



BAC Library

**Pooling and Superpooling
< Matrix Technology >
One Plate Superpool**

Users Manual

Manual Version 3.2e4
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Table of Contents

Overview	3
Graphical Overview	4
Contents of Kit and Storage Conditions	5
Quick Start User Guide	6
Troubleshooting FAQ	9
Screening Recommendations	
PCR protocols	11
Internal Standard	11
Detailed Descriptions	
Superpools	12
Matrix Pools	12
Example Clone Identification	
Superpool	14
Matrix Plate Pools (not needed)	15
Matrix Row Pools	16
Matrix Column Pools	17
Intentionally Left Blank	18
Keys	
Intentionally Left Blank	19
Key to Superpool	20
Key to Matrix Pools	23
Key to Matrix Plate Pools (not needed)	24
Key to Matrix Row Pools	25
Key to Matrix Column Pools	26
Intentionally Left Blank	27

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Overview:

The Matrix pooling and superpooling system enables researchers to use PCR for screening a BAC library. The exact plate and well position containing a BAC clone of interest can be correctly identified in 50% fewer PCR experiments (compared with a traditional plate/row/column pooling strategy). The Matrix method improves a researcher's ability to identify false positives, by requiring two positive bands be observed after gel electrophoresis.

Each kit is custom built for the researcher and the total number of Superpools in the kit will depend on the total number of BAC clones in the library. Each Superpool will have a corresponding 96-well plate of Matrix Pools. These Matrix Pools are composed of clones from one 384-well BAC library plates and cleverly positioned into a matrix of Row and Column (RD) pools. The Matrix Pools are aliquoted onto TWO identical 96-well plates (to help reduce the risk of contamination).

Screening is done in two separate rounds of PCR on extracted DNA from independently grown, then separately pooled, BAC clones ('Round I PCR' and 'Round II PCR').

The Round I PCR is performed on all of the Superpools (containing all BAC clones in the library). Each Superpool contains 384 individual BAC clones. The results from Round I of PCR will identify which Superpool contains BAC clone(s) with the sequence of interest (there may be more than one Superpool identified). The researcher may choose to pursue one or more positive hits from the Round I PCR.

The Round II PCR will be performed on the Matrix Pools for the specific Superpool identified in Round I PCR. Round II PCR requires 18 PCR experiments plus controls (for each positive hit pursued from Round I PCR). The results from Round II PCR should allow the researcher to identify the well position of a single positive hit. In comparison, using a traditional row/column strategy, Round II PCR screening of PRC pools requires 40 PCR reactions plus controls. The Matrix system reduces the PCR experiments by 50%.

The Matrix Pools are Row and Column (RC) pools combined so that EACH of these PRC pools is contained in TWO unique Matrix Pools (see pages 12 and 24-27 for details on Matrix Pool Construction). There are a total of 18 Matrix Pools for each Superpool eight Matrix Row Pools (MRP) and 10 Matrix Column Pools (MCP). There are at most 96 individual BAC clones inside each Matrix Pool well (see pages 12 and 13 for details).

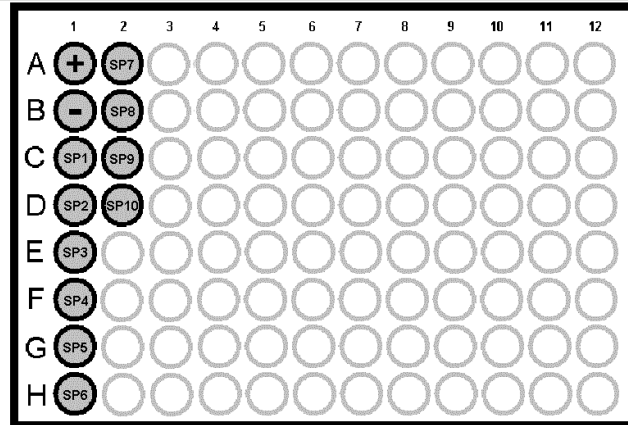
Interpretation of positive hits from Round II PCR (screening the Matrix Pools) is done by comparing the positive hits seen on the electrophoresis results to the Matrix Pool keys (pages 24-27). The keys are necessary to provide the location (row and column) of all positive clones from Round II PCR.

GRAPHICAL OVERVIEW

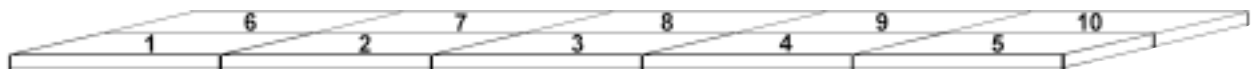
The researcher will receive 2 identical Superpool Collection Plates which will be used for Round I PCR.

Each Superpool plate will provide template for at least 750 PCR experiments.

After viewing Round I PCR gel electrophoresis results, the researcher will determine which superpool(s) to screen in Round II PCR.



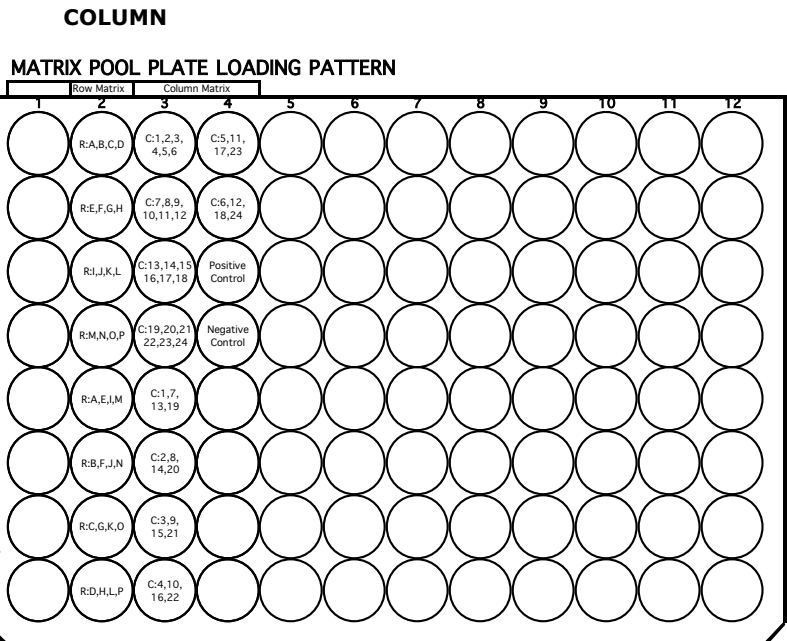
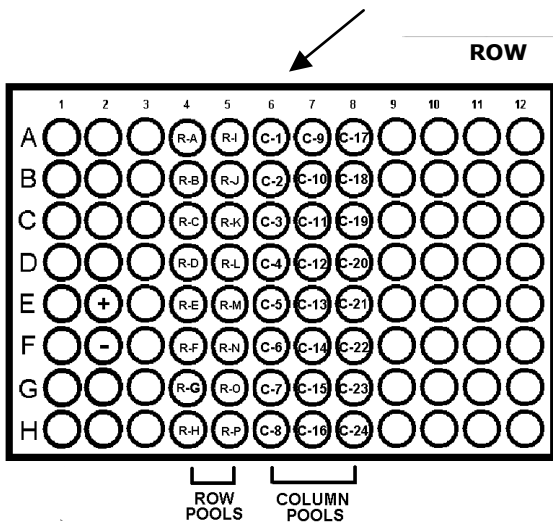
Library Code Superpool Collection Plate Copy #1
Library plates 001-70



SUPER POOL

The BAC library is separated into sequential Superpools of one 384-well plate.

Each Superpool of 1 plate is further separated into 16 Row pools and 24 Column pools.



The RC pooled DNA is further combined onto the Matrix Pool Plate. The researcher will receive two identical Matrix Pool Plates for each Superpool to perform Round II PCR.

Contents of Kit (Biological Materials and Documentation Supplied):

All plates (Superpool & Matrix) should be stored at -20°C. It is best to reduce the number of freeze/thaw cycles as much as possible. If the plates are going to be used at least one time per week, they should be refrigerated rather than frozen to reduce the freeze/thaw cycles.

Each Pool & Superpool kit comes with the following items:

1. Instruction Manual with a detailed examples, graphics, Superpool Key and Matrix Keys, (Matrix Row and Matrix Column).
2. TWO identical sets of Superpool DNA (on 96-well plates) for the Round I PCR. The Superpools are aliquoted and delivered on FOUR 2 ml 96-well plates. This helps reduce the risk of contaminating the entire collection.
3. TWO identical sets of Matrix Row and Matrix Column DNA pools for each Superpool (delivered on TWO 1 ml 96-well plates) to be used in Round II PCR. Remember- each Superpool will have a corresponding Matrix Pool Plate 96-well plate (two aliquots to help reduce the risk of contamination).
4. Two identical 2.0 ml tubes each containing 1.0 ml of both PCR control primers for the positive PCR control at a concentration of 10 μ M for each primer. There is enough primer mix for about 1,000 positive PCR control reactions.

Note: Each of the two sets are packaged separately, so the reserve set can be conveniently stored at -20°C until needed.

There is 5600 μ L (5.6 mL) of Superpool DNA supplied for each Superpool (1,400 μ L (1.5 mL) aliquoted on four identical 2ml 96-well plates), enough for approximately 3,000 Round I PCR experiments.

There is 1600 μ L (1.6 mL) of DNA supplied for each of the Matrix Pools (800 μ L (0.8 mL) aliquoted on two identical 1 ml 96-well plates), enough for approximately 800 Round II PCR experiments on each Superpool.

For technical support please contact Keith Stormo <keith@genomex.com> or Robert Bogden <bogden@genomex.com> or call 1-509-332-8080 ext. 36 (9am to 5pm US Pacific Time).

Quick Start Users Guide

Checklist For Using This Kit:

Initial steps

1. Identify the 'sequence of interest' by a variety of methods (i.e. similarity to other genes, or organisms, etc.).
2. Design robust PCR primers to amplify the sequence of interest (i.e. no hairpins, matching annealing temperatures and minimal duplex formation).
3. Perform BLAST search for known sequences using the primer sequences to identify potential problems (i.e. primers designed on a repetitive element).
4. Have newly designed PCR primers manufactured (we recommend <http://www.mwg.com>).

Preliminary Steps

5. Test amplification with above primers on genomic DNA and run positive and negative genomic controls (chosen by the researcher) and other controls for identifying proper Mg^{++} concentration and other PCR reagents according to the directions supplied with each.
6. Identify appropriate PCR annealing temperature, amplification, cycling and electrophoresis conditions that generate the appropriate band on gel electrophoresis (see page 11 for suggested PCR conditions for the included PCR amplification controls).
7. Once a robust PCR protocol has been determined and tested on genomic DNA and controls are favorable, the researcher is ready for screening the Superpool Collection Plate.
8. Make a PCR master mix with information gained in step 5 and tested in step 6 to screen the Superpool DNA. Note- there are 10 Superpools in the following example plus controls.

IT IS IMPORTANT TO KEEP THE **PCR SET-UP AREA COMPLETELY SEPARATE** FROM ALL AMPLIFIED PCR PRODUCTS.

Superpool Screening

9. Prepare the PCR set-up area with careful attention to insure NO contamination of the Superpool Collection Plate can occur. If the wells are accidentally contaminated with genomic template, the pooled DNA source has been irreversibly contaminated, the complete plate must be discarded, and one of the reserve plate used.
10. Thaw the Superpool Collection Plate and keep it on ice.
11. Spin down Superpool Collection Plate to remove any liquid on the mat (lid). Carefully remove the mat and be certain that the mat or plate are not contaminated.
12. Prepare PCR tubes using the PCR master mix prepared in step 8 and the Superpool Collection Plate DNA template and any additional controls needed.
13. Carefully reapply the mat paying close attention to the mat label and orientation and refrigerate (4°C) or refreeze the Superpool Collection Plate DNA template (-20°C).
14. Run the PCR experiment for the appropriate cycles determined in step 6.

Gel Electrophoresis of Superpool Collection Plate PCR Experiment

15. Prepare appropriate gel for observing PCR products of interest (usually 0.8 to 2.0 % agarose).

16. Load gel with ladder and PCR products.
17. Electrophores with standard conditions determined in step 6.
18. Stain gel and record which Superpool(s) have bands "positive hits" (sequences of interest).

IT IS IMPORTANT TO KEEP THE **PCR SET-UP AREA** COMPLETELY SEPARATE FROM ALL AMPLIFIED PCR PRODUCTS.

Superpool of interest Screening

19. Prepare a PCR master mix (like in step 8) for 23 reactions plus controls to follow one superpool positive hit. If additional superpool hits are to be tracked, then make sufficient PCR master mix for all interested superpools.
20. Clean the PCR set-up area with careful attention to assure NO contamination of the Superpool or Matrix plates can occur. If the wells are accidentally contaminated with genomic template or Superpooled template, the pooled DNA source has been irreversibly contaminated and the complete plate must be discarded and the (one) reserve plate used.
21. Thaw the Superpool Matrix Plate identified in step 18 and keep it on ice.
22. Spin down Superpool Matrix Plate to remove any liquid on the mat (lid). Carefully remove the mat and be certain that the mat and plate are not contaminated.
23. Prepare PCR tubes using the PCR master mix prepared in step 19 and the Superpool Matrix Plate DNA template (thawed in Step 21) and any additional controls needed.
24. Carefully reapply the mat paying close attention to the mat orientation and refreeze (-20°C) the Superpool Matrix Plate DNA template.
25. Run the PCR experiment for the appropriate cycles determined in step 6.

Gel Electrophoresis of Superpool Matrix Plate PCR Experiment

26. Prepare appropriate gel for observing PCR products of interest (usually 0.8 to 2.0 % agarose).
27. Load gel with ladder and PCR products.
28. Electrophores with standard conditions determined in step 6.
29. Stain gel and record which Matrix Plate Pool wells have bands "positive hits".

Interpret the Matrix gel data to identify the specific Row and Column

30. Compare the positive Matrix Row Pool gel bands to the Matrix Row Pool keys on page 25. Record all possible intersections of the gel bands on the Matrix Row Key. These intersections are the row(s) pools with the hit(s). Alternately, use the gel banding patterns on page 25 to identify the original source row(s) location.
31. Compare the positive Matrix Plate Column gel bands to the Matrix Column Pool keys on page 26. Record all possible intersections of the gel bands on the Matrix Column Key. These intersections are the column(s) pools with the hit(s). Alternately, use the gel banding patterns on page 26 to identify the original source column(s) location.

Final identification of the Clone of Interest (If ONLY 1 Plate, 1 Row, and 1 Column are identified during matrix interpretation)

32. Use the Row Pool data and Column Pool data determined in steps 30 and 31 to identify the clone well location on the library plate identified in step 34.

Final identification of the Clones of Interest (If MULTIPLE Plate, Row and Column hits are identified during matrix interpretation)

33. Use the data recorded in steps 30, 31 and write down all the possible Rows and Columns the clones could possibly be located.

34. The ambiguities can be resolved in the clone confirmation test.

Clone Confirmation Test

35. Prepare antibiotic media for growing the individual clones identified in steps 32 and 33.

36. Carefully remove the clone of interest from the BAC Library Source Plate and inoculate media prepared in step 35. The BAC Library Source Plates are not included in the Pool & Superpool Kit, if you do not have a copy of the BAC library, please contact Robert Bogden bogden@genomex.com to obtain a copy. Many researchers have a variety of methods for aseptically removing a single clone from 384 well plates without thawing the entire plate. These methods include sterile toothpicks, a sterilized loop that is still warm enough to melt the top of the well of interest, and a number of other individual preferences.

37. Grow up the clones and make a confirmatory test with the primer pair on lysed cells to be certain that the clone of interest has been located. We also recommend including another clone in this confirmation test that is not identified as a hit for a negative control.

Troubleshooting FAQ

1. Can I use smaller volumes of template or reduced PCR reaction volumes?

We have tested for ample template at 35 rounds of PCR. Allowing for some template degradation with multiple freeze/thaw cycles, we would not recommend less than 2 μL of template. We have included sufficient template pools for about 3,000 PCR primer pair screenings of the Superpool Collection Plates and about 800 screenings of the Matrix Plates. Some researchers have reduced the volume with good success but we do not offer technical support or troubleshooting support for templates volumes below 2 μL (or final PCR volumes below 20 μL).

2. All the Superpools are showing hits, what can I do and what could this mean?

Check the positive and negative genomic controls, if the PCR primers are not stringent enough, there can be broad amplification. Try increasing the PCR annealing temperature. If this does not help, the primer sequences could be part of a repeating element or other common sequence that would have multiple copies in the genome.

Blast your primers against databases to look for possible common sequence homology. You could also have genomic DNA contamination in your Superpool Collection Plate.

3. The bands are not all the same intensity, does that mean something is wrong?

There are a number of factors that influence amplification efficiency. Most matrix pools have 672 different clones but some have 1152 clones in each matrix pool and others have only 448 clones (See the Matrix Keys sections for clone counts). If the key indicates the bands would be from one specific plate, it is likely that you have found a hit. If they would indicate different source plates, it could be that PCR amplification parameters in Quick Start User Guide step 6 are not as robust as initially thought.

4. There are 3 bands in one of my matrix gels, what could this mean?

It could mean that there are two clones that have been identified as hits. Use the keys to identify what two sources they came from. There could be as many as 4 clones identified with 5 bands if they all were in one matrix pool and then they would have to be in four other separate matrix pools. You should see more than 2 bands in other matrices as well. Follow Quick Start User Guide steps 35, 36, 37 and 38 for multiple hit resolution.

5. I still cannot resolve the issue of 3 bands in only one of my matrix gels, what can I do?

Follow the Clone Confirmation Test in Quick Start User Guide steps 37, 38 and 39. If the Clone Confirmation Test does not resolve the issue, call technical support for further suggestions.

6. Is there any way to screen all the Superpools in one round of PCR with fewer PCR experiments than running all the Superpool collection and all the Superpool Matrix Plates at the same time?

Yes, please call technical support for additional information.

7. I think I may have contaminated my plates, is there any way to check?

Rerun an earlier primer pair that did not have hits in the questionable Superpool or a primer pair that had correctly been screened.

8. The Matrix Keys are too confusing to follow each time I have a hit, what can I do?

There are two options, the first is to call technical support for pointers and a phone tutorial. Please email a list of at least 10 hits that you have found but have had difficulty in resolving. The second solution is to do more PCR experiments by using our standard PCR system where there are 47 reactions for each superpool instead of the 23 reactions in the Matrix system for the same superpool.

9. I have used up all of my Superpool Collection stock, can I get more?

Yes, we are able to supply additional pooled stocks at a discounted price for original purchasers of our Pooling and Superpooling System. Please contact technical support for further information and costs.

Screening Recommendations:

We highly recommend researchers develop a robust PCR protocol for proper amplification of positive and negative controls before screening the Superpool DNA. There is a positive control DNA template included on all plates (Superpools and Matrix Pools). The positive control DNA templates are at the same relative concentrations as the corresponding pools. The positive control primers have a 57 °C annealing temperature and produce a 607 bp.

The positive PCR control primers are:

AM001-C12-M13-F (20 mer) Td=57.3 5' ATATAATGCAAAAAGTGGCT 3'

AM001-C12-M13-R (20 mer) Td=57.6 5' GTAATGACCCTTTTCTCTCC 3'

A recommended PCR protocol for amplifying the positive control template follows:

2µL DNA positive control from Superpool plate or a Matrix Pool plate

2µL Primers for positive control (both primers are in the tube and give a 607 bp product)

2µL 10X PCR buffer (supplied with TAQ polymerase)

4 µL [10mM] Mg²⁺ (2mM final concentration)

1 µL TAQ Polymerase (~1 Unit)

9 µL H₂O

20 µL final volume

Thermocycler Conditions:

Step 1 94 °C for 120 seconds

Step 2 94 °C for 30 seconds

Step 3 57 °C for 30 seconds

Step 4 72 °C for 60 seconds

Step 5 Go to Step 2 for 35 cycles total

Step 6 72 °C for 600 seconds

Step 7 Hold at 4 °C

Internal Standard

As part of our Quality Assurance protocol, we choose a missed well in one of the Superpools of each BAC Library and replace the missed well with a Positive Control BAC clone. This is the same BAC clone that we use for our positive control. The superpool, plate and well location of this positive control is different for each library. This positive clone is a BAC clone of approximately 130 Kb from *Anaplasma marginale*. If you have a positive hit from your primers of interest in the QA control well identified for your library, the hit is not from your organism. Please contact Technical Support if you identify a hit from your primers on this QA control BAC clone.

Detailed Description of Pools & Superpools:

The system consists of a collection of multiple Superpools that are screened during First Round PCR, to determine which set of Matrix Pools to screen during Second Round PCR. The total number of Superpools is determined by the total number of clones in the BAC library. **Each Superpool has its own 96-well plate of corresponding Matrix Pools.**

Superpools:

Each Superpool consists of one consecutive 384-well plates from a BAC library (see Superpool Key pages 20-22). DNA is prepared by growing EACH BAC CLONE separately (to avoid growth competition between BAC clones) then combining the 384 cultures into one large-scale BAC prep. The Superpool of BAC DNA is then aliquoted onto two identical 96-well plates.

Superpool SP-1 has all the BAC clones in the first plate of the BAC library (Plate).

Superpool SP-2 has all the BAC clones in the second plate of the BAC library (Plate 2).

This naming continues for the entire library.

Matrix Pools:

For each Superpool there is one set **Matrix Pools** (this set of 18 Matrix Pools are aliquoted onto two identical **Matrix Pool Plates** to help reduce the risk of contamination). The **Matrix Pools** of Superpool #1 are named:

Matrix Row Pools 1MRP-A2 through 1MRP-H2 for the 8 wells that contain the matrix of rows A-P in Superpool one. Each Matrix Row Pool contains 96 clones. See the Matrix Row Pool Key on page 25 for exact composition of each well.

(1 plate X 4 different rows X 24 row wells/plate=96 clones per Matrix Row Pool)

Matrix Column Pools 1MPP-A3 through 1MPP-B4 for the 10 wells that contain the matrix of columns 1-24 in Superpool one. See the Matrix Column Pool Key on page 26 for exact composition of each well. The Matrix Column Pools in wells A3 through D3 have 96 clones (1 plate X 6 different columns X 16 column wells/plate=96 clones per Matrix Column Pool). The Matrix Column Pools in wells E3 through B4 contain 64 clones (1 plate X 4 different columns X 16 column wells/plate=64 clones per Matrix Row Pool).

Please see the **Key to Matrix Pool Plates** and **Matrix Pool 96-Well Plate Contents** on pages 24-26 for the exact composition of each well in the **Matrix Pools**.

Remember that each Superpool has it's own 96-well plate of corresponding Matrix Pools.

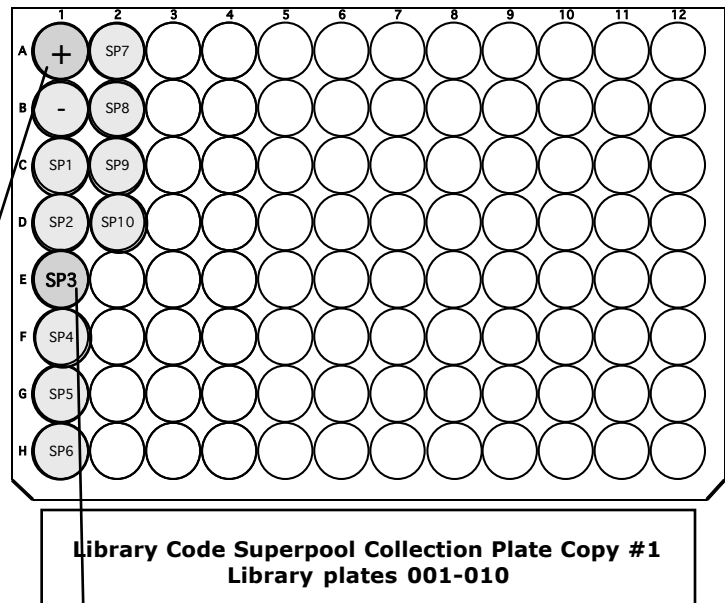
For technical support please contact Keith Stormo <keith@genomex.com> or Robert Bogden <bogden@genomex.com> or call 1-509-332-8080 ext. 36 (9am to 5pm US Pacific Time).

Example Clone Identification (Round I PCR):

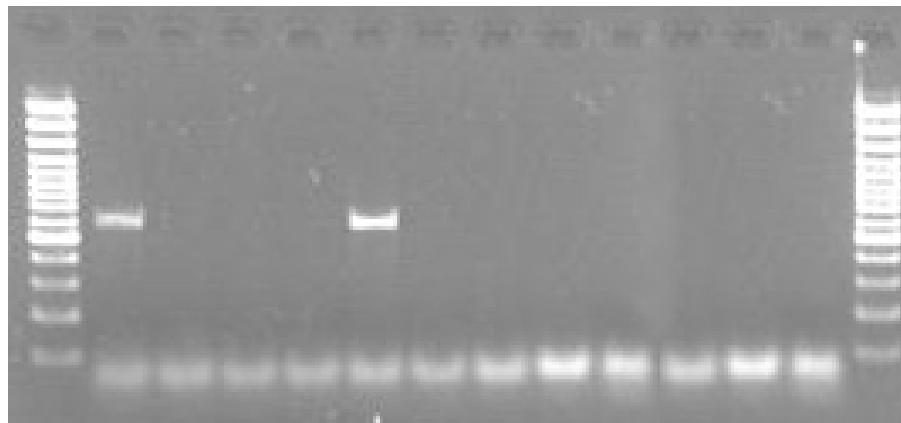
SCREENING THE SUPERPOOLS

In this example, the Round I PCR gel results show "hits" in the positive control (using positive control primers with a 607 bp product) and Superpool 3 (to find the plate and well location of our QA control). This QA control is placed in all libraries to provide a Quality Assurance test and to enable researchers to test the Pool and Superpool method on a known control.

The researcher will now proceed with Round II PCR on the Superpool 3 Matrix Pool Plate.



100 bp ladder
A1; + control
B1; - control
C1; SP1
D1; SP2
E1; SP3
F1; SP4
G1; SP5
H1; SP6
A2; SP7
B2; SP8
C2; SP9
D2; SP10
100 bp ladder



Example Clone Identification (Round II PCR):

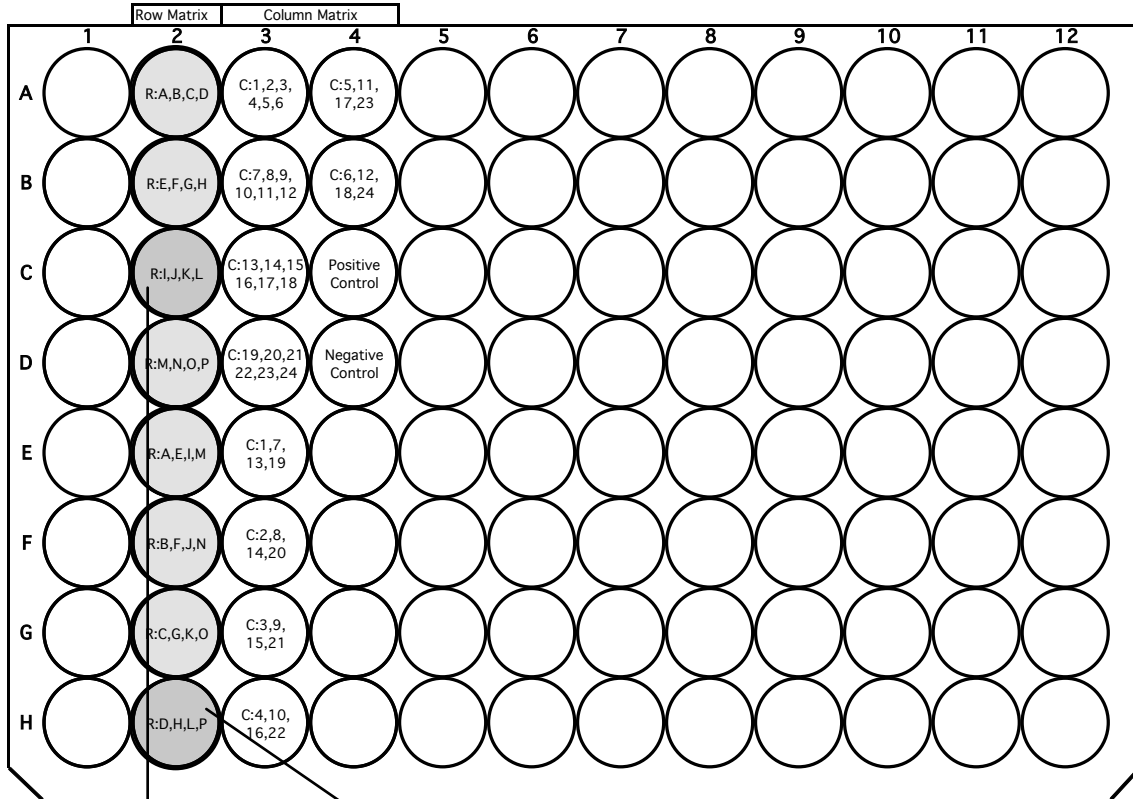
SCREENING THE MATRIX PLATE POOLS

NOT USED IN THE ONE PLATE SUPERPOOL DESIGN

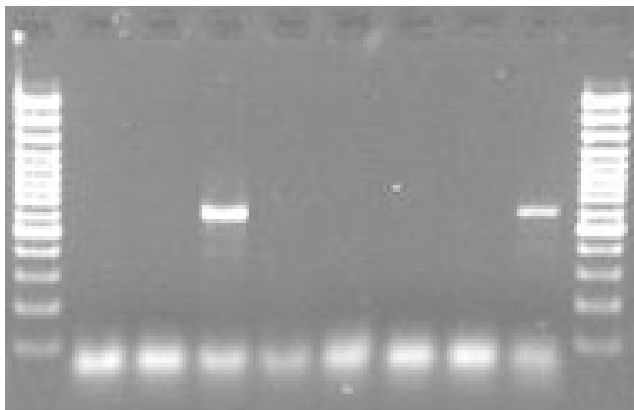
Example Clone Identification (Round II PCR):

SCREENING MATRIX ROW POOLS

MATRIX POOL PLATE LOADING PATTERN



100bp ladder
 3MRP; A2
 3MRP; B2
 3MRP; C2
 3MRP; D2
 3MRP; E2
 3MRP; F2
 3MRP; G2
 3MRP; H2
 100 bp ladder



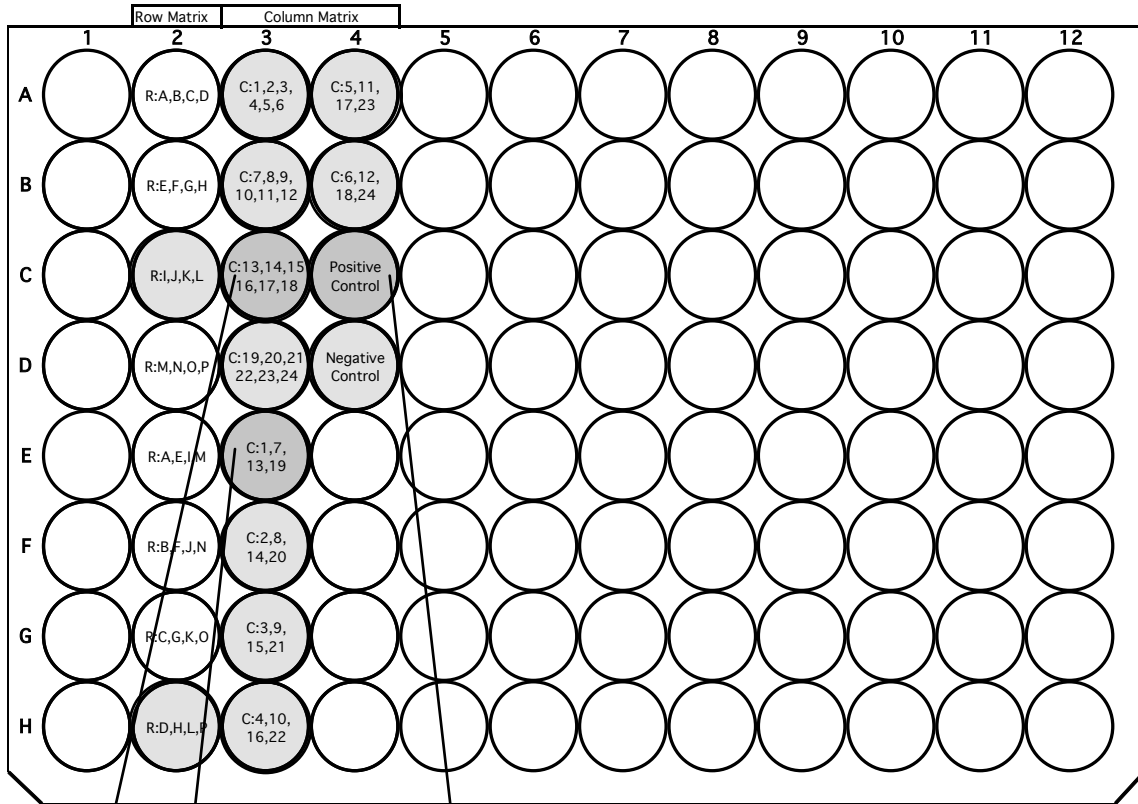
The results of Round II PCR gel electrophoresis for the SP-3 Matrix Row Pools show a positive hit in wells C2 and H2 of the SP-3 Matrix Row Pools.

The Matrix Plate Loading pattern and the Matrix Row Keys (page 25) show that the clone of interest is in Row L.

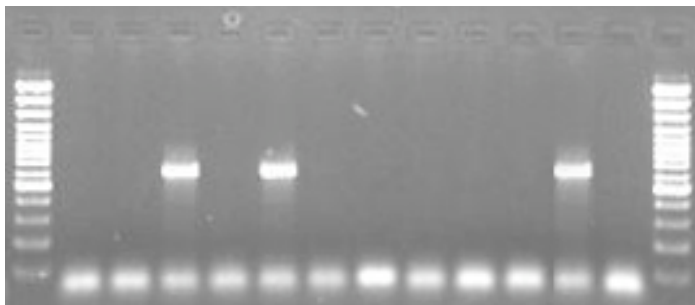
This result, combined with the Round I Superpool result, show the clone of interest is in:
 Library plate #3, Row L.

Example Clone Identification (Round II PCR):

MATRIX POOL PLATE LOADING PATTERN



100 bp ladder
 3MCP; A3
 3MCP; B3
 3MCP; C3
 3MCP; D3
 3MCP; E3
 3MCP; F3
 3MCP; G3
 3MCP; H3
 3MCP; A4
 3MCP; B4
 C4; + control
 D4; - control
 100 bp ladder



The results of Round II PCR gel electrophoresis for the SP-3 Matrix Column Pools show a positive hit in wells C3, E3 and C4 of the SP-3 Matrix Column Pools.

The Matrix Plate Loading pattern and the Matrix Column Keys (page 26) show that the clone of interest is in Column 13.

This result, combined with the Round I and Round II Row Pool results, show the clone of interest is in:

Library plate #03, Row L, Column 13.

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This page is for custom tools to further resolve multiple hits.

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This page is for custom tools to further resolve multiple hits.

Key to Superpool Plate:

The Superpool number is the corresponding Source Plate number.

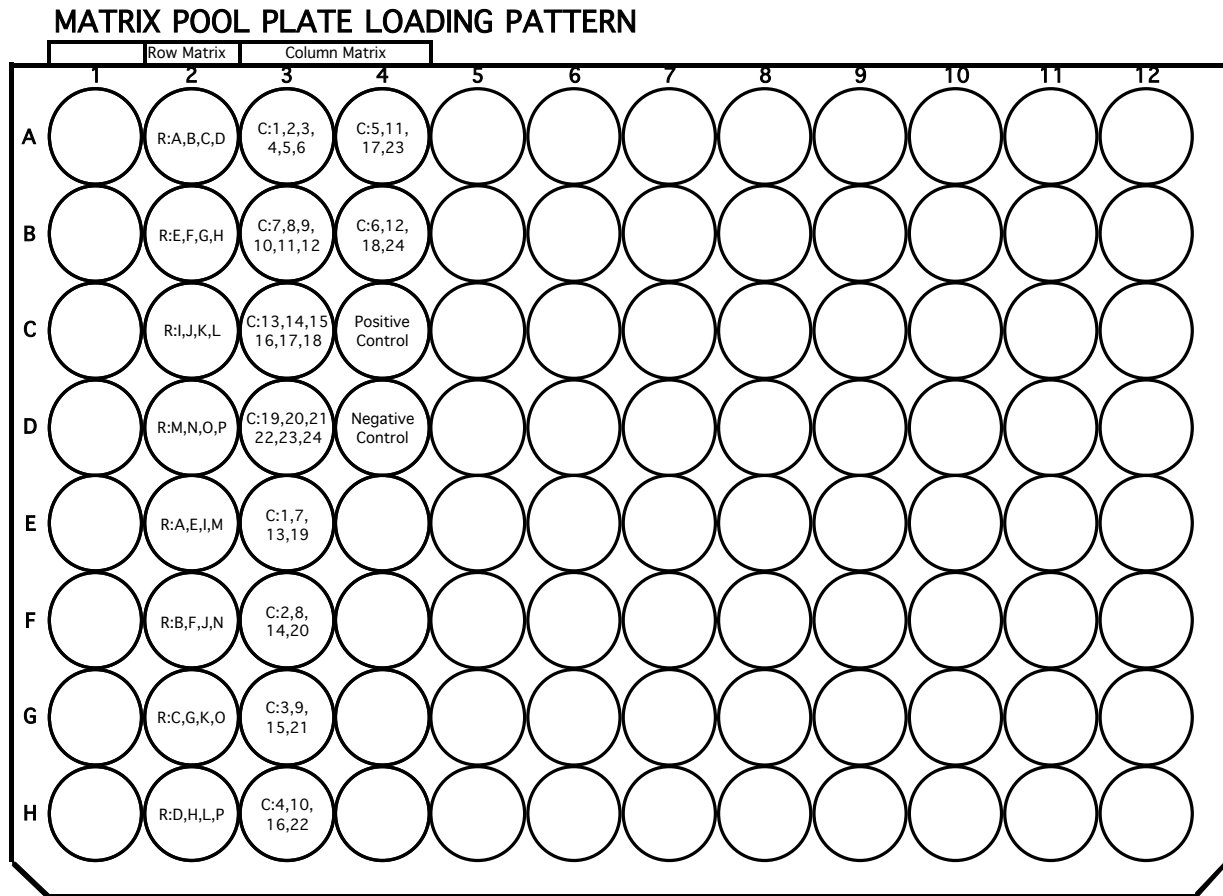
Key to Superpool Plate (continued):

Intentionally left blank

Key to Superpool Plate (continued):

Intentionally left blank

Key to Matrix Pool Plates for Each Superpool



There is nothing in column one for the One Plate Superpool System.

Please disregard any mention or graph that shows plates in column 1. It is from our 7 plate Superpool system.

Matrix Plate Pools:

Not needed in the One Plate Superpool System

Matrix Row Pools:

The Row Pools from one Superpool are put into a matrix as shown below. The rows are pooled across and the columns are pooled down. By looking at the location of positive hits on the electrophoresis gel in the construction matrix, the identity of the plate(s) that contain the clone of interest can be determined.

↓

MATRIX ROW POOLS	E 2	F 2	G 2	H 2
A 2	A	B	C	D
B 2	E	F	G	H
C 2	I	J	K	L
D 2	M	N	O	P

→

An alternate point of view is to look at all of the possible gel banding patterns that could possibly be formed. The shaded bands show all possible potential patterns and the black bands show the example from our gel photos indicating a hit in Row L.

Superpool Row Pools pooled together to form the Matrix Row Pools. All Matrix Row Pools have 672 clones.
Matrix Pool Plate, row wells ran on a gel.

MATRIX ROW POOLS KEY	R:A,B,C,D	R:E,F,G,H	R:I,J,K,L	R:M,N,O,P	R:A,E,I,M	R:B,F,J,N	R:C,G,K,O	R:D,H,L,P
Source Row	A2	B2	C2	D2	E2	F2	G2	H2
R-A	Shaded				Shaded			
R-B	Shaded					Shaded		
R-C							Shaded	
R-D	Shaded							Shaded
R-E		Shaded			Shaded			
R-F		Shaded				Shaded		
R-G							Shaded	
R-H		Shaded						Shaded
R-I			Shaded		Shaded			
R-J			Shaded			Shaded		
R-K			Shaded				Shaded	
R-L			Black					Black
R-M				Shaded	Shaded			
R-N						Shaded		
R-O				Shaded			Shaded	
R-P				Shaded				Shaded



Matrix Column Pools:

The Column Pools from one Superpool are put into a matrix as shown below. The rows are pooled across and the columns are pooled down. By looking at the location of positive hits on the electrophoresis gel in the construction matrix, the identity of the plate(s) that contain the clone of interest can be determined.

↓

MATRIX COLUMN POOLS	E3	F3	G3	H3	A4	B4
A3	1	2	3	4	5	6
B3	7	8	9	10	11	12
C3	13	14	15	16	17	18
D3	19	20	21	22	23	24

→

An alternate point of view is to look at all of the possible gel banding patterns that could possibly be formed. The shaded bands show all possible potential patterns and the black bands show the example from our gel photos indicating a hit in column 13.

Superpool Column Pools pooled together to form the Matrix Column Pools. Wells A3-D3 have 672 clones and wells E3-B4 have 448 clones. Matrix Pool Plate, column wells ran on a gel.

MATRIX COLUMN POOLS KEY	C:1,2,3, 4,5,6	C:7,8,9, 10,11,12	C:13,14,15, 16,17,18	C:19,20,21, 22,23,24	C: 1,7,13,19	C: 2,8,14,20	C: 3,9,15,21	C: 4,10,16,22	C: 5,11,17,23	C: 6,12,18,24
Source Column	A3	B3	C3	D3	E3	F3	G3	H3	A4	B4
C-1										
C-2										
C-3										
C-4										
C-5										
C-6										
C-7										
C-8										
C-9										
C-10										
C-11										
C-12										
C-13										
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C-19										
C-20										
C-21										
C-22										
C-23										
C-24										



Notes: