timer 1 : 900 sec

29	Comment	***Heat Shock Plate 1***	
30	ThermalCycler	OpenLid(1)	
31	Transfer Labware	Source: Grid '20,' Site '3'; Destination: Grid '46', Site '1'; User defined (Narrow (ROMA 1)	See Note 4
32	ThermalCycler	CloseLid(1,1)	
33	ThermalCycler	StartProgram(1,AN-HS,BLOCK,ON,10)	See Note 5
34	ThermalCycler	WaitForProgram(1)	
35	ThermalCycler	OpenLid(1)	
36	Comment	move plate to left carrier	
37	Transfer Labware	Source: Grid '46,' Site '1'; Destination: Grid '4', Site '2'; User defined (Narrow (ROMA 1)	
38	User Prompt	"add warm LB to trough" sound : no	See Note 6
39	Comment	add warm LB to plate 1	
40	Begin Loop	3 times "add LB over plate"	
41	Aspirate	400 µl Water 500 SP Visc in Trough "LB" (Col. 1, Rows 1-8)	
42	Begin Loop	4 times "add LB 4x"	
43	Dispense	100 µl Water 500 SP Visc NO DETECT "Labware2" (Col. 1, Rows 1-8) , 2 options	
44	End Loop	"add LB 4x"	
45	Wash Tips	5 + 5 ml	
46	End Loop	"add LB over plate"	

47	Comment	***Shake the plate for 2 hrs***
48	Transfer Labware	Source: Grid '4,' Site '2'; Destination: Grid '13', Site '1'; Narrow (ROMA 1)
49	Te-Shake Shaker	SetFrequency(1800)
50	Begin Loop	12 times "shakeit"
51	Te-Shake Shaker	Start(1)
52	Start Timer	2
53	Wait for Timer	Timer 2 : 60 sec
54	Te-Shake Shaker	Stop()
55	Start Timer	3
56	Wait for Timer	Timer 3 : 180 sec
57	Te-Shake Shaker	Start(1)
58	Start Timer	2
59	Wait for Timer	Timer 2 : 60 sec
60	Te-Shake Shaker	Stop()
61	Start Timer	4
62	Wait for Timer	Timer 4 : 300 sec
63	End Loop	"shakeit"
64	Notification	Send email now Mayo Lab

See Note 7

See Note 8

Notes

1. Script line 3 controls how many columns of the "sdm1" plate to add to the "transf1" plate. For example, if you only had 9 columns of digested product, change the number in line 3 to 9 loops.
2. The amount of volume in the 1.5mL tubes is key to the script proceeding properly. If there is an input error, hit "Move tips to Z-Max" to aspirate from the bottom of the tube. Script lines 14 and 22 control how many columns of the "transf1" plate the robot will dispense into. For example, if you wish to only dispense into 9 columns and not 12, change script line 22 to 3 loops. Don't forget to change the aspiration volumes of lines 10–13 and 18–21 to reflect the smaller amount of competent cells needed. Continuing the example, change each aspiration volume to 54µL.
3. Common in most transformation protocols, script lines 26–28 incubate the plate for 15 min at 4°C.
4. The Transfer Labware commands used in lines 31 and 37 employ custom made vectors that handle the tricky BIO-RAD PCR plates. The plates are difficult for the RoMa to work with primarily because of the restricted space around the thermalcycler loading area.
5. The actual thermalcycler program call occurs here on line 33. Adjust the program name as necessary.
6. Script line 38 is a user prompt that stops the script and only continues when a user clicks OK in the software. Ideally, this is when the user will take warm LB and add it to the 100mL trough.
7. The Te-Shake is addressed by a Start command, then a timer, and finished by a Stop command. In lines 49–63, the Te-Shake's frequency is set (1800 is the maximum), followed by commands for 12 cycles of 1 min shake, 3 min rest, 1 min shake, and 5 min rest. This was programmed to keep the cells suspended in media over 2 hr.
8. At the end of the transformation script there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

QTray Plating

Immediately after the bacterial transformation step is completed, the outgrowth cultures are plated onto 48-segmented Qtrays. This protocol is the most improvised procedure in the entire chapter because the Qtray is not a standard microtiter plate. After finding a consistent location for the Qtray on the deck, a simple and convenient spreading method is used, which pipettes just enough culture in the plate segment to both spread by gravity and dry in a reasonable amount of time.

Materials

Transformed bacterial culture. This is typically carried over from the "Bacterial Transformation" procedure as a 96-well BIO-RAD PCR plate.

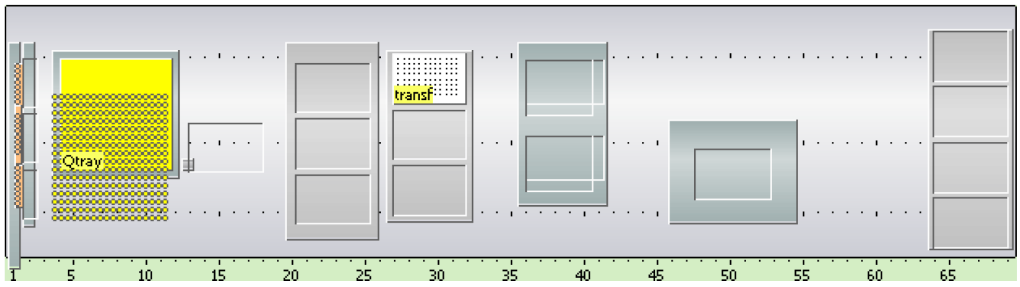
Qtray(s). This specialty petri plate, available from Genetix (x6029), has 48 subdivisions upon which different bacterial cultures can be plated. The plate was chosen because it is the only input to the Genetix Qbot, a colony picking robot located on the 2nd floor of the Beckman Institute.

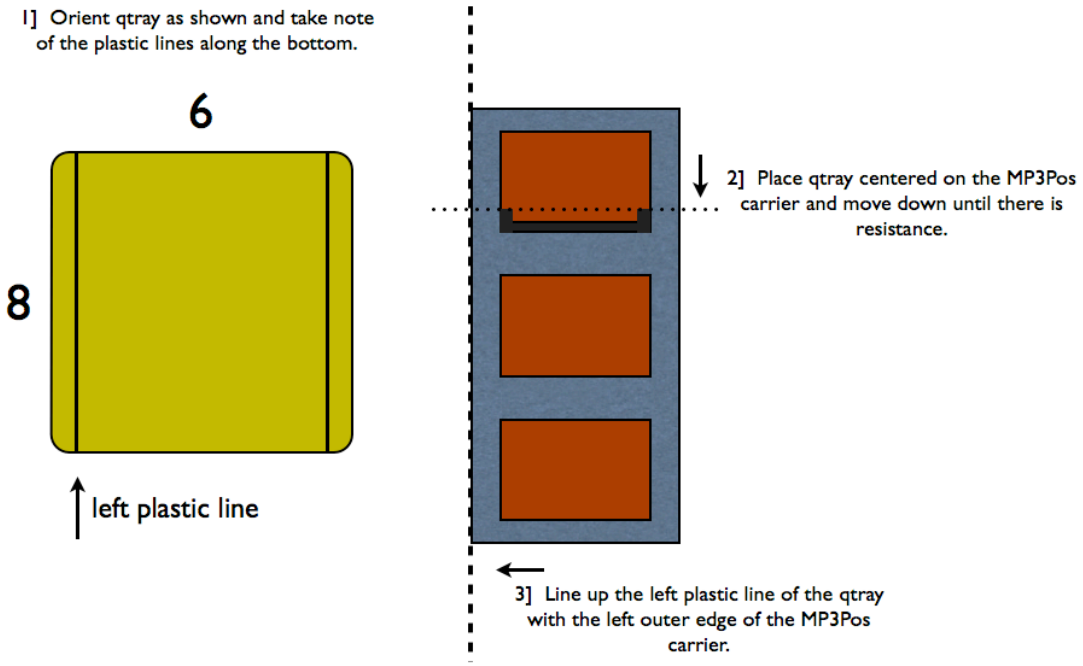
Methods

Setup

The template script is named "AN_qtrayplating" and takes less than 5 min to complete. Load the Qtray precisely as shown on the following page and organize the deck as seen in the figure below. After the procedure is finished, cover the Qtray and shake slightly to spread the drops in each segment. Remove the cover and let the solutions air dry. Replace the cover and incubate overnight.

Qtray: 48-segment Qtray
transf: 96-well BIO-RAD PCR plate



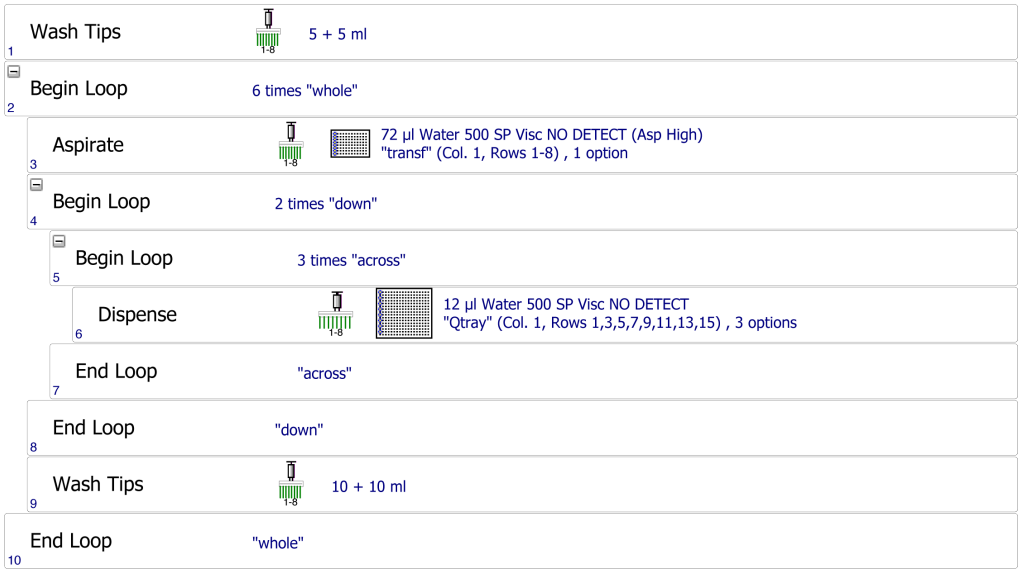


QTray Loading

Procedure

See Note 1

See Note 2



Notes

1. Change the loop "whole" to reflect the number of columns you wish to plate. Typically, leave this at 6, the maximum number of columns you can plate onto a 48-segment Qtray.
2. Double click this LiHa aspirate command on line 4 and change the starting column from column 1 to column 7 when plating the second half of "transf." Save the changes and run the altered protocol.

Culture Rearray

The colony picking robot, or Qbot, is located on the 2nd floor of the Beckman Institute and is available for supervised use by all members in the Mayo Lab. Contact Autumn (email: qiuy@caltech.edu) to schedule a session. The preferred output plate of the Qbot is a Genetix 384-well plate. However, it is beneficial for us to generate 96-well glycerol stock plates because it gives the experimentalist more volume to pull from when it is time to inoculate expression plates. For the site-directed mutagenesis experimental pipeline, 8 colonies are picked per reaction (48 different reactions per 384-well plate) and 2 colonies are sent for initial sequencing to Agencourt Bioscience. Hence, this robot protocol takes 2 colony cultures per reaction from a 384-well plate and rearrays them into 2 96-well plates.

Materials

384-Well Genetix Picking Culture Plate(s). These plates, available from Genetix, are used exclusively with the Qbot to inoculate picked cultures into liquid media. They can be filled with LB/antibiotic on the colony picking day by the Qfill instrument, located in the Qbot room. For this protocol, the plate should contain overnight cultures.

96-Well Round-Bottom Plates(s). These polystyrene (PS) plates, available from Falcon (351177), are used to send samples to Agencourt Bioscience for sequencing and to keep as frozen stock from which to inoculate downstream volumes of media. These plates were chosen because of their optical clarity, which allows visual checks of bacterial growth, and for their sterility right out of the box. They are typically sealed with aluminum sticker seals. Fill with 250µL of LB/10% glycerol before use on the robot.

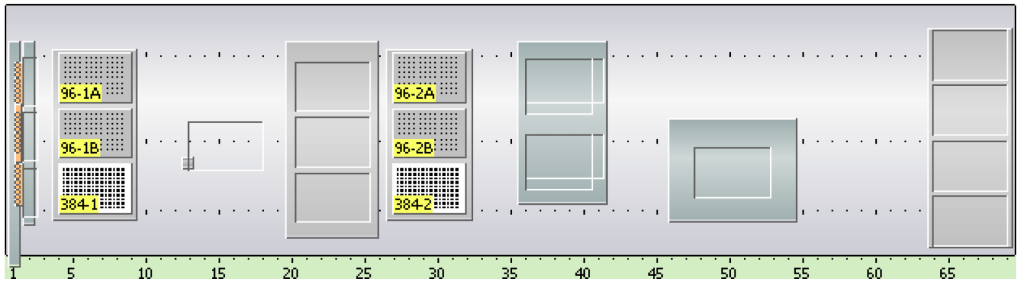
LB With 10% Glycerol. Four 96-well plates (2 384-well plates, or looking back, 1 96-well site-directed mutagenesis plate) require about 100mL of this media. We recommend making 1L in a 2L Corning bottle by mixing 100mL of glycerol with 25g of LB granules and filling with 900mL of MilliQ water.

Methods

Setup

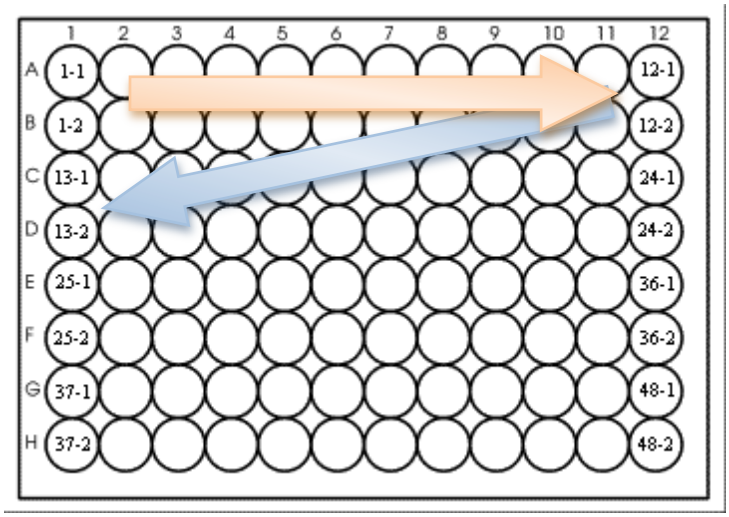
The template script is named "AN_Qbot_rearray" and takes roughly X min to complete. It will inoculate the first two picked colony cultures from each reaction of the 384-well plate into a fresh 96-well plate. It then creates a duplicate 96-well plate to send for sequencing and continues to do the same procedure on another 384-well plate. Organize the deck as shown in the figure below.

96-1A, 96-1B, 96-2A, 96-2B: 96-well round-bottom plates
384-1, 384-2: 384-well Genetix picking culture plates



Colony Placement:

Execution of the script inoculates the first two colony cultures (X-1, X-2) from the 384-well plate into the 96-well array shown below. The 96-well plate uses the column-style numbering from the 48-well Qtray the 384-well plate was picked from. The arrows are provided as guides for possible manual inoculations and the subsequent sequencing sample identification.



Procedure

	1	Wash Tips	5 + 5 ml
	2	Comment	rearray 1st 384-well plate
See Note 1	3	Begin Loop	2 times "duplicate plate"
	4	Begin Loop	12 times "across plate"
See Note 2	5	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-1" (Col. 1, Rows 1,3) , 1 option
	6	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-1" (Col. 2, Rows 1,3) , 1 option
	7	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-1" (Col. 1, Rows 2,4) , 1 option
	8	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-1" (Col. 2, Rows 2,4) , 1 option
	9	Dispense	15 µl Water 500 SP Visc NO DETECT "96-1A" (Col. 1, Rows 1-8) , 2 options
	10	Wash Tips	5 + 5 ml
	11	End Loop	"across plate"
	12	End Loop	"duplicate plate"
See Note 3	13	Comment	rearray 2nd 384-well plate
	14	Begin Loop	2 times "duplicate plate"
	15	Begin Loop	12 times "across plate"
	16	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-2" (Col. 1, Rows 1,3) , 1 option
	17	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-2" (Col. 2, Rows 1,3) , 1 option
	18	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-2" (Col. 1, Rows 2,4) , 1 option
	19	Aspirate	15 µl >> Water 500 SP Visc NO DETECT << "384-2" (Col. 2, Rows 2,4) , 1 option
	20	Dispense	15 µl Water 500 SP Visc NO DETECT "96-2A" (Col. 1, Rows 1-8) , 2 options
	21	Wash Tips	5 + 5 ml
	22	End Loop	"across plate"
	23	End Loop	"duplicate plate"
See Note 4	24	Notification	Send email now Mayo Lab

Notes

1. This loop on script line 3 controls the creation of a duplicate plate to send to Agencourt for sequencing.
2. Script lines 5–8 control which colony cultures to pick from out of the 384-well plate. Currently, they are pulling the top two colony cultures from each reaction. If you wanted colony cultures 3 and 4, line 5 should read (Col. 1, Rows 5,7), line 6 should read (Col. 2, Rows 5,7), line 7 should read (Col. 1, Rows 6,8) and line 8 should read (Col. 2, Rows 6,8). Also notice that the liquid class name is encapsulated by ">> <<" notations, indicating a custom liquid class. In this case, the liquid class "Water 500 SP Visc NO DETECT" is prefixed with a tip mixing routine to resuspend bacterial culture that may have settled. This feature was implemented in this way to avoid having an extra four Tip Mixing script lines in the code before each aspiration block.
3. The comment on line 13 separates the script into two parts, indicating the completion of the first 384-well plate.
4. At the end of the rearray script, there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

Expression Plate Inoculation

For experimental assays that require a large amount of protein, it is very beneficial to grow 5mL bacterial cultures in 24-well plates as opposed to 1mL cultures in 96-well deep-well plates. To assist with this task, a robotic procedure was developed to inoculate 24-well plates from a 96-well liquid stock plate. Incubate the 24-well plates for 16 hr with shaking and at the appropriate temperature for optimal protein expression.

Materials

96-Well Frozen and Liquid Stock Plate(s). Typically, 96-well frozen stocks are kept in Falcon round-bottom PS plates (351177) with aluminum seals. These are then replicated into identical plates filled with 200µL of LB/antibiotic and grown statically overnight. This liquid stock plate is then used to inoculate the 24-well plates.

24-Well Expression Plate(s), Lid(s), and Capmat(s). These round-bottom Whatman plates (7701-5110) are made of polypropylene (PP), arrive sterile, and can be autoclaved and reused. The BugStopper capmats from Whatman (7704-0014) are porous to air, autoclavable, and keep the wells sealed from each other during incubated shaking.

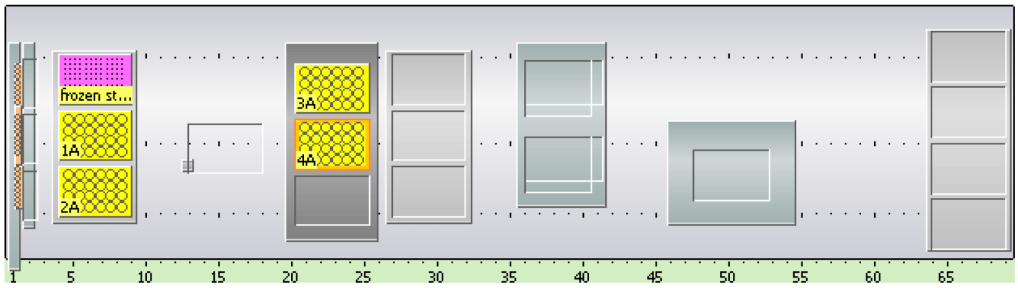
Auto-Induction TB Media and Antibiotic. This media, available from Novagen (71491-4), obviates the need for an induction agent such as IPTG and can be prepared in the autoclave or microwave. Any leftover media is refrigerated. For 1L, add 1 packet (60g) to 10mL of glycerol and fill with water. Add the appropriate antibiotic before dispensing into the 24-well plates.

Methods

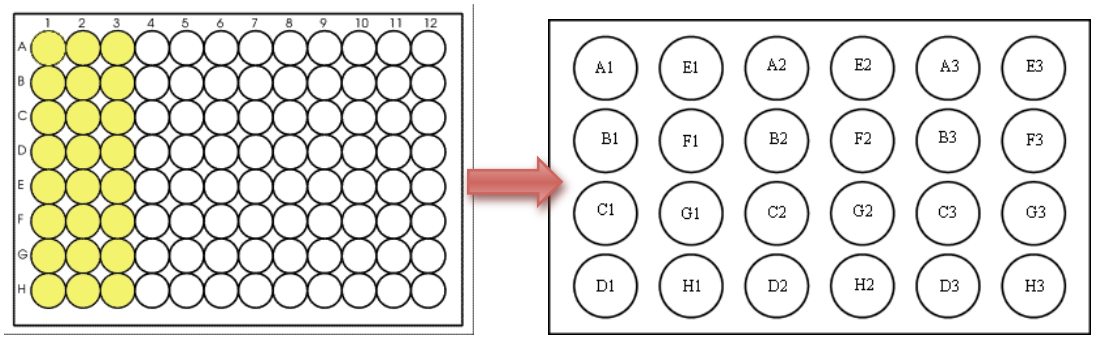
Setup

The template script is named "AN_96inoculate_1x5mL" and takes roughly 10 min to complete. Organize the deck as shown in the figure below.

Frozen/liquid stock plate: 96-well Falcon round-bottom PS plate
1A,2A,3A,4A: 24-well Whatman round-bottom plates



Example:



Every three columns of the 96-well plate is moved to a 24-well plate following the labeling shown above.

Procedure

1	Wash Tips	1-8	5 + 5 ml
2	Comment		inoculate 1 96well plate into 4 24well plates
3	Comment		inoculate plates 1A and 2A
4	Begin Loop		2 times "diffplate"
5	Begin Loop		3 times "aspdisp"
6	Mix	1-8	2 x 75 µl LiHa Tip Mixing AN "frozen stock" (Col. 1, Rows 1-8) , 2 options
7	Aspirate	1-8	25 µl Water 500 SP Visc NO DETECT "frozen stock" (Col. 1, Rows 1-8) , 2 options
8	Dispense	1-4	25 µl Water 500 SP Visc NO DETECT submerged dispense "1A" (Col. 1, Rows 1-4) , 2 options
9	Dispense	5-8	25 µl Water 500 SP Visc NO DETECT submerged dispense "1A" (Col. 2, Rows 1-4) , 2 options
10	Wash Tips	1-8	10 + 10 ml
11	End Loop		"aspdisp"
12	End Loop		"diffplate"
13	Comment		inoculate plate 3A
14	Begin Loop		3 times "aspdisp"
15	Mix	1-8	2 x 75 µl LiHa Tip Mixing AN "frozen stock" (Col. 7, Rows 1-8) , 1 option
16	Aspirate	1-8	25 µl Water 500 SP Visc NO DETECT "frozen stock" (Col. 7, Rows 1-8) , 1 option
17	Dispense	1-4	25 µl Water 500 SP Visc NO DETECT submerged dispense "3A" (Col. 1, Rows 1-4) , 1 option
18	Dispense	5-8	25 µl Water 500 SP Visc NO DETECT submerged dispense "3A" (Col. 2, Rows 1-4) , 1 option
19	Wash Tips	1-8	10 + 10 ml
20	End Loop		"aspdisp"
21	Comment		inoculate plate 4A
22	Begin Loop		3 times "aspdisp"
23	Mix	1-8	2 x 75 µl LiHa Tip Mixing AN "frozen stock" (Col. 10, Rows 1-8) , 1 option
24	Aspirate	1-8	25 µl Water 500 SP Visc NO DETECT "frozen stock" (Col. 10, Rows 1-8) , 1 option
25	Dispense	1-4	25 µl Water 500 SP Visc NO DETECT submerged dispense "4A" (Col. 1, Rows 1-4) , 1 option
26	Dispense	5-8	25 µl Water 500 SP Visc NO DETECT submerged dispense "4A" (Col. 2, Rows 1-4) , 1 option
27	Wash Tips	1-8	10 + 10 ml
28	End Loop		"aspdisp"

See Note 1

See Note 2

See Note 3

See Note 4

Notes

1. The mixing steps are based on there being at least 100µL in each well of the stock plate. It is OK to be more aggressive and add more cycles if the cells have precipitated.
2. The "2 options" loop tag on script lines 6–9 inform the LiHa to (1) move over 1 column every "aspdisp" and (2) either move over 3 columns in every "diffplate" in lines 6 and 7 or move down 1 labware in every "diffplate" in lines 8 and 9. The first option is consistent with the "1 option" tag in script lines 15–18 and 23–26.
3. The liquid class has been defined to dispense liquids under the presumed volume level in the destination plate. This minimizes cross-contamination from ricochet droplets.
4. The next two 24-well plates (plates 3A and 4A) are inoculated in separate blocks of script code, which helps to visualize what is going on.

Protein Purification

Automated protein purification has long been a major goal of high-throughput methodology, hence the existence of several commercial applications. Several companies offer 96-well nickel filter plates and readily available lysis and elution reagents. Much of the procedure described here was developed by balancing the simplicity of ordering materials against the cost of those materials.

Purification protocols vary from protein to protein, and the procedure here is no different. All conditions were developed with the β1 domain of protein G, and preparation of other protein domains will likely need some optimization. In addition, the vacuum steps in the procedure are not entirely robust and currently require human supervision. This procedure is fed directly from the "Expression Plate Inoculation" protocol described earlier. This signifies that the unpacking scheme used to inoculate 24-well plates is used to transfer the cell lysates to the 96-well nickel filter plate.

Materials

Expression Cultures With Lids. Typically, automation experiments use 24-well round-bottom Whatman PP plates (7701-5110) filled with 5mL cultures of bacteria. Autoclavable BugStopper capmats from Whatman (7704-0014) are used as lids during overnight growth.

Centrifuge Plate Adapters. The Sorvall Legend RT centrifuge with a swing-out rotor in Broad 140 (autoclave room) has four hanging bucket adapters for microtiter plates.

Lysis Buffer. For four 24-well plates, make 45mL of lysis buffer consisting of (1) 4.5mL of 10× CelLytic B (Sigma-Aldrich), (2) 0.9mL of 10mg/mL lysozyme (Sigma-Aldrich), (3) 9µL of HC benzonase (Sigma-Aldrich), (4) 0.45mL of elution buffer, and (5) enough equilibration buffer to fill to 45mL.

His-Select Filter Plate(s). These nickel plates, available from Sigma-Aldrich (H0413), are the most expensive reagent in the purification process. However, the purity in all purification schemes performed so far has been excellent.

96-Well Reservoir Plate. Available from Seahorse Scientific (#S30014), these are pyramid-bottom PP reservoirs.

Equilibration, Wash, and Elution Buffers in 100mL Troughs. These troughs, available from Tecan (10 613 049), only fit in the carrier located at grid 2. To purify one 96-well plate, prepare 57.6mL of equilibration buffer (50mM $NaPO_4$ buffer, 300mM NaCl at pH 8), 115.2mL of wash buffer (equilibration buffer + 5mM imidazole at pH 8), and 48mL of elution buffer (equilibration buffer + 250mM imidazole at pH 8). Be sure to have at least 15% more volume than required in the appropriate troughs.

200µL Robot Tips. These automation tips are available from USA Scientific (TipONE #1188-1700). They are stacked with yellow inserts.

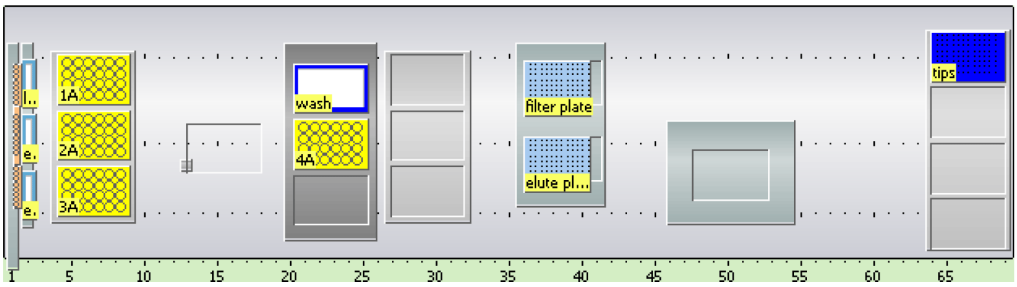
96-Well Collection Plate(s) and Plate Seal(s). These plates are manufactured by Axygen and are available from VWR (P-DW-11-C-S). They are 1mL deep-well PP plates and arrive sterilized. These plates fit and work well with the Tecan-provided spacer #2 and are used to minimize cross contamination during elution. The plastic sticker seal is then applied to prevent evaporation.

Methods

Setup

The template script is named "AN_ProtPurif_1x5ml_96" and takes roughly 1 hr to complete. Organize the deck as shown in the figure below.

1A,2A,3A,4A: Expression cultures in 24-well Whatman round-bottom plates
Lysis/equilibration/elution buffers: Stored in 100mL troughs at grid 2
Wash buffer: 96-well reservoir plate
Filter plate: 96-well His-Select filter plate, on top of separation block
Elute plate: 96-well collection plate on top of spacer #2
Tips: 200µL robot tips (blue box)



Prior to the robot procedure:

1. Centrifuge 24-well expression cultures at 3060 ×g and dump the supernatant.
2. Freeze plates at –20°C for at least 15 min. Bring back to 25°C. Once pellets are sufficiently thawed, proceed with the robot protocol.

Procedure

See Note 1

See Note 2

1	Wash Tips	1-8	10 + 10 ml
2	Comment		Lyse all plates on left carrier
3	Begin Loop		3 times "lyse all plates on carrier"
4	Begin Loop		3 times "lyse plate"
5	Aspirate	1-8	400 µl Water 500 SP Visc in Trough "lysis buffer" (Col. 1, Rows 1-8)
6	Dispense	1-4	400 µl Water 500 SP Visc NO DETECT "1A" (Col. 1, Rows 1-4) , 2 options
7	Mix	1-4	5 x 440 µl LiHa Lysate Resuspension AN "1A" (Col. 1, Rows 1-4) , 2 options
8	Dispense	5-8	400 µl Water 500 SP Visc NO DETECT "1A" (Col. 2, Rows 1-4) , 2 options
9	Mix	5-8	5 x 440 µl LiHa Lysate Resuspension AN "1A" (Col. 2, Rows 1-4) , 2 options
10	Wash Tips	1-8	10 + 10 ml
11	End Loop		"lyse plate"
12	End Loop		"lyse all plates on carrier"
13	Comment		Lyse plate on middle carrier
14	Begin Loop		3 times "lyse plate"
15	Aspirate	1-8	400 µl Water 500 SP Visc in Trough "lysis buffer" (Col. 1, Rows 1-8)
16	Dispense	1-4	400 µl Water 500 SP Visc NO DETECT "4A" (Col. 1, Rows 1-4) , 1 option
17	Mix	1-4	5 x 440 µl LiHa Lysate Resuspension AN "4A" (Col. 1, Rows 1-4) , 1 option
18	Dispense	5-8	400 µl Water 500 SP Visc NO DETECT "4A" (Col. 2, Rows 1-4) , 1 option
19	Mix	5-8	5 x 440 µl LiHa Lysate Resuspension AN "4A" (Col. 2, Rows 1-4) , 1 option
20	Wash Tips	1-8	10 + 10 ml
21	End Loop		"lyse plate"
22	Comment		Add equlibration buffer to filter plate and vacuum
23	Begin Loop		12 times "across plate"
24	Begin Loop		2 times "600ul"
25	Aspirate	1-8	300 µl Water 500 SP Visc in Trough "equil buffer" (Col. 1, Rows 1-8)
26	Dispense	1-8	300 µl Water 500 SP Visc NO DETECT "filter plate" (Col. 1, Rows 1-8) , 1 option
27	End Loop		"600ul"
28	Wash Tips	1-8	5 + 5 ml
29	End Loop		"across plate"

30	Comment	run equlibrium buffer	
31	Vacuum Separation	ApplyVacuumRear(700)	See Note 3
32	Start Timer	1	
33	Wait for Timer	Timer 1 : 25 sec	
34	Vacuum Separation	VentRear()	
35	Vacuum Separation	DeactivateSystem()	
36	Comment	Add cell lysates to filter plate and vacuum	
37	Group	cell lysate pipetting	
38	Comment	plates1-3	
39	Begin Loop	3 times "carrier"	See Note 4
40	Begin Loop	3 times "addlysate"	
41	Mix	1-4 5 x 440 µl LiHa Lysate Resuspension AN "1A" (Col. 1, Rows 1-4) , 2 options	
42	Aspirate	1-4 425 µl Water 500 SP Visc NO DETECT "1A" (Col. 1, Rows 1-4) , 2 options	
43	Mix	5-8 5 x 440 µl LiHa Lysate Resuspension AN "1A" (Col. 2, Rows 1-4) , 2 options	
44	Aspirate	5-8 425 µl Water 500 SP Visc NO DETECT "1A" (Col. 2, Rows 1-4) , 2 options	
45	Dispense	1-8 425 µl Water 500 SP Visc NO DETECT "filter plate" (Col. 1, Rows 1-8) , 2 options	
46	Wash Tips	1-8 10 + 10 ml	
47	End Loop	"addlysate"	
48	End Loop	"carrier"	
49	Comment	plate4	
50	Begin Loop	3 times "addlysate"	
51	Mix	1-4 5 x 440 µl LiHa Lysate Resuspension AN "4A" (Col. 1, Rows 1-4) , 1 option	
52	Aspirate	1-4 425 µl Water 500 SP Visc NO DETECT "4A" (Col. 1, Rows 1-4) , 1 option	
53	Mix	5-8 5 x 440 µl LiHa Lysate Resuspension AN "4A" (Col. 2, Rows 1-4) , 1 option	
54	Aspirate	5-8 425 µl Water 500 SP Visc NO DETECT "4A" (Col. 2, Rows 1-4) , 1 option	
55	Dispense	1-8 425 µl Water 500 SP Visc NO DETECT "filter plate" (Col. 10, Rows 1-8) , 1 option	
56	Wash Tips	1-8 10 + 10 ml	
57	End Loop	"addlysate"	
58	Group End	cell lysate pipetting	

59	Comment	run lysate	
60	Vacuum Separation	ApplyVacuumRear(150)	
61	Start Timer	1	
62	Wait for Timer	Timer 1 : 60 sec	
63	Vacuum Separation	VentRear()	
64	Vacuum Separation	DeactivateSystem()	
65	User Prompt	"Check if all lysate went through." sound : no	See Note 5
66	Comment	Add Wash Buffer	
67	Begin Loop	2 times "2x wash step"	
68	Group	Move_MCA_Safe_to_Right_Back_Corner	See Note 6
69	Command	"W1PAA,,2362," wait	
70	Command	"W1PAA,10,," wait	
71	Move Vector	Vector "Safe-RightBackCorner", Grid 64, Site 1(MCA 1` move from Safe to End Positior	
72	Group End	Move_MCA_Safe_to_Right_Back_Corner	
73	Get DiTis	Grid 64; Site: 1 (DiTi 200ul MCA) Fetch 8 rows and 12 columns	
74	Move	Positioning with global Z-Trave "wash" (Col. 1, Rows 1-4)	See Note 7
75	Begin Loop	3 times "wash buffer"	
76	Aspirate	200 µl MCA96 trough "wash" (Col. 1, Rows 1-8)	
77	Dispense	200 µl MCA96 trough "filter plate" (Col. 1, Rows 1-8)	
78	End Loop	"wash buffer"	
79	Drop DiTis	Back to Source	
80	Comment	run wash buffer	
81	Vacuum Separation	ApplyVacuumRear(500)	
82	Start Timer	1	
83	Wait for Timer	Timer 1 : 30 sec	
84	Vacuum Separation	VentRear()	
85	Vacuum Separation	DeactivateSystem()	
86	End Loop	"2x wash step"	

87	User Prompt	"Wipe filter plate nozzles if necessary." sound : no
88	Comment	move filter plate to front Vac position
89	Comment	Pickup block at rear position
90	ROMA Vector	Vector "Te-VacS_Narrow_1", Grid 36, Site 2 (ROMA 1) open, from Safe to End Position, grip, from End to Safe Position
91	Comment	Deliver block to front positior
92	ROMA Vector	Vector "Te-VacS_Narrow_1", Grid 36, Site 5 (ROMA 1) move from Safe to End Position, open, from End to Safe Positior
93	Move ROMA	Move to home position (ROMA 1)
94	Comment	Add elution buffer to filter plate and vacuum
95	Begin Loop	12 times "across plate"
96	Begin Loop	2 times "500ul"
97	Aspirate	250 µl Water 500 SP Visc in Trough "elute buffer" (Col. 1, Rows 1-8)
98	Dispense	250 µl Water 500 SP Visc NO DETECT "elute plate" (Col. 1, Rows 1-8) , 1 option
99	End Loop	"500ul"
100	Wash Tips	5 + 5 ml
101	End Loop	"across plate"
102	Comment	run elution buffer
103	Vacuum Separation	ApplyVacuumFront(300)
104	Start Timer	1
105	Wait for Timer	Timer 1 : 45 sec
106	Vacuum Separation	VentFront()
107	Vacuum Separation	DeactivateSystem()
108	User Prompt	"Check if all elution volumes went through.' sound : no
109	Notification	Send email now Mayo Lab

See Note 8

See Note 9

Notes

1. The "2 options" loop tags in lines 6–9 refer to the loops defined in lines 3 and 4. The loop in line 3, "lyse all plates on carrier," moves through the labware on the current carrier. The loop in line 4, "lyse plate," moves through every 2 columns on the 24-well plates. Script lines 14–21 define an isolated "lyse plate" loop for the fourth 24-well plate on the middle carrier at grid 20.

2. All mixing steps use the special liquid class "LiHa Lysate Resuspension AN" developed exclusively for this protein purification procedure. Lysates are mixed immediately after the lysis buffer dispense command and again before being added to the nickel filter plate. These steps do an adequate to superb job of resuspending the pellets.

3. All the Vacuum Separation commands in the script follow the same structure. The first (line 31) tells which of the two blocks to pull from (rear or front) and at what pressure (mbar). Then the script counts off a timer, after which the pressure is vented (line 34) and the vacuum is turned off (line 35). All vacuum pressure and timer parameters were determined empirically and can and should be adjusted depending on what the user sees during a run.

4. The loops within the cell lysate pipetting steps are identical to those described in Note 1.

5. Line 65 (and later on, lines 87 and 108) has user prompts that stop the run, unlock the shield, and require the user to click OK. Due to the non-robustness of the current purification protocol, these prompts are necessary.

6. The Group "Move_MCA_Safe_to_Right_Back_Corner" is a set of commands that orient the MCA96 into a safe position starting from anywhere on the deck. This helps to avoid crashes when executing the "Get DiTis" command.

7. Line 74 instructs the MCA96 to move to a far site so that it won't collide with the thermalcycler as it moves between the MP3Pos carriers and its DiTi carrier.

8. Lines 89–93 are very specific RoMa vectors that were developed to move the separation block from the rear to the front position.

9. At the end of the purification script there is a command to send an email to the user. This is useful since the script runs longer than 15 min.

HT Agarose Gel Electrophoresis

In order to aid with the high-throughput visualization of nucleic acid product, Invitrogen has developed 48- and 96-well agarose gel systems for use on liquid handling platforms. The 96-well version was purchased and adapted for use on the Tecan Freedom EVO. Check the Invitrogen website for questions concerning what percentage agarose is appropriate for the size of the products being separated.

Invitrogen provides the MS Windows program "E-Editor" that aids in visualization by aligning EGel images into a variety of convenient arrays.

Materials

Nucleic Acid Product. Typically, this is DNA product from a site-directed mutagenesis procedure in a 96-well BIO-RAD PCR plate.

E-Gel Low Range Quantitative DNA Ladder. This ladder is from Invitrogen (12373-031). For one 96-well agarose gel, 200µL of a 1:1 mixture of ladder and water is required.

E-Gel 96 2% Agarose Gel(s). These are available in packs of eight from Invitrogen (G7008-02). This version uses ethidium bromide for staining, but Invitrogen also offers non-carcinogenic versions.

Mother Ebase. This is the base (EB-M03) for the 48- and 96-well agarose and PAGE gels that Invitrogen offers. They can be daisy chained, but we currently only have one. Plug it into the power strip underneath the deck.

50µL Robot Filter Tips. These automation tips are the ART BioRobotix tips available from Molecular BioProducts (#906-021). They are individually wrapped in green boxes.

1.5mL Eppendorf Tubes in 24-Well Cooled Block. These are the lab standard autoclavable tubes available in any biochemistry lab placed in the 24-well cooled block. For this method, the block doesn't need to be cold.

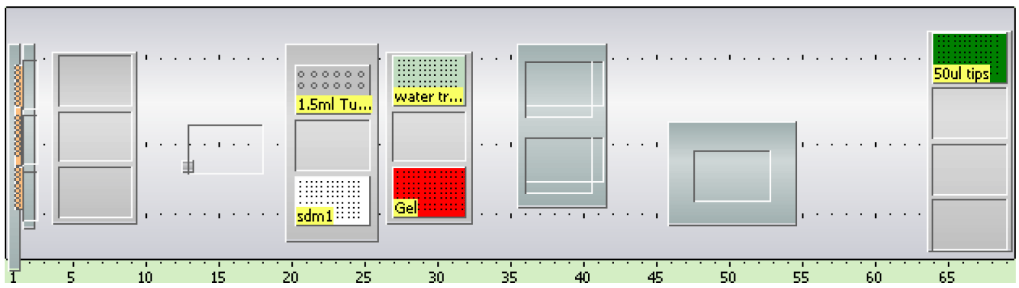
Sterile H_2O in 96-Well Low Profile Reservoir Plate. Available from Seahorse Scientific (S300-18), these are pyramid-bottom PP reservoirs. Although the low profile is not required, the method was developed when only this reservoir was available. Fill with standard sterile DI water.

Methods

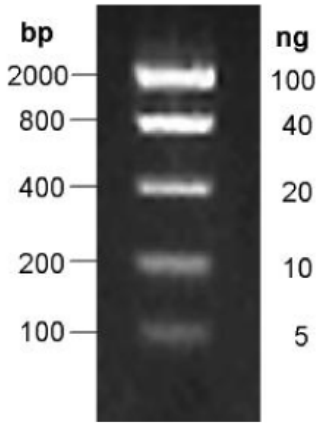
Setup

The template script is named "AN_EGel_load_econ" and takes roughly 20 min to complete, including the 12-min electrophoresis. Organize the deck as shown in the figure below. Plug in the Mother Ebase and make sure it is set to "EG" for E-Gel. Open the E-Gel package and load the E-Gel. The display on the Mother Ebase should switch to a 12-min timer.

1.5ml tubes: Put the 1:1 DNA ladder/water mix into A4 of the 24-well block
sdm1: 96-well BIO-RAD PCR plate
Water trough: 96-well low profile reservoir plate
Gel: Mother Ebase loaded with an E-Gel 96
50µL tips: 50µL robot filter tips (black box)



E-Gel Low Range Quantitative DNA Ladder



Procedure

1	Group	Move_MCA_Safe_to_Right_Back_Corner	See Note 1
2	Command	"W1PAA,,2362," wait	
3	Command	"W1PAA,10,," wait	
4	Move Vector	Vector "Safe-RightBackCorner", Grid 64, Site 1(MCA 1) move from Safe to End Positior	
5	Group End	Move_MCA_Safe_to_Right_Back_Corner	
6	Comment	Dilute the DNA samples 1:1 inside the tip and dispense into the EGe	
7	Get DiTis	Grid 64; Site: 1 (DiTi 50ul MCA) Fetch 8 rows and 12 columns	
8	Move	Positioning with global Z-Trave "water trough" (Col. 1, Rows 1-8)	See Note 2
9	Aspirate	10 µl MCA96 trough "water trough" (Col. 1, Rows 1-8)	
10	Aspirate	10 µl MCA96 trough (2nd aspirate) "sdm1" (Col. 1, Rows 1-8)	See Note 3
11	Dispense	20 µl MCA96 Diti egel "Gel" (Col. 1, Rows 1-8)	
12	Move	Positioning with global Z-Trave "water trough" (Col. 1, Rows 1-8)	
13	Drop DiTis	Back to Source	
14	Comment	Add the DNA Ladder to the eGel96; 200uL of 1:1 water/ladde	
15	Wash Tips	5 + 5 ml	
16	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
17	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
18	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
19	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
20	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
21	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
22	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
23	Aspirate	20 µl Water 500 SP Visc NO DETECT "1.5ml Tubes" (Col. 4, Row 1)	
24	Dispense	20 µl Water 500 SP Visc NO DETECT "Gel" (Col. 13, Rows 1-8)	
25	Wash Tips	5 + 5 ml	

See Note 4

Notes

1. The Group "Move_MCA_Safe_to_Right_Back_Corner" is a set of commands that orient the MCA96 into a safe position starting from anywhere on the deck. This helps to avoid crashes when executing the "Get DiTis" command.
2. Lines 8 and 12 instruct the MCA96 to move to a far site so that it won't collide with the thermalcycler as it moves between the MP3Pos carriers and its DiTi carrier.
3. The liquid class "MCA96 trough (2nd aspirate)" used in line 10 was developed to allow the MCA96 to introduce a small airgap between successive aspirations. By doing this, the script obviates the need for a separate dilution plate and instead dispenses 10µl each of water and DNA product directly into the E-Gel.
4. After the script is finished, check the E-Gel for any missed dispenses. If everything is satisfactory, run the 12-min electrophoresis program.

Chapter 3: Site-Directed Mutagenesis

Overview

Although there are numerous site-directed mutagenesis (SDM) procedures available, it can be difficult to select and effectively employ the protocol that best serves your application. In this case, an automated SDM protocol should be cost-effective, simple in terms of enzymatic steps, and robust enough to avoid manual intervention. The method described by Tseng *et al,* Anal. Biochem, 2008 titled "A novel megaprimed and ligase-free, PCR-based, site-directed mutagenesis method" satisfies all of these prerequisites. The single mutagenic primer halves oligo costs when compared against the standard quickchange method. The absence of phosphorylation or ligase steps simplifies the overall procedure and with some tweaking the method can be robust enough to not incur a large number of repeat reactions.

Some modifications were made to the procedure described in the paper in order to further accommodate the needs of automated protocols. The most important change was to the choice of polymerase from Pfu Turbo to NEB's Hot Start Phusion Polymerase. The new enzyme is faster (speeds reaction times from 8 hours to 2 hours), advertised as higher fidelity and its Hot Start feature prevents it from modifying template and primer DNA before the initial melting step. Polymerase choice has a large effect on the successful outcome of this particular SDM protocol, and therefore should be investigated for each new system. A minor modification to the overall procedure was the usage of smaller mutagenic oligos which can function as well as larger oligos so long as the annealing temperature has been optimized. The financial benefits of ordering hundreds of shorter oligos are quickly realized.

The SDM protocol is outlined below in Figure 1. First, megaprimers are amplified by annealing the mutagenic and constant flanking oligos to the template. Then, full length product is generated by annealing the megaprimers to the template and completing the extension. Finally, a Dpn1 digestion step is necessary to remove the parental wild-type template.

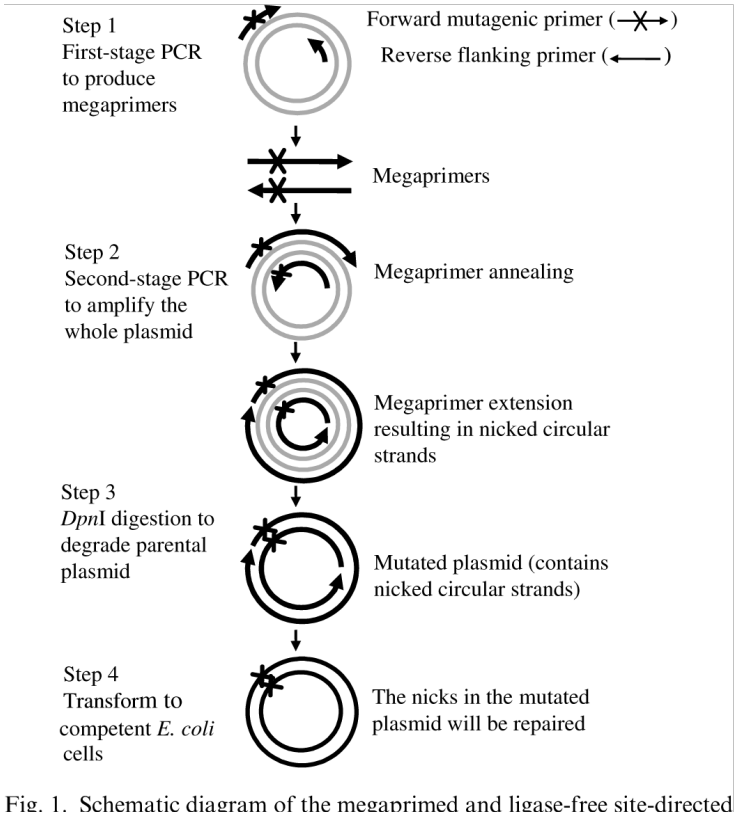


Fig. 1. Schematic diagram of the megaprimed and ligase-free site-directed

Figure 1. Schematic of the megaprimed and ligase-free SDM method. Adapted from Tseng *et al*, Anal. Biochem. 2008.

Oligonucleotide Design

In the original paper, an oligo Tm calculation is described that includes a "%mismatch" variable in order to account for the number of basepairs that won't anneal exactly to the template strand. Initial oligo design work was guided by this Tm value, and five amino acid scans (A, V, S, T, and M) of the small model system GB1 were tested. Upon experimental construction of the variants it was noticed that the calculated Tm values had no bearing on successful amplification of the mutagenic megaprimer and therefore subsequent full length mutated plasmid. Instead, successful amplification and mutagenesis correlated better with the Tm values calculated by IDT, done by the nearest neighbor method. In fact, a later review of the data showed that a simpler Tm calculator was almost as good as the complex nearest neighbor method in terms of predicting successful amplification.

The first few amino acid scans were designed manually in an excel spreadsheet. In order to facilitate the entire high-throughput scheme, a python script was developed (oligotm.py) that scans a given nucleotide sequence and generates a mutagenic oligo for each codon in the sequence while keeping the Tm within a user-defined range. This range should reflect the annealing temperature used in the first step of the SDM reaction. The Tm calculators implemented so far are the basic Tm calculator (oligo tm = 64.9+41*(number of gc bp)-16.4)/(number of total bp) and the mismatch Tm calculator (oligo tm = 81.5+0.41*(oligo gc%)-675/(number of total bp)-mismatch%). The script

output can easily be tailored to allow for a simple copy/paste operation into an IDT excel order form, thus simplifying oligonucleotide design.

As is the script is ideal for site-directed scanning mutagenesis, facilitating the design of oligos for small projects such as alanine scans all the way to larger projects like total site saturation mutagenesis of a protein domain. Alternatively, automated oligonucleotide design for codon saturation mutagenesis schemes (all codons at non-continuous sites) can be adapted from the existing functions defined in the python script.

Experimental Pipeline

The methods for automated site-directed mutagenesis were developed in response to the desire for a database containing stability data for every single-mutant of the GB1 domain. The project was initiated in the spring of 2009 and saw completion by the summer of 2010. A simple schematic of the experimental pipeline is shown in Figure 2. Protocols were first developed as separate modules and later strung together into an efficient system. Over the course of the project, clustered groups of samples fed to the experimental pipeline provided opportunities to individually refine and optimize each module. Currently, 192 single mutants can be constructed and sent for confirmation sequencing in five days with minimal experimentalist strain.

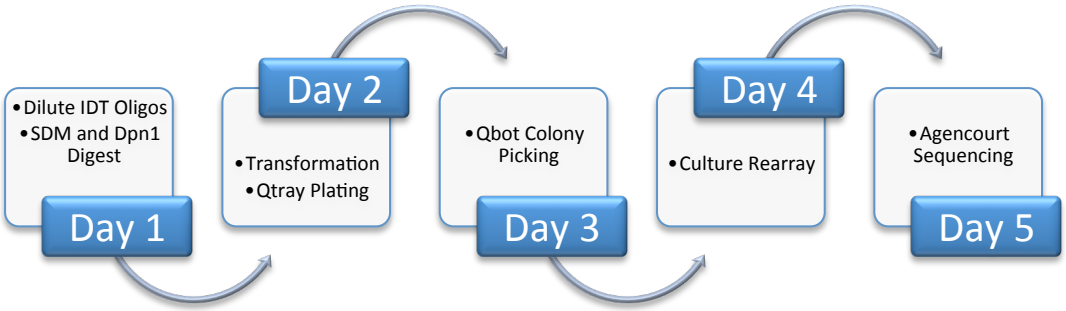


Figure 2. The 5 day SDM experimental pipeline. The majority of the procedure is automated by stringing together the relevant robotic procedures described in chapter 2.

Day 1

Allow the oligos delivered from IDT to come to room temperature. Prepare and run the protocols "Dilute IDT Oligos", "Site-Directed Mutagenesis", and "Dpn1 Digest". For maximum efficiency, generate "2plate" versions of SDM and Dpn1 Digest and then combine them into one ~8hr script that can be run overnight.

Day 2

In the afternoon of the second day, prepare and run the protocols "Bacterial Transformation" and "Qtray Plating". For maximum efficiency, generate a "2plate" version of Bacterial Transformation.

Day 3

Colony picking can be performed manually at the bench or robotically by contacting Autumn (qiuy@caltech.edu) in the Beckman Institute (BI) to assist with the Qbot colony picker. If done robotically, on the morning of the third day bring the Qtray plates and an appropriate volume of LB/antibiotic (153.6ml for 4 Qtrays) to the BI. Incubate the picked 384-well Genetix plates overnight.

Day 4

On the morning of the fourth day prepare and run the "Culture Rearray" protocol for the four 384-well plates. Incubate the generated 96-well plates for 12 hours.

Day 5

On the morning of the fifth day, prepare the 96-well plates for shipping by first professionally labeling them, then affixing aluminum seals and finally freezing them with dry ice. Fill a Styrofoam box with freezer packs, the plates, and the Agencourt order form and ship it via Fedex. In approximately one week, a representative will contact with data downloading instructions.

The fantastic increase in experimental productivity provided by these automation methods should not be overlooked. As an example, consider the time courses shown in Table 1. Before the introduction of automation methods to the lab, only handfuls of single mutants could be generated at any single time for stability studies. It is unrealistic for one experimentalist to routinely construct and verify any more than 5 mutants with these manual methods, especially considering the large time commitment required in performing stability determination by circular dichroism. The robotics assisted method for constructing single mutants presented here, coupled with the plate-based stability assay described in Allen, Nisthal and Mayo, 2010 is able to generate and analyze 192 mutants in about the same amount of time. The limiting step in this procedure is the week long turnaround time of confirmation sequencing. However, this turnaround time should remain constant if more mutants are sent for sequencing, ensuring the superb scalability of the automated methods.

Table 1. Time Required to construct and measure stabilities for single mutants. The lab procedure in 2007 (manual construction) was only feasible for a handful of mutants. The current state of automation in the lab allows for ~40x more mutants to be constructed and analyzed in roughly the same amount of time.

Procedural Step	Manual Construction (5 mutants)	Robotics Assisted Construction (192 mutants)
SDM and Dpn1 Digest	1.5 days	Overnight
Transformation and Plating	0.5 day	0.5 day
Colony Picking	0.5 day	0.5 day
Protein Expression and Purification	1.5 days	1 day
Sequencing Turnaround	1 day	7 days
Dialysis	Overnight	Overnight
Stability Determination	5 days	1 day
Total Time Required	**10 days**	**10 days**

Chapter 4: Automated Gene Assembly

Co-written with Samy Hamdouche

Overview

The chief benefit to developing automation technology in the lab is to expedite the interplay between theory and experiment. Having the ability to take the results from a protein design calculation and in a minimal amount of days produce viable, sequence verified mutant genes should greatly improve the productivity of the lab.

The project was first approached from the experimental side, as it was necessary to determine whether it was possible to perform the molecular biology needed to construct and clone genes into an expression plasmid in an automated fashion. After failing to adapt the complex gene construction methods detailed in Allen *et al,* PNAS 2010 for automation, more simplified protocols were tested. To assist with constructing multiple variants the DNAworks source code was acquired and installed onto our local computer cluster. By ordering completely overlapping oligonucleotides (as designed by DNAworks), performing a PCR assembly reaction, and then amplifying the product with flanking primers we were able to recover the correct sequence-verified gene more than half the time. The next step required automating molecular cloning for further protein expression and production.

The traditional cloning methods of restriction enzymes and overnight ligation reactions are already not very robust when done on the bench, so in their stead we first adapted the PCR cloning method called PIPE, for polymerase incomplete primer extension (Klock *et al*, Proteins, 71, 2008). As shown in Figure 4-1, the PCR products from an insert amplification (IPIPE) and a plasmid linearization/amplification (VPIPE) are mixed together and transformed directly into cells, without any modifications. Any wild-type background is eradicated by limiting the choice of plasmid to those containing a suicide gene such as ccdB, where upon transformation the gene product kills the cell. Although this method performs admirably under benchtop conditions (~40 colonies

per 300uL transformation), attempts to transfer that success to the robotic platform have failed to produce enough colonies in a robust fashion. To address this problem a second PCR cloning method was introduced into the lab called CPEC, or circular polymerase extension cloning (Quan and Tian, PLoS ONE, 2009). Figure 4-2 shows that the method is essentially a site-overlap extension (SOE) PCR reaction that uses the IPIPE and VPIPE

products as templates for each other in order to create a fully assembled plasmid. Benchtop testing with this method has shown a direct correlation between number of PCR cycles and amount of transformed bacteria, with 15 cycles of CPEC producing ~800 colonies from a 300uL transformation. The primary reason for the twenty-fold improvement in transformable material is that where the PIPE procedure uses the minor product of a PCR reaction (incomplete PCR products) to form complete plasmid, the CPEC method's major product is our desired end product.

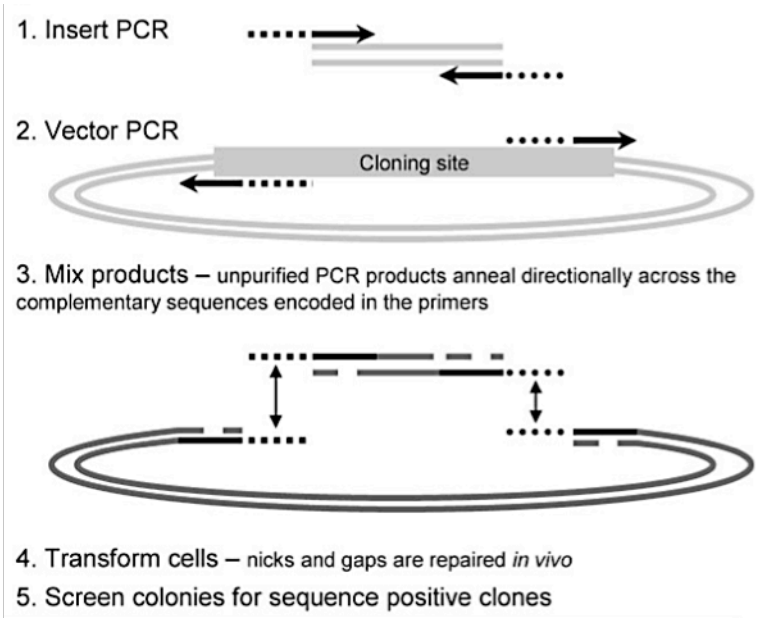


Figure 4-1. Schematic of the polymeriase incomplete primer extension method, or PIPE. Adapted from Klock *et al*, 2008.

Plugging in the transformation, plating and picking routines developed for automated site-directed mutagenesis (Chapter 3) completes the experimental pipeline for gene assembly. Detailed robotic protocols for the steps mentioned above can be found in Chapter 2. Finally, a significant amount of scripting was required in order to bridge the gap between the sequence lists produced by typical protein design software and robot-compatible worklists. We now describe our method for taking sequence lists from standard or library designs and ultimately outputting an IDT oligonucleotide order form and an oligo assembly worklist for the robot.

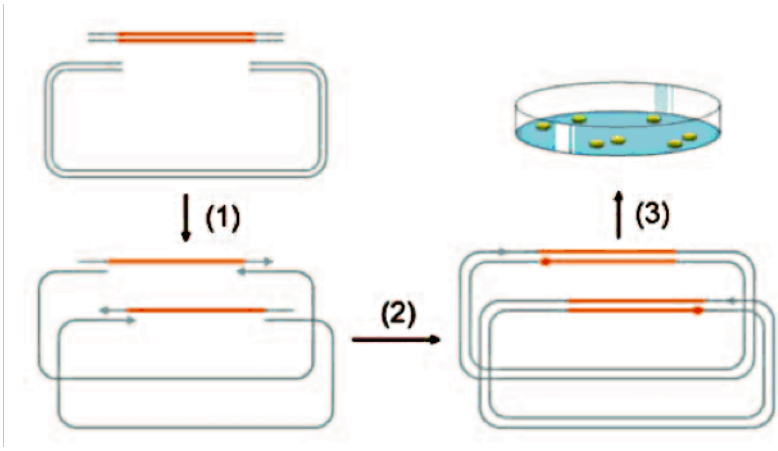


Figure 4-2. Schematic of Circular Polymerase Extension Cloning, or CPEC. Adapted from Quan and Tian, PLoS ONE, 2009.

Python Scripting: From the Computer Screen to Reality

Once a standard or library design calculation is made, a python script is available to generate both an IDT order form for the required oligonucleotides, and the robot work list that assembles the oligonucleotides into the pre-mixes for gene assembly. There are several options for how the order form and worklist is generated, including whether the sequence list is generated from standard or library design output, whether a replacement oligonucleotide or degenerate oligonucleotide method of library construction is desired, and whether the wild-type oligonucleotides have previously been designed by DNAworks and are arrayed onto a 96-well plate. All of these options are specified in the input file to the script. A call to the script is of the form

> python geneassem.py input.txt

where the argument "input.txt" simply designates the name of the input file.

The Input File

The input file specifies the parameters for the python script and consists of five lines. The parameter of the first line can either be "previous" or "new." The argument "previous" specifies that the wild-type oligonucleotides have already been constructed using non-gapped design by DNAworks and are arrayed column-wise onto a 96-well plate. In this case, the second line of the input file specifies the name of the DNAworks output file for the wild-type sequence, and the solution number from the DNAworks output that is used, separated by whitespace. The argument "new" specifies that the script should design the wild-type oligonucleotides and generate an order form including both wild-type and mutant oligos. In this case, the second line of the input file is simply the wild-type amino-acid sequence from which oligos are to be designed. The third line of the input file is the name of the PDB file for the wild-type protein (i.e. from input to PHOENIX). The parameter of the fourth line can either be "standard" or "library," specifying whether sequences are taken from standard or library PHOENIX design output, respectively. In the former case, the PHOENIX output files "design.out" and "design.phoenix" are expected in the same

directory as the script. In the latter case, the library number desired is also specified on the fourth line, with a whitespace separator, and the PHOENIX library design output file "design.out" is expected in the same directory as the script. Finally, parameter on the fifth line can be either "replacement" or "degenerate," designating whether the replacement oligonucleotide (separate assembly and construction of each individual mutant) or degenerate oligonucleotide (libraries that sample multiple mutations at a position are assembled via degenerate oligonucleotides) method of library construction is used, respectively.

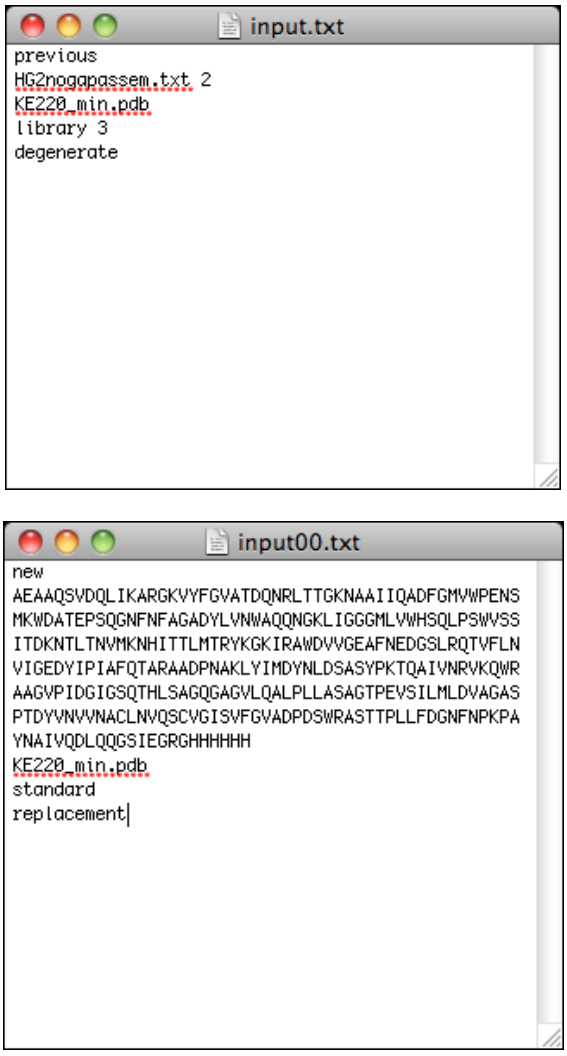


Figure 4-3. Top: an example input file, where wild-type oligos have previously been designed and plated. Bottom: an example input file, where the wild-type oligos have not been designed or ordered.

Output

The output of "geneassem.py" is two files. If the replacement oligonucleotide method is used, the files will be named "idt_order.xls" and "wl.csv." The file "idt_order.xls" is the excel-formatted IDT order form for the replacement oligonucleotides on the required number of 96-well plates needed to construct the library (which includes wild-type oligos if the "new" parameter is specified). The second output file is the robot-formatted work

list, “wl.csv.” In the case that the wild-type oligos have previously been plated, the work list directs the robot to assemble the pre-mixes for all the mutants in the library from the wild-type oligonucleotides in 96-well plates (labeled “wt plate *,” where “*” denotes the plate number) and the replacement oligonucleotides on separate 96-well plates (labeled “mut plate *,” where “*” denotes the plate number). In the case that the wild-type oligonucleotides are ordered along with the mutant oligos, the work list directs the robot to assemble the pre-mixes for all the mutants in the library from the wild-type oligonucleotides and the replacement oligonucleotides from the 96-well plates ordered as per “idt_order.xls” (labeled “idt plate *,” where “*” denotes the plate number).

In the case that the degenerate oligonucleotide method is used, output of “geneassem.py” will be the files “idt_order_deg.xls” and “wl_deg.csv,” which are the IDT order form and the robot work list, respectively, in which degenerate oligonucleotides are used.

Test Case: Rational Stabilization of a Designed Enzyme

An example of this powerful methodology is exhibited in the recent construction and analysis of two degenerate oligonucleotide libraries that attempted to stabilize the HG-2 enzyme reported in Privett *et al* while maintaining it’s activity (in preparation).

The two libraries came from a design that repacked the hydrophobic space between the inner beta sheets and outer alpha helices of the enzyme’s fold. One library used the output design structure from the enzyme design calculation as a structural input (deslib) while the other library used the experimentally determined crystal structure of the designed enzyme (xtallib). Each 32-member library was designed and chosen using the protein design software and parameters as described in Allen *et al*, PNAS 2010.

First, the wildtype HG-2 enzyme was constructed in DNAworks and its oligos were ordered from IDT. Then, as in Figure 4-3, the appropriate inputs were submitted to libseqgen.py, followed by writedeg.py, and finally wlgen.py. The oligos necessary to construct the two libraries were ordered using the output idt_order.xls file and once the materials were received, the libraries were assembled on the robot using the output wl.csv file.

A PCR assembly reaction was then performed on this pre-mix of oligos, producing a great number of different construct sizes that can be visualized as a smear on an agarose gel. The correctly sized construct was then amplified out of the smear by running an IPIPE PCR reaction with the appropriate flanking oligos. An agarose gel confirmed a single band of the correct size was amplified after this reaction. Mixing the IPIPE PCR product in a 1:1 ratio with previously prepared VPIPE product and subsequently transforming the solution with chemically competent cells produced an adequate amount of colonies for simple molecular cloning. An additional transformation was performed the next day to get the required amount of colonies for sequencing a 4x oversampling of the library size.

Analysis of the sequencing results showed that we had recovered ~75% of the members of each library. These mutants were then tested for stability by first following the inherent tryptophan fluorescence (chemical denaturation) and then by adding the fluorescent dye SYPRO Orange (thermal denaturation). Unfortunately, no stabilizing mutants were found in the libraries.

Including the weeklong turnaround required for high-throughput sequencing, the experimental construction and verification of the two libraries spanned less than one month. We would not expect the time to increase significantly when considering the simultaneous construction of many more libraries.

Appendix

BL21 Gold DE3 Competent Cell Preparation

This strain grows extremely fast and is useful for both plasmid and protein expression and recovery. When transforming mutagenic protein libraries into this strain of competent cells be sure to keep a glycerol stock of the picked colonies; this simplifies and expedites protein expression after plasmid sequencing as there is no need to perform miniprep or transformation procedures.

On the day of competent cell preparation, be sure to clear space in the cold room for all 4°C manipulations and to autoclave a 1L centrifuge container. Alternatively, prechill all pipets, volumes, and containers to -20°C and perform all procedures on ice.

$MgCl_2$ solution. 100mM $MgCl_2$/water, filter sterilized. Store at 4°C.

$CaCl_2$ solution. 100mM $CaCl_2$/15% glycerol/water, filter sterilized. Store at 4°C.

1. Start a 10mL overnight culture in LB at 37°C for a 1L batch.

2. Seed a 1L volume with the 10mL overnight culture and shake at 37°C until OD600 reaches 0.4-0.6. All further operations need to be done at 4°C.

3. Spin down the culture at 2500xg for 20 min at 4°C in a liter centrifuge container. Pour off the supernatant and add 100mL (1/10th of initial 1L culture) of the $MgCl_2$ solution. Resuspend the pellet gently, ideally by shaking at 200-250 rpm on ice. Split the volume between two pre-chilled 50mL tubes.

4. Incubate the resuspended cells at 4°C for 30 min.

5. Spin down the cultures at 2500xg for 20 min at 4°C. Pour off the supernatant and add 20mL (1/50th of initial 1L culture) of the $CaCl_2$/glycerol solution. Resuspend the pellet gently, ideally by shaking/inversion/nutating.

6. Divide the cells into 1.2mL aliquots in 1.7mL sterile eppendorf tubes. Don't use a repeater pipet. Flash freeze the aliquots in liquid nitrogen. Store at -80°C.

48-Well Qtray Preparation

The Genetix 48-well QTray (x6029) is the perfect petri dish for high-throughput bacterial transformation experiments. The 8 row by 6 column format is amenable to the Freedom EVO's LiHa, and two Qtrays represent exactly one 96-well plate. They are prepared in much the same way as normal petri dishes.

1. Prepare the Qtrays ideally the day before you expect to plate the transformation reactions. Turn on the 55°C water bath located at the end of the Bay 3/4's sink.
2. For four Qtrays, or conversely, 2 96-well Site-Directed Mutagenesis plates, autoclave 1L of LB agar (mix 25g of LB broth miller with 15g of Bacto-Agar and add 1L of MilliQ water).
3. Move the flask out of the autoclave and into the prepared water bath. After ~25 min, the LB agar should be cool enough to add antibiotic and pour into the Qtrays.
4. Turn on your Bunsen burner. Add antibiotic, mix, and then using a 50mL sterile pipet add 200mL of LB agar to the open Qtray. The best location found so far is the bench in Bay 2 or Bay 3 as it is more level then the bench in Bay 1. Flame off any bubbles, replace the 48-well divider, and then put on the Qtray lid in a skewed position to allow air exchange.
5. Let the plates cool to room temperature and dry, ideally overnight in this position. When storing the plates before or after transformation, make sure to keep them upside down to prevent moisture from collecting on the agar surface. If the plate was stored in the cold room, remove any moisture on the surface of the agar before transformation by removing the lid and letting them air-dry under a Bunsen burner for ~30min. This same drying process is also highly recommended after plating.

EasyPress Operation

product manual

AXYGEN SCIENTIFIC

EasyPress, silicone sealing mat applicator

Axygen EasyPress is a manual press which provides simple and effective application of Axymats to 96 and 384 microplates, deep well plates, microtiter tube systems and PCR plates. The EasyPress can handle any size plate of varying heights (384 PCR to 2 ml deep well blocks) by quick adjustment of the large thumbwheel.

The procedure is simple .....

Compatible with the following Axygen Multiwell Plates and Sealing Mats

Plates

- 2.0ml deep well plates, 96- round wells
- 2.0ml deep well plates, 96- square wells
- 1.1ml deep well plates, 96- round wells
- 600µl deep well plates, 96- round wells
- 120µl microwell plates, 384- square wells
- 240µl microwell plates, 384- square wells

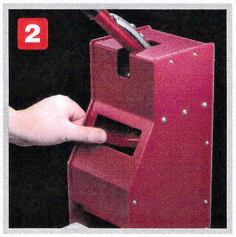
Sealing Mats

- ImpermaMat for 384 well, 120µl and 240µl, microplates with square wells
- ImpermaMat for 96 well, 1.1ml and 2.0ml, deep well plates with round wells
- Axymat for 600µl 96 well deep well plates
- Axymat for 1.1ml and 2ml 96 well round deep well plates
- Axymat for 2ml 96 well square deep well plates
- Axymat for 384 well, 120µl and 240µl, microplates with square wells

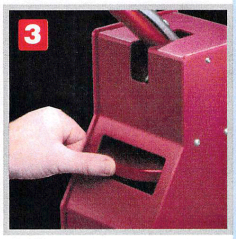
Request an Axygen catalog for full technical ordering information

Pre-adjustment of EasyPress

To adjust for the first time or for a new a plate, lower the handle and place a plate on the 'plate slide' with no Axymat.



Adjust the large thumbwheel (spin thumbwheel to right side to lower the press head and left to elevate) until the press head is above the height of the plate and slide the plate into the press.



Adjust the thumbwheel to lower the press head until the press head makes light contact with the plate, return the handle the top position. You are now ready to seal plates.

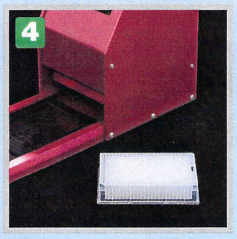
Caution, improper adjustment may cause damage to the EasyPress, do not adjust thumbwheel to exert excess force when attempting to seal plates.

Operation of EasyPress

With the 'plate slide' in the out position, place the appropriate Axymat on top of the plate, align 2 - 3 corners of the Axymats and place the plate with the AxyMat into the 'plate slide'.

Move the 'plate slide' into the press.

Holding the outer edge of the 'base plate', gently pull down on the handle to the bottom of its stroke and then return the handle to the top. Slide the plate tray out of the EasyPress and remove your plate.



You are now ready to continue sealing plates of the same size.

AXYGEN SCIENTIFIC, INC. 33170 CENTRAL AVENUE, UNION CITY, CA 94587 USA tel: 510-494-8900 fax: 510-494-0700 e-mail: info@axygen.com web: www.axygen.com

E-Gel 96 Operation

QUICK REFERENCE CARD

E-Gel® *96* Gels

Catalog nos. G7008-01, G7008-02
25-0419 Version I; 18 April 2005

Instructions are provided below for using E-Gel® *96* Gels with the E-Base™. For more details, refer to the E-Gel® Technical Guide available at www.invitrogen.com or contact Technical Service.

Preparing Samples

- Use 20-100 ng DNA per band for samples containing one unique band or up to 500 ng per lane for samples containing multiple bands.
- Prepare DNA samples in a **total sample volume of 20 µl for E-Gel® *96* gels** in deionized water or loading buffer (recommended final loading buffer concentration is 10 mM Tris-HCl; 1 mM EDTA, pH 7.5; 0.005% bromophenol blue; and 0.005% xylene cyanol FF).
- Dilute **high salt samples** (samples with >50 mM NaCl, >100 mM KCl, >10 mM acetate ions, >10 mM EDTA), 2- to 20-fold in deionized water, TE, or loading buffer in final volume of 20 µl.

Selecting Program On E-Base™

The recommended program for E-Gel® *96* gel is EG and the run time is 12 minutes.

1. Plug the Mother E-Base™ into an electrical outlet. Connect the Daughter E-Base™ to a Mother E-Base™ or another Daughter E-Base™ connected to a Mother E-Base™.
2. Press and release the pwr/prg (power/program) button on the base to select program EG.

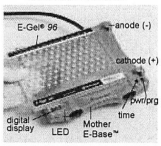
Note: The E-Gel® *96* gels are also compatible with the E-Gel® *96* base available previously from Invitrogen. For using E-Gel® *96* gels with E-Gel® *96* base, refer to the E-Gel® Technical Guide.

Loading and Running E-Gel® *96* Gels

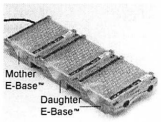
Load each gel within 30 minutes of removing gel from the package and run within 15 minutes of loading.

1. Remove gel from the package and remove plastic comb from the gel.
2. Slide gel into the two electrode connections on the Mother or Daughter E-Base™. If gel is properly inserted, a fan in the base begins to run, a red light illuminates, and digital display shows 12 minutes.
3. Load 20 µl prepared DNA sample into the well. Keep all sample volumes uniform. Load samples manually, with a multichannel pipettor, or use robotic loading devices (8-, 12-, 96-tip).
   Note: To ensure proper sample loading with robotic loading device, align the robotic tip assembly (see E-Gel® Technical Guide for details).
4. Load appropriate DNA markers in the marker wells. Be sure the marker salt concentration is similar to that of the adjacent samples.
   1% gel: E-Gel® *96* High Range DNA Marker
   2% gel: E-Gel® Low Range Quantitative DNA Marker
5. Load 20 µl sample buffer containing the same salt concentration as the sample into any empty wells.
6. To begin electrophoresis, press and release the pwr/prg button on the E-Base™. The red light changes to green.
7. At the end of the run (signaled with a flashing red light and rapid beeping), press and release the pwr/prg button to stop the beeping.

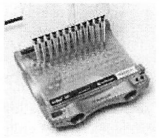
Mother E-Base™



Daughter E-Base™



Loading Gels



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Contact Information for Other Countries:
See our Website: www.invitrogen.com

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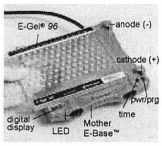
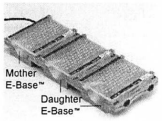
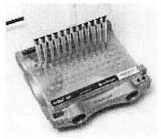


Daughter E-Base™



Loading Gels



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Contact Information for Other Countries:
See our Website: www.invitrogen.com

DNA Ladder for E-Gel 96

Invitrogen
life technologies

E-Gel® Low Range Quantitative DNA Ladder

Cat. No. 12373-031 **Conc. 175 ng/10 µl**

Introduction

The E-Gel® Low Range Quantitative DNA Ladder is designed for use as a molecular weight standard and for estimating the amount of DNA. The important features of the ladder are listed below:

- Consists of five linear double-strand DNA fragments (100-2000 bp)
- Suitable for estimating molecular weight and quantity of DNA fragments in agarose gel electrophoresis
- Provided in a ready-to-use format
- Visualized by ethidium bromide staining

Specifications

Contents: 1 ml of E-Gel® Low Range Quantitative DNA Ladder. Sufficient ladder is provided for 100 applications.

Storage Buffer: 8 mM Tris-HCl, pH 7.5; 1 mM EDTA; 5% glycerol; and 0.005% Orange G.

Storage: Store at room temperature or 4°C.

Directions for Use

The E-Gel® Low Range Quantitative DNA Ladder is provided in a ready-to-use format. There is no need to heat the ladder prior to loading. Use loading volumes listed below to obtain the best results:

Gel	Ladder	Water	Total Volume
Standard agarose	10 µl	--	10 µl
E-Gel® *96*	10 µl	10 µl	20 µl
E-Gel® double comb			
Marker lane	5 µl	5 µl	10 µl
Sample lane	10 µl	10 µl	20 µl
E-Gel® single comb	10 µl	10 µl	20 µl

Part No. 12373031.PPS Doc. Rev. 081602

This product is distributed for laboratory research only. CAUTION: Not for diagnostic use. The safety and efficacy of this product in diagnostic or other clinical uses has not been established.

For technical questions about this product, call the Invitrogen Tech-Line℠ U.S.A. 800 955 6288

Invitrogen
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