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Plate Design for and Cherry Picking of Bacterial RNAi Clones for Systematic Error Detection in High-Throughput *C. elegans* RNAi Screens

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Abstract

Automated or semi-automated high-throughput RNAi screens are highly prone to systematic errors because of multistep repetitive protocols and extensive use of automated instruments. A well-designed RNAi library can help detect and minimize systematic human and robotic errors. In this Unit, we described how to design an RNAi bacterial library used in conjunction with the well-studied nematode *Caenorhabditis elegans* for automated phenotypic screens. We provide strategies to design and assemble RNAi libraries to reduce or eliminate systematic errors. These strategies serve as a good quality control (QC) check and facilitate obtaining high-quality data in a genome-wide and sub-library RNAi screen.

Keywords

RNAi; RNAi library design; feeding RNAi; reverse genetics; automated high-throughput screening; *C. elegans*

INTRODUCTION

The discovery of RNA interference (RNAi) in *Caenorhabditis elegans* by Andrew Fire, Craig Mello and colleagues (Fire et al., 1998) revolutionized the reverse genetics approach to study gene function in multicellular organisms. The availability of whole genome RNAi bacterial libraries in 96- and 384-well format plates and the ease of silencing most target genes by RNAi feeding (Fraser et al., 2000; Kamath, Martinez-Campos, Zipperlen, Fraser, & Ahringer, 2001; Rual, Hill, & Vidal, 2004) made *C. elegans* a highly facile model system for high-throughput RNAi screening. In the RNAi-feeding method, the bacteriovorous *C. elegans* animals, which are normally fed on *E. coli*, are simply fed on an engineered *E. coli* that produces a double-stranded RNA molecule corresponding to a particular *C. elegans* gene. Typically, for whole-genome RNAi screening in *C. elegans*, the protocol involves the primary screening of ~23,269 *E. coli* clones, most of which express a distinct dsRNA

corresponding to ~87% of the *C. elegans* genome. The hits identified in the primary screen are then validated using one or more secondary assays, which are mostly done in replicate.

Performing genome-wide screens is expensive and time consuming. An inherent problem with whole-genome screens is the fact that it is challenging to generate high-quality genome-wide datasets with biological significance. In our experience, a key factor in successfully carrying out a whole-genome RNAi screen in *C. elegans* with a minimal amount of troubleshooting depends on several key factors. One such key factor is the ability to identify, investigate and correct the inevitable systematic errors that occur in high-throughput screening. The systematic errors caused by the experimenter, machines, or robotic tools can be identified efficiently by designing effective RNAi library plates. Appropriate positioning of each control RNAi and sample RNAi in a screening plate enables the screener to identify systematic errors, which is almost always well-position dependent. Thus, an effective RNAi screen plate design improves the overall quality of data and minimizes costs and time spent in troubleshooting when a screen fails. This Unit describes a protocol for designing a plate map in 96-well format, which is adaptable to 384-well formats as well. The protocol also describes how to make a custom bacterial RNAi library for high-throughput screening to obtain high-quality datasets.

STRATEGIC PLANNING

Almost all of the primary screens are done using commercially available RNAi libraries in which RNAi clones are preloaded in 384-well format. While commercially available RNAi libraries come in 384-well plates, a majority of phenotypic screens are either performed in 96-, 48- or 24-well plates. It is relatively straightforward to change the formatting from 384-well format to a 96-, 48-, or 24-well format using a manual multi-pronged replicator. It is much more complicated, however, from both technical and bookkeeping perspectives, to add custom negative and positive controls to the plates in which the assays will take place. Thus, a primary screen when arrayed directly from the commercial library often relies on controls that are placed in independent plates. A good strategy in these scenarios is to identify RNAi clones that score as positive hits in the particular assay that is being employed. This can usually be accomplished by screening a limited number of the plates from the whole-genome library or by screening a sub-RNAi library, consisting, for example, of all annotated transcription factors, kinases, or phosphatases, etc. Using these control RNAi clones, one can create a digital map for the control plate for their primary screening. In addition, it is also helpful to identify several RNAi clones from widely distributed plates in the whole-genome library that yield distinct reproducible phenotypes, for examples RNAi clones that cause vulval bursts, delayed development, or developmental arrest. Specifically, we found that the RNAi clone corresponding to the gene *klf-3* causes a vulval burst phenotype in 100% of assays. Such RNAi clones scattered throughout the genome library, not only help check the quality of the screen and detect systematic errors, but also serve as a quality control for the RNAi efficiency.

Furthermore, in primary whole-genome screens, one can use most of the sample wells in each plate as negative controls to identify systematic errors. The reason is that it is very unlikely that a majority of the dsRNAs will score as positive hits in a particular assay.

Because a relatively high hit rate for a biological screen using *C. elegans* is ~5–10%, one would anticipate that ~90–95% of the clones would behave like negative controls. In our laboratory, for example, when we carried out a whole-genome RNAi screens for RNAi clones that modulated the *C. elegans* innate immune response to pathogen attack, our hit rate in the primary screen was ~10%. Thus, it is reasonable to use the majority of the sample wells as negative control for systematic error detection measure in primary screen.

Although it is generally not possible to add appropriate controls to assay plates when carrying out a primary whole-genome RNAi screen, a custom plate design is feasible in secondary screens or screens that use a pre-selected, enriched gene-set for a particular biological function (customized by the screener). In secondary screens, particular RNAi clones are preselected, and it is usually inappropriate to use the majority of the sample wells as negative controls. It is very important to design these secondary assay plates with proper positive and negative controls at appropriate well positions in each plate to generate reliable quantitative data and to detect systematic errors.

BASIC PROTOCOL 1. Plate Design for Effective Systematic Error Detection in High-Throughput *C. elegans* RNAi Screens

Basic Protocol 1 gives guidelines to design and assemble control and sample RNAi clones in 96-well format plates for both primary and secondary RNAi screens in *C. elegans*. The guidelines provided here are also applicable to 384-well format plates.

Control RNAi plate design for screens using commercial RNAi libraries

As most of the commercially available RNAi libraries come preloaded in 384- or 96-well format, the primary screens using these libraries often depend on control RNAi clones that are assembled in separate plates. The first step is to select appropriate negative and positive controls for the assay that is being employed for the screen. In addition to control RNAi clones for the assay, it is also prudent to add one or two controls that can be used to detect RNAi efficiency. The controls can be selected either from published literature or by performing a mini-screen using random library plates from genome library or from pre-selected sub-library plates. Using these control RNAi clones, one can create a digital map for the control plate. The screen is then carried out using the control plate as described below.

An example control plate design is illustrated in Fig. 1. The important points to consider while positioning your controls in particular wells in the plate are as follows:

- Make sure each control is present in more than one column and row. In the example shown in Fig. 1, each control is distributed in more than one column or row. It is hard to detect systematic errors, if the controls are clustered in one column or one row.
- Leave some wells free to add additional controls during the course of a screen. For example, screening of genome libraries is often done in batches. If possible, choose a few additional control genes from the library plates that are being

screened in a particular batch and add them to the free wells in the control plate. This way, you can compare the phenotypes of genes in the control plate to the phenotypes of the same genes in the library to check the quality of the screen.

- The example control plate illustrated in Fig. 1 is a simplified design with one positive and one negative control with some free wells for additional controls that can be added during the course of screening. When additional controls are used, distribute them in the plates in accordance with the points mentioned above.
- When more controls are used consider having at least 8–10 wells per control distributed throughout the plate.

Designing custom assembled RNAi libraries with control RNAi clones

The following guidelines for RNAi library design is mostly applicable for secondary screens in which a sub-library of RNAi clones is assembled, sometimes based on data obtained from a primary screen. The guidelines can also be used to generate a sub-RNAi library with which to carry out a primary screen. For instance, in our lab, we have designed a “human orthologs” library from the full-genome source library and used it for primary screening. That is, we cherry picked the RNAi clones from the full-genome library that correspond to genes that have clear human homologs. Following are the important points to consider when determining the placement of RNAi clones in the screening plates:

- The selection and placement of effective positive and negative biological controls plays a crucial role in the successful design of an RNAi bacterial plate. There should be at least one positive RNAi control, which reproducibly causes a specific phenotypic effect in the assay conditions and at least one negative control with no specific knockdown phenotype. Ideally, as described above, it is best to include a positive control for RNAi efficiency that generates a clear developmental phenotype independent of the specific assay that is being employed for screening, as well as positive RNAi control that scores as a hit in the screening assay. Generally, it is recommended to have at least 10–16 wells dedicated to controls per 96 well plate, usually 5–8 wells per control (Zhang, 2008). Both positive and negative should be distributed throughout the plate to detect edge effects and other systematic errors. When using multiple plates for screening, human errors such as mixing up the order of the plates or mixing up the orientation of a particular plate while feeding it into an automated screening microscope are inevitable. This type of error can be detected and corrected by distributing the controls in each plate in different patterns and using the positions of the controls as an internal barcode for each plate.
- Figure 2 shows a few plate design patterns to help you design plate maps for custom-made libraries. A typical plate map contains both positive and negative controls on both edges and sample RNAi clones in the remaining wells (Designs A & B in Fig. 2). The drawback of design A is that all of the negative controls are in one column, which makes it insensitive to edge effects or other types of errors, such as pipetting and liquid handling machine errors. In contrast, design B efficiently detects systematic errors using the negative controls distributed in two

columns. For example, if the negative controls in column 1 score lower than those in column 12, it indicates the presence of a systematic error linked to the position of the wells. Neither design A and B, however, can detect systematic errors that occur in the middle of the plate and this design should not be used in secondary screens where the sample wells are enriched for phenotypes similar to the positive control. In contrast to designs A & B, designs C & D (Fig. 2) have positive and negative controls distributed throughout the plate to efficiently detect systematic errors. In addition, designs C and D employ three empty wells (A2–A4, B2–B4), which can be used as internal “pattern-based barcodes” to correctly associate the output data with the appropriate plate. In addition, designs C and D can also detect cross-contamination of RNAi from well-to-well in the experiment. In this latter case, empty wells (A2–A4) that should not contain bacterial food that would support worm growth will contain bacteria that promote worm growth, indicating cross contamination and that the assay needs to be repeated.

- The plate design patterns shown in Fig. 2 are just example patterns, which can be customized further. For example, if you have relatively few sample RNAi clones, increase the number of controls in each plate and *vice versa*, but try to have at least 8–10 wells for controls and do not compromise on negative controls, especially for secondary screens. If you are planning to include other controls later in the screen, leave extra empty wells.

In conclusion, a well-designed RNAi plate allows the detection and potentially the identification of systematic errors and thus improves the quality of the screen by reducing assay variability as well as false positive and false negative hits.

BASIC PROTOCOL 2. Making of a control plate or a sub RNAi library by Cherry-picking RNAi clones

The protocol below describes how to make a library in 96-well format by cherry-picking from a commercially available or a pre-existing library using the plate designs described above as template and how to prepare them for long-term storage at -80°C .

Materials

Luria-Bertani agar

Luria Broth

Ampicillin or carbenicillin

RNAi library in 96-well format frozen at -80°C

96 Solid Pin Multi-Blot Replicators (V&P Scientific, catalog number: VP 408)

96-deep-well assay blocks (1 ml or 2ml) (Costar; catalog numbers: 3958 or 3960)

96-well-flat-tissue culture treated sterile plates for RNAi library glycerol stock preparation (Corning, catalog number: 353916)

Breathe-Easy-Gas permeable sealing membranes for 96 well plates

50 ml sterile disposable reagent reservoirs for media (Corning, catalog number: 4870)

Aluminum seals for 96-well plates used to freeze RNAi stocks (Costar, catalog number:

6570)

Hand roller for attaching the aluminum foil seals to assay plates

Dry ice

Multi-channel pipette (5–50 μ l and 50–200 μ l)

Incubators (15°C, 20°C and 25°C)

NOTE: All solutions and equipment must be sterile and proper sterilization methods should be used accordingly.

Cherry-picking RNAi clones to make a control plate or a sub RNAi library to be stored as frozen stocks

1. Prepare a sufficient number of rectangular LB agar plates (128x86mm) containing 200 μ g/ml carbenicillin and 15 μ g/ml tetracycline.
 - a. Dry plates with the lids off on a clean bench for 10 min and make sure there are no condensed water droplets on the plates or lids before using them.

Condensed water will drop on the colonies and cross-contaminate the library.
 - b. Label plates before spotting them and always mark the position of the A1 well, which will help keep the plates orientated correctly and minimize labeling errors in subsequent steps.

Note: We have found that it is extremely helpful to label the bottom of the plates using a black Sharpie in mirror image writing so that you can easily read the label from the top of the plate when you are stamping the plates in the subsequent steps below. This allows you to easily maintain the correct orientation of each plate. It will take some practice writing in mirror image, but the time spent learning how to do this will definitely pay off in the long run. It is almost inevitable that you will make some mistakes when handling potentially hundreds of plates. The mirror image labeling technique helps reduce these errors, which can prove extremely costly in wasted time and supplies.

2. Collect the appropriate frozen RNAi library stock plates that contain the RNAi clones of interest from the –80°C freezer and put them on dry ice.

3. Remove the aluminum foil covers from the frozen plates carefully to avoid aerosols that might cross contaminate the wells.
4. Replicate the RNAi library stock plates onto the square agar plates using a sterile 96 well pin replicator (Solid Pin Multi-Blot Replicator). The 96 well pin replicator must be sterilized after each inoculation. Use three sterile empty tip boxes to sterilize the replicator: fill the first reservoir with 10% bleach, the second with sterile water, and the third with 70% ethanol. Dip the pin replicator successively in the three reservoirs and then flame thoroughly. Let the replicator cool down before use.
5. Seal the library stock plates with new aluminum foil covers using a hand roller to thoroughly seal the foil to the plate to prevent cross-contamination between wells and return the plates immediately to -80°C .
6. Incubate the inoculated rectangular LB agar plate upside down at 37°C overnight.

Incubating the plates upside down mitigates condensed water cross contaminating the RNAi clones.
7. The next day, check the spotted plate and make a note of overlapping clones.

When replicating an RNAi library plate, it is customary to discard plates that have overlapping clones. When cherry picking, overlapping clones may not be an issue as long as the relevant clones are not overlapping. RNAi clones grown on agar plates can be stored at 4°C , upside down for 3 weeks. After 3 weeks of storage, RNAi efficiency may decrease. RNAi efficiency can also be decreased by replicating an agar plate to a new agar plate as well as by replicating RNAi clones grown in liquid to agar plates.
8. The next day, prepare an appropriate number of 96 deep well assay plates (2 ml) containing 1 ml of LB broth containing 200 $\mu\text{g/ml}$ of carbenicillin and 15 $\mu\text{g/ml}$ tetracycline to grow the cherry-picked clones.
9. Print the plate map of the library plates to be cherry picked. An example plate map template in table format is illustrated in Fig. 3 for easy and error-free cherry picking.
10. Pick the clone(s) to be cherry-picked from the appropriate plate position using the plate map as a blueprint using 1 ml pipette tips with filters and inoculate the appropriate well in the recipient 96-well deep-well assay plates containing LB with 200 $\mu\text{g/ml}$ carbenicillin and 15 $\mu\text{g/ml}$ tetracycline. Keep the pipette tip in the appropriate recipient well in the 96 deep well assay plate until the entire plate have been inoculated. As each RNAi clone is picked into the recipient plate, cross off the clone on the plate map. Incubate at 37°C with shaking, overnight.

Keeping the tips in the appropriate wells until an entire plate has been cherry-picked minimizes the chances of inoculating multiple clones/ well or inoculating clones in the wrong wells. This method drastically

reduces the human error in cherry-picking the clones. Care should be taken while taking the tips out to avoid any cross-contamination. Also make note of the clones that did not grow and update your plate map whenever clones are missing for future reference. While screening, the lack of clones can also be used as an internal barcode for a particular plate.

11. The next day, centrifuge the bacterial culture at 4000 rpm for 10 min and discard the supernatant carefully without causing cross contamination.
12. Add 500 μ l of LB containing 20% glycerol to each well and resuspend the pellets using a multichannel pipette.
13. Transfer 200 μ l of the bacterial cultures with glycerol from the 96-deep well plates to 96-well flat bottom sterile plates (in duplicate).

Care should be taken to avoid cross-contaminating the wells.

14. Seal the plates with a new aluminum foil cover using a hand roller to thoroughly seal the foil to the plate to prevent cross-contamination between wells and carefully place them in a -80°C rack. The plates should not be disturbed for at least 12 hours after being placed in the -80°C freezer.
15. Use one frozen glycerol stock control plate as a working stock and store another as a master plate stock. If RNAi efficiency goes down in the control plate, likely due to damage caused by frequent freezing and thawing, use the master plate to and a new glycerol working stock plate.

As there is typically up to a ~20% error rate in RNAi genome libraries, sequence the clones to confirm their gene targets.

COMMENTARY

Background Information

In high-throughput screening, systematic errors are inevitable. The early detection and restoration of these errors are critical for obtaining high-quality data with biological significance. Unlike random errors, which might not affect the overall output of the screen, systematic errors, which are mostly well-position dependent, significantly over- or under-represent the output of the screen. For example, recurrences of row or column systematic error through all the plates of the assay can result in highly biased raw data, consequently leading to either select many false positive hits or might fail to identify many positive hits (Dragiev, Nadon, & Makarenkov, 2011). Thus, an effective RNAi screen plate design with proper controls distributed throughout plate facilitates detection of systematic errors efficiently. In addition, an effective plate design in conjunction with appropriate statistical methods can detect and correct the errors. (Dragiev et al., 2011; Zhang, 2008). Hence designing an effective RNAi screen plate for systematic error detection improves the quality of hits obtained from the screen.

Critical Parameters

The protocol described in this unit are relevant to two types of RNAi screens: screens in which commercial RNAi libraries are replicated directly into the 96- or 384-well assays plates being employed in a liquid-based high-throughput RNAi screen, and second, screens in which individual RNAi clones are cherry-picked from a commercial library to make customized assay plates. For the first type of screen, it is typically not feasible to customize the arrangement of the RNAi clones in the assay plates, for example by including appropriate positive and negative controls in each plate. It would be technically challenging to re-array the library without sophisticated liquid handling robots that are not available in most academic laboratories. Thus, it is necessary to design a separate control plate and include the plate while screening the library.

A major advantage of having a separate control plate when carrying out a genome-wide screen is that a variety of controls can be run in the same plate. Another advantage is that the control plate can be used to identify errors caused by malfunctions of an automated microscope, especially malfunctions of the robotic arm. In our laboratory, a common problem is mis-feeding of a plate or two into the microscope, thereby causing an interruption in the pipeline. When dealing with hundreds of plates this can cause a serious problem. Even though most microscopes use a barcode reader, it is sometimes impossible to figure out which plate was missed. This happens when the barcode on the plate is read but the plate is not fed into the microscope. This potential problem can be mitigated by adding control plates at regular intervals in the pipeline. If the robotic arm misses a plate, the position of the control plate in the stack can be used to realign the data in the pipeline with the appropriate plates. The control plates are readily identified by the particular pattern of controls in the plate. In addition to using the control plates to realign the data pipeline, we also take advantage of the fact that typically not of all the RNAi clones grow in a particular library plate. We always make note of “missing” clones for each plate and use their pattern as an “internal barcode” to cross-check plate identification. We use missing clones as internal barcode for secondary screens as well, where the controls are embedded inside the screen plate itself.

Other critical factors in making cherry-picked RNAi libraries are to cherry-pick from the bacterial plate containing replicated RNAi clones within 10 days and to make two frozen RNAi library copies, a working copy and one as a main stock. Only use the working copy for repeated freezing and thawing. If you observe a reduction in RNAi efficiency, make another working copy from the frozen stock.

Understanding the Results

In conclusion, an effectively designed RNAi screening plate allows the detection of systematic errors caused by various factors including robotic failure, pipette-related errors, edge effects due to uneven evaporation of reagents, which might otherwise go unnoticed and only be detected at the validation stage after the screen is complete. This often leads to poor quality data that cannot be used resulting in loss of time and resources.

Time Considerations

Plate designing for RNAi library may take from one day to a week according to the number of plates. Typically, cherry-picking the library for glycerol stock takes only 3 days. The number of days will vary according to the number of plates to be cherry-picked. If multiple plates have to be cherry-picked, first stamp all the plates from glycerol stock that needs to be cherry-picked and then start cherry-picking the plates. In our hands, cherry-picking clones into one 96-well recipient plate takes between 30–60 minutes.

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Figure 1.
Control plate design template for screens using commercial RNAi libraries.

Old plate	Old plate well	RNAi clone	New plate	New well
PGS-1	A9	F23C8.6	ZSS-1	A1
PGS-1	A3	F53G12.10	ZSS-1	A2
PGS-1	F11	ZC328.1	ZSS-1	A3
PGS-1	G10	ZC581.1	ZSS-1	A4
PGS-1	A1	R119.4	ZSS-1	A5
PGS-1	G2	K06A5.4	ZSS-1	A6
PGS-1	D12	F56A3.3	ZSS-1	A7
PGS-1	D1	T21E12.4	ZSS-1	A8
PGS-1	H1	T21G5.5	ZSS-1	A9
PGS-1	F9	C10G11.4	ZSS-1	A10
PGS-1	D11	Y110A7A.m	ZSS-1	A11
PGS-1	F5	T09B4.9	ZSS-1	A12
PGS-1	D4	D1007.12	ZSS-1	B1
PGS-1	D3	D1007.6	ZSS-1	B2
PGS-1	A2	F56C11.1	ZSS-1	B3
PGS-1	F2	T19B4.2	ZSS-1	B4
PGS-1	A10	W01B11.3	ZSS-1	B5
PGS-1	H7	F22D6.3	ZSS-1	B6
PGS-1	A4	F53G12.3	ZSS-1	B7
PGS-1	F12	F37E3.1	ZSS-1	B8
PGS-1	H10	R06C7.8	ZSS-1	B9
PGS-1	A6	C53D5.i	ZSS-1	B10
PGS-1	C4	C50F2.3	ZSS-1	B11

Figure 3.

An example of plate map in table format to use while cherry-picking RNAi clones to make a control plate or a sub-RNAi library.