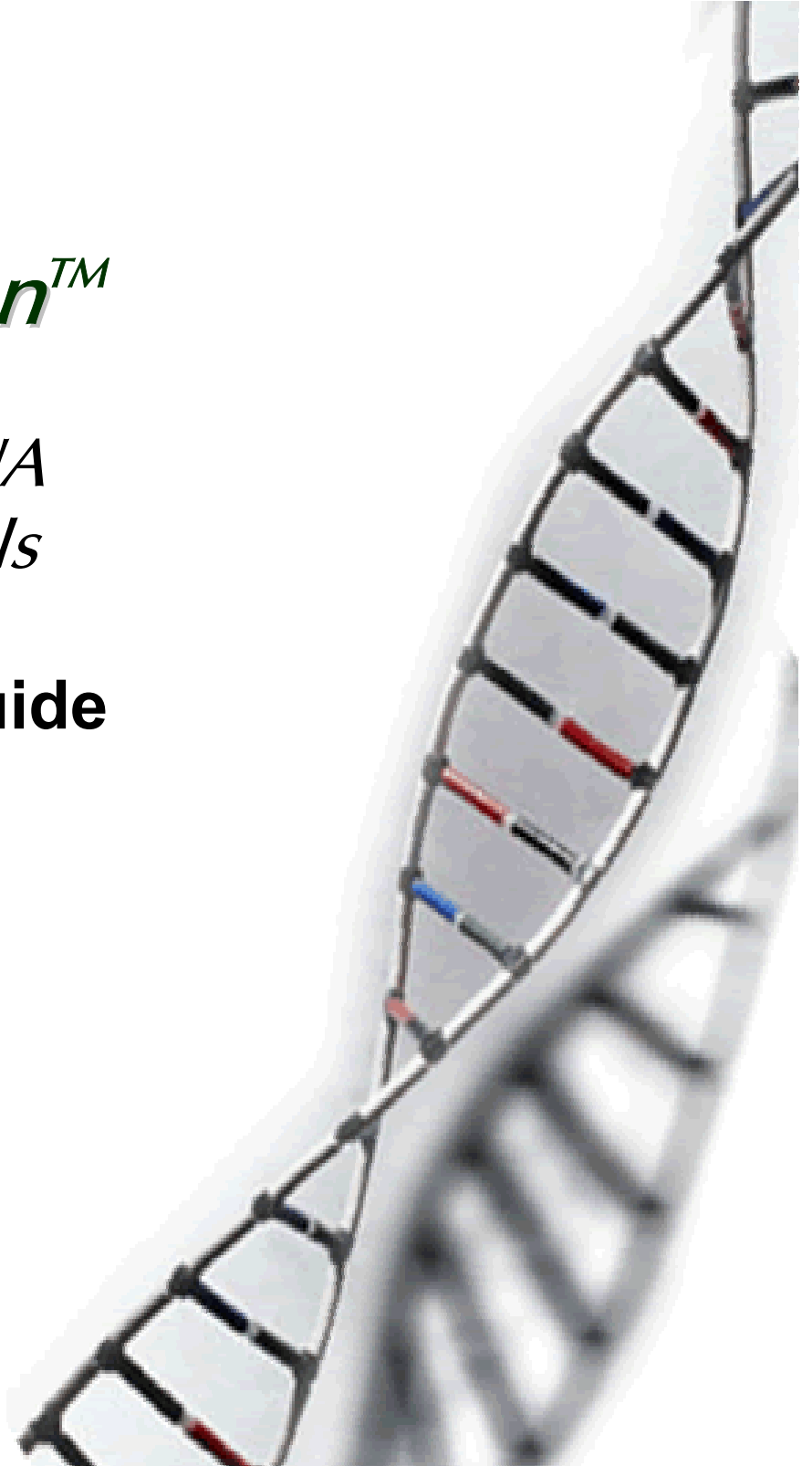


RapidScreen™

Arrayed cDNA Library Panels

Application Guide



RapidScreen™ Arrayed cDNA Library Panels – Application Guide

Table of Contents

Table of Contents.....	1
Package Contents and Storage Conditions.....	3
Included components of Product:	3
Additional required components:	3
Introduction	4
Table 1. List of available Rapid-Screen™ Arrayed cDNA Library Panels.....	4
Library Construction and Design.....	5
Library vector	5
cDNA synthesis and fractionation	5
Library arraying and preparation	5
Quality control testing	5
Screening Procedures	5
Overview	5
Gene Specific-primer PCR.....	6
Fig. 1. Overview of RapidScreen™	7
Fig. 2. RapidScreen™ PCR approaches.	8
Vector specific primer PCR.....	9
Positive and negative controls	9
Ordering of Sub-Plates	10
Screening of Sub-Plates	10
Colony screening	10
Colony screening by PCR — Method I — Microscreen	10
Colony screening by PCR — Method II — Macroscreen	11
Colony screening by hybridization	12
Positive cDNA library clone.....	12
Sample Library Screen	12
Screening by gene-specific primer PCR	12
Fig. 3. Use of RapidScreen™ panels to screen for full-length cDNA.....	13
Longest clone detection	14
Fig. 4. Detection of longest clones with RapidScreen™ panels.....	14
Fig. 5. RapidScreen™ analysis using different Taq DNA polymerases.....	15
Pooling of wells (additional Protocols)	15
Fig. 6. Performing Rapid-Screen™ using pooled wells.....	16
Fig. 7. Detection of alternatively-spliced transcripts with Rapid-Screen™	17
Trouble-shooting Guide	17
A. No positive signal on Master Plate screen	17
B. Every well is positive	17
C. Smears on the gel	18
Published Papers Using Rapid-Screen™ Arrayed cDNA Library Panels.....	18
Appendices	19
Appendix A. Map of pCMV6-XL4	19
Appendix B. Polylinker Sequence of pCMV6-XL4	20
Appendix C. Primer Testing	21
Appendix D. Standard PCR Conditions	22
Appendix E. pCMV6-XL4 Sequence	23
Appendix F. pCMV6-XL4 Restriction Map	25
Appendix G. Limited License Agreement.....	30

Rapid-Screen™ is covered by pending U.S. patent applications. This product is optimized for use in the Polymerase Chain Reaction ("PCR") covered by patents owned by Hoffmann-La Roche Inc. and F. Hoffmann-La Roche Ltd. No license under these patents to use the PCR Process is conveyed expressly or by implication to the purchaser by the purchase of this product.

Package Contents and Storage Conditions

Included components of Product:

- 96-well Master Plate. Store upright at -20°C .
- Vector Primer 3. Store at -20°C .
- Vector Primer XL39. Store at -20°C .
- Positive control DNA. Store at -20°C .
- Positive control primer-pair. Store at -20°C .
- Four sheets of aluminum foil sealing tape. Store at room temperature.

Additional required components:

- LB broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl. Adjust pH to 7.0 with 1 N NaOH and autoclave.)
- Ampicillin
- LB agar + ampicillin plates
- PCR reagents (gene-specific primers, *Taq* DNA polymerase, buffer, dNTPs)
- Pipettors used exclusively for PCR, to avoid inadvertent contamination
- Barrier pipette tips, to avoid aerosol contamination from the pipettors
- PCR-grade water

Introduction

Rapid-Screen™ panels were generated with a bias towards longer cDNAs, so they are ideal for identifying long cDNAs and the previously hard-to-clone 5' ends of cDNAs. Each panel is a series of arrayed cDNA library clones. Currently 20 panels from different tissues are available (see Table 1). The Master Plate for each library panel contains DNA from 500,000 cDNA clones and is initially screened in one simple 96-well PCR. This first set of PCRs identifies the Sub-Plate(s), which contains the clone of interest. The Sub-Plates are escherichia coli glycerol stocks contained in standard 96-well microtiter dishes. Each well contains 50 cDNA clones with approximately 5,000 clones per plate. The Sub-Plates are screened with a single 96-well PCR to identify the positive well(s) in the Sub-Plate. Cells from a positive well(s) are then plated on LB/ampicillin agar and the resulting bacterial colonies are screened by PCR in order to obtain the desired clone. It is that simple and that easy!

Table 1. List of available Rapid-Screen™ Arrayed cDNA Library Panels.

Human	Cat. No.
Brain (adult)	LAB-1001
Brain (fetal)	LLFB-1001
Colon	LCO-1001
Heart	LHT-1001
Kidney	LKD-1001
Liver	LLI-1001
Lung	LLU-1001
Peripheral blood leukocyte	LPBL-1001
Placenta	LLPC-1001
Skeletal muscle	LMU-1001
Small intestine	LSI-1001
Spleen	LLSP-1001
Testis	LTS-1001
Mouse	
Brain (adult)	MAB-1001
Embryo (12.5-day)	MEB-1001
Embryo (19-day)	MEA-1001
Liver	MLI-1001
Testis	MTS-1001
Thymus	MTM-1001
Rat	
Brain (adult)	RAB-1001

Library Construction and Design

Rapid-Screen™ Arrayed cDNA Library Panels are cDNA libraries arrayed in a 96-well format. This unique design allows for easy and quick screening of the cDNA library. However, the power of using the Rapid-Screen™ Arrayed cDNA Library Panels lies in the design and construction of the cDNA libraries themselves.

Library vector

Rapid-Screen™ libraries are constructed in the vector pCMV6-XL4 (see **Appendix A**) and the cDNA is cloned unidirectionally between the *EcoRI* and *SaI* restriction sites (see **Appendix B**).

NOTE: The *SaI* site is destroyed in the cloning process.

cDNA synthesis and fractionation

The cDNA used in making the library panels is synthesized from double-purified mRNA using an oligo(dT) primer and MMLV reverse transcriptase. The cDNA is size fractionated in an agarose gel. The sample lanes of the gel are cut into multiple slices each containing a distinct set of sizes and then individually processed. These fractionated cDNAs are then ligated separately into the library vector pCMV6-XL4. The final plasmid library is then assembled from the resulting clones from each ligation and transformation. The libraries are arrayed into the plates in such a way as to give larger-sized cDNA clones high representation. The directionally-cloned cDNAs are positioned downstream of the CMV promoter which can later be used to transcribe the cloned cDNAs.

Library arraying and preparation

The arrayed cDNAs in the Master Plate are purified plasmid DNA isolated from each clone represented in the combined libraries. Each well on the Master Plate contains DNA from approximately 5,000 clones, with about 500,000 clones in each panel. Each individual well on the Master Plate corresponds to a single Sub-Plate. The Sub-Plates are 96-well plates containing glycerol stocks of library plasmid-harboring *E. coli*. The bacterial clones in each well are amplified from an original 50 clones. Unlike standard cDNA libraries, in which $1-5 \times 10^6$ cDNA clones are normally screened in order to identify positive clones, Rapid-Screen Arrayed cDNA Library Panels require a screen of only 500,000 clones. Because the cDNA used in Rapid-Screen™ is subject to size selection before its ligation there is no bias toward the cloning of small cDNAs, as is the case in traditional cloning. Each of the cDNA ligations are performed and transformed independently of each other to assure maximum representation in the final library. Additionally, the larger cDNAs are purposefully over-represented in the arrayed library relative to their expression levels in the cell to favor the cloning of large cDNA inserts.

Quality control testing

Rapid-Screen™ Arrayed cDNA Library Panels are subjected to a series of quality control measures prior to their distribution. Both the insert size range and percentage of clones containing cDNA inserts for each size fraction in each library are determined. In all of the library panels, greater than 90% of the clones contain cDNA inserts. The “clonability” of each library panel is ensured, this is achieved by carrying out at least three library screens for known genes such as transferrin receptor, HPRT, neurofilament subunit NF-L, amyloid-beta protein (APP), phospholipase A2, integrin beta subunit, norvegicus protein tyrosine phosphatase gene and recombination activating protein.

Screening Procedures

A clone of interest can be easily identified using Rapid-Screen™ with a minimum of three rounds of PCR. An overview of the screening procedure is shown in Fig. 1.

Overview

There are two basic ways to screen a Rapid-Screen™ library by PCR (Fig. 2). The first method involves the use of a pair of gene specific primers directed to a gene of interest. The second approach requires the use of a library vector specific primer and a single gene-specific primer. This latter method can be used to identify the longest cDNA for a given gene present in the library. The sequence of the vector primer corresponds to a site located upstream (5') of the multiple cloning site in the library vector. The choice of methods depends on the size of the mRNA of interest,

location of the known sequence in the gene and the quality of the known cDNA sequence information. The following sections will describe both screening methods and some general considerations concerning PCR.

Gene Specific-primer PCR

This method is ideal for use with cDNA of lower abundance or when the location of the known sequence is not known within a large gene (>3 kbp). Two gene-specific primers are required that should amplify a product between 300 and 800 bp in size.

Primer design

Primer design is a crucial factor in achieving success in PCR. A general set of guidelines for primer design is reported to aid in the amplification of specific product. Primers should be between 21 and 26 nucleotides in length, contain approximately 50% G/C content and not form strong secondary structures. Avoid sequences that are susceptible to primer-dimer formation. There are several commercially available computer programs for designing PCR primers as freeware, shareware and on the Internet.

Primer testing

Perform PCR according to standard protocols, such as **Appendix C** and determine the optimal conditions for PCR amplification with the chosen primer pair. The test PCR can be done using a known template, such as first strand cDNA or a cloned cDNA fragment.

Screening of the Master Plate with gene specific primers

Remove the Master Plate from –20°C storage.

- Resuspend the dried DNA in each well of the Master Plate with 28 µL of sterile distilled water. Leave the plate at room temperature for 30 min. before use.
- Prepare a PCR pre-mix for 96 reactions of 25 µL each taking into account 5 µL of template DNA. Aliquot 20 µL of the PCR pre-mix into each well of a 96-well PCR plate. See **Appendix D** for a recommended protocol.
- Transfer 5 µL of the resuspended DNA from each well of the Master Plate into the corresponding wells of the 96-well plate for PCR (Pipette resuspended DNA up-and-down several times to facilitate mixing, before transferring the DNA to the PCR plate).
- Re-seal the Master Plate with a new sheet of sealing tape (provided) and store the plate at –20°C.
- Perform a 35-cycle PCR following standard protocols (see **Appendix D**). The annealing temperature should be compatible with the T_m of the gene-specific primers.

Identify the positive wells by agarose gel electrophoresis of the PCR products.

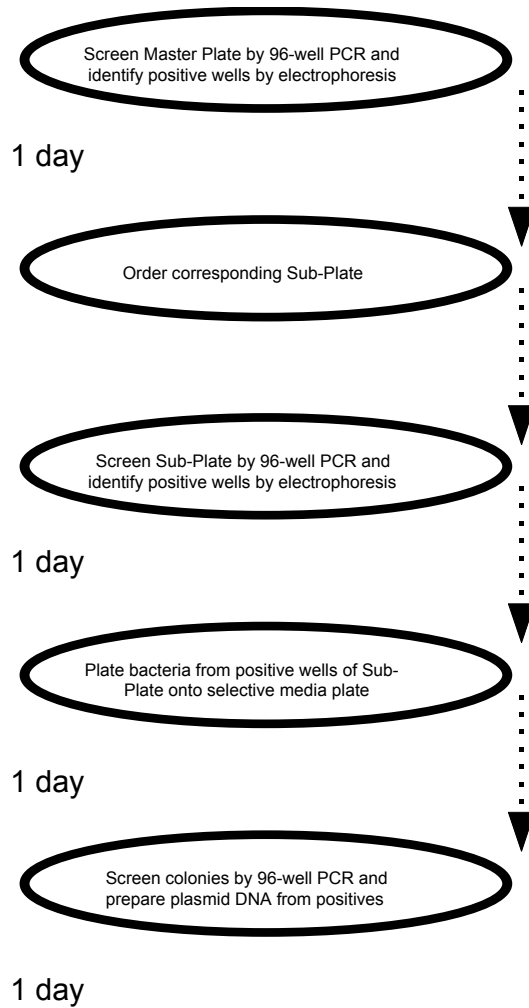
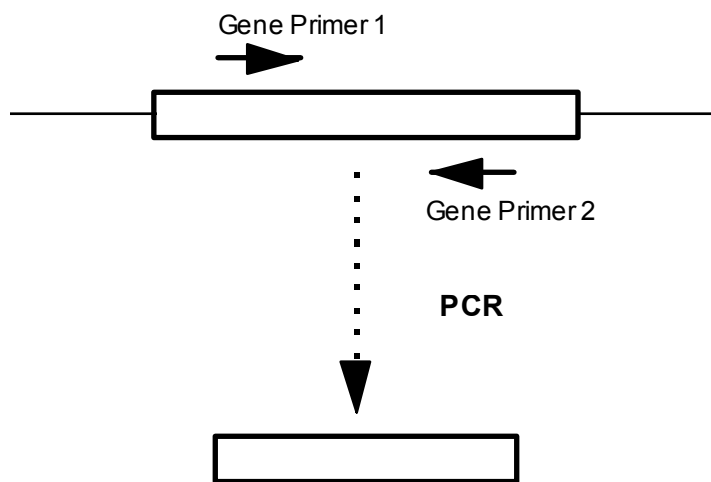


Fig. 1. Overview of RapidScreen™.

Gene Specific Primer PCR



Vector Specific Primer PCR

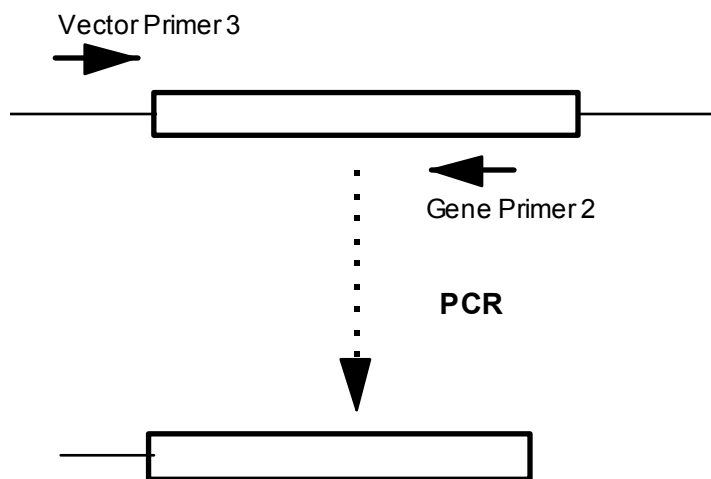


Fig. 2. RapidScreen™ PCR approaches.

Vector specific primer PCR

This method is best used when there are multiple gene specific positives. By using a reverse gene specific primer with the provided Vector Primer 3, one is able to identify the clone with the longest 5' end.

Primer design

For the vector PCR screening, primers should be designed with annealing temperatures of 68°C. Please refer to section 4.i.a (Primer design) above for more details about designing primers.

Primer testing

Perform PCR according to standard protocols, such as **Appendix D** and determine the optimal conditions for PCR amplification with the chosen primer pair. A suitable forward (5') primer is required to test the reverse (3') gene-specific primer. This partner primer can be complementary to either a cloned cDNA sequence or cloning vector sequence.

Screening of Master Plate with vector specific primer

Remove the Master Plate from -20°C storage. Care should be taken when removing the sealing tape from the Master Plate not to contaminate one well with another.

Follow the protocol as in 4.i.c (Screening of the Master Plate with gene specific primers) using Vector Primer 3 and a reverse (3') gene-specific primer but using the touchdown program suggested in **Appendix D**.

Positive and negative controls

Positive control

If no positive signals are observed in the PCR screen, it is recommended that the PCR reagents be tested in a control reaction using the Positive Control DNA and Primer Mix contained in this kit.

- Set up the following reaction:

10× PCR reaction buffer	2.5 µL
dNTPs (2 mM each)	2.5 µL
Positive Control DNA*	5.0 µL
Primer Mix*	1.0 µL
<i>Taq</i> DNA polymerase	0.5 U
sterile ddH ₂ O	to 25 µL

*The Positive Control DNA (6 ng/µL) is a sample Master Plate well. The Primer Mix (10 pmol/µL) contains oligonucleotides to amplify a specific PCR product from the Positive Control DNA.

- The following cycling parameters are recommended:

Pre-soak	94°C for 5 min
Denaturation	94°C for 30 sec
Annealing	60°C for 30 sec
Extension	72°C for 1.0 min
Cycles	35
Final Extension	72°C for 5 min

Analyze the reaction by agarose gel electrophoresis. A product of 483 bp in size should be observed. Failure to detect this band indicates that the PCR reagents used are inadequate.

Negative control

Each of the gene-specific primers should be tested separately in a PCR using a cloned cDNA as template to ensure the absence of false amplification products.

Ordering of Sub-Plates

The Sub-Plate number corresponds to the column number (1-12) and row letter (A-H) of the positive well on the Master Plate. For example, if well 10F of the Master Plate gives a positive signal then the corresponding Sub-Plate is number 10F. Once a positive well(s) is identified on the Master Plate, simply send a fax of the **Rapid-Screen™ Sub-Plate Order Form**, found enclosed with this manual, to OriGene. The requested Sub-Plate(s) will be shipped on the next business day.

Screening of Sub-Plates

The Sub-Plates contain 30 μ L of glycerol stocks of *E. coli* culture. The bacterial clones in each well are amplified from the original 50 clones. The plates need to be stored at -80°C .

Perform a 96-well PCR using 1 μ L of glycerol stock from each well of the Sub-Plate as template. Use the same number of cycles and annealing temperature, but increase the extension time to 3 minutes. (see **Appendix D**) Identify the positive well(s) by agarose gel electrophoresis of the PCR products. Once the identity of the well(s) which contains the specific cDNA clones are known, proceed to colony screening.

Colony screening

Individual wells of the Sub-Plate are screened for the presence of the positive signal(s) that was previously identified by PCR. Since each well contains bacterial cultures of about 50 different clones, the cDNA of interest can easily be pinpointed by either PCR screening or filter hybridization. Two methods for PCR screening are presented. The microscreen is the standard protocol, whereas the macroscreen is used when the cDNA of interest is thought to be either unstable or large and, in turn, under-represented in the amplified *E. coli* stocks.

Colony screening by PCR — Method I — Microscreen

- Remove the Sub-Plate from -80°C storage. Care should be taken when removing the sealing tape from the plate, not to contaminate one well with another. Thaw the Sub-Plate, mix the well of interest, remove 1 μ L of suspension and dilute 1:1000 in LB broth.
- Spread 0.5 μ L, 1 μ L and 4 μ L of the dilution onto two separate LB agar + ampicillin plates. It is easier to place these small volumes in a larger (approx. 100 μ L) volume of LB to make spreading less difficult.
- Incubate the plates overnight at 37°C . If the desired clone is large, incubate at 30°C overnight. This will help avoid the selective growth of clones with short insert sizes.
- The following day, prepare a PCR pre-mix for a 96-well plate using the appropriate primer pair for a 25 μ L reaction (see **Appendix D**). Aliquot 25 μ L of the pre-mix into each of the 96 wells of the PCR plate.
- Pick 95 individual colonies from the overnight selection plate and transfer onto a gridded LB agar + ampicillin plate and into a corresponding well of the PCR plate containing the aliquoted pre-mix.
- Incubate the spotted LB agar + ampicillin plate at 37°C overnight.
- Transfer 1 μ L of glycerol stock from the Sub-Plate of the well that produced the positive signal from the screening into well 12H (#96). This serves as a positive control PCR.
- Perform PCR following the same parameters used in the screening of the Master Plate and the Sub-Plate, as in **Appendix D**.
- Identify the positive clones by separating the reaction products using agarose gel electrophoresis.
- Refer back to the gridded LB agar + ampicillin plate to identify positive bacterial clone(s) and inoculate a culture for plasmid preparation the following day.

Colony screening by PCR — Method II — Macroscreen

- Remove the Sub-Plate from -80°C storage. Care should be taken when removing the sealing tape from the plate, not to contaminate one well with another. Thaw the Sub-Plate, mix the well of interest, remove 1 µL of suspension and dilute 1:1000 in LB broth.
- Spread 0.5 µL, 1 µL and 4 µL of the dilution onto two separate LB agar + ampicillin plates. It is easier to place these small volumes in a larger (approx. 100µL) volume of LB to make spreading less difficult.
- Incubate the plates overnight at 37°C. If the desired clone is large, incubate at 30°C overnight. This will help avoid the selective growth of clones with short insert sizes.
- The following day, prepare a PCR pre-mix for 24 reactions of 80 µL each using the appropriate primer pair. Aliquot 80 µL of the pre-mix into 24 wells of a 96-well PCR plate. Individual tubes may also be used.
- Depending on the number of colonies obtained (ideally between 250 and 500), group the colonies into 23 groups of 11–20 colonies each. Label each group with a number that will correspond to the number on the PCR tube.
- Using a fresh pipette tip for each colony, pick all the colonies from one group into one well or tube of the 80 µL PCR mix.
- Include one reaction as a positive control. Add 2 µL of the glycerol stock from the positive well of the Sub-Plate screen to tube 24.
- Perform PCR following the same parameters used in the screening of the Sub-Plate, as in **Appendix D**.
- Identify the positive groups by separating the reaction products (run only 20-30 µL of reaction) using agarose gel electrophoresis. A longer than average run time and length of run on gel may be necessary, especially for small products to separate them from the large amount of RNA from the bacteria.
- Perform PCR using the individual single colonies (11–20) in the positive group(s) as template using the same primer pair as above for the screening. Use a final reaction volume of 20 µL aliquoted from a PCR pre-mix.
- Identify the positive clones by separating the reaction products using agarose gel electrophoresis.
- Refer back to the LB agar + ampicillin plate to identify positive bacterial clone(s) and inoculate a culture for plasmid preparation the following day.

Colony screening by hybridization

If many positive Sub-Plate wells need to be screened, it may be preferable to screen by colony hybridization. This procedure takes several days longer than the PCR approach, but many samples can be processed in parallel. A detailed protocol for colony hybridization can be found in *Molecular Cloning: A Laboratory Manual*, 2nd ed. (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press).

Positive cDNA library clone

The positive cDNA library clone that is recovered with Rapid-Screen™ is obtained in the form of a library plasmid. The insert size of the positive clone(s) should be determined by restriction enzyme analysis. Cloned cDNA in pCMV6-XL4 can be excised with *NotI* (see **Appendix B**). Once the plasmid containing the longest desired cDNA clone has been identified amongst the positives, it can then be prepared on a larger scale starting from culture derived from the gridded LB agar + ampicillin plate. Prepare glycerol stocks of the positive bacterial clones and store at -80°C.

The sequence of the 3' end of the cDNA insert in pCMV6-XL4 can be derived using the XL39 vector reverse primer. Sequencing of the 5' end of the cDNA inserts can be performed using Vector Primer 3.

Sample Library Screen

Screening by gene-specific primer PCR

A Rapid-Screen™ Arrayed cDNA Library Panel was used to isolate full-length cDNA using a pair of gene-specific primers for Gene X (Fig. 3). After the first round of PCR, all 96 samples were separated in a 2% agarose gel to identify wells in the Master Plate that contain a clone with insert encoding Gene X, in this case, A4, B8, D3, F8 and G11 (panel A).

A Sub-Plate corresponding to a well in the Master Plate that gave a positive signal (in this case, D3) was then screened by a second round of PCR using the same two gene-specific primers for Gene X. All 96 reactions were then separated in an agarose gel to determine which wells in the Sub-Plate contain the positive clone (panel B). One of the positive wells for Gene X from the Sub-Plate was then used in the final screening process. Bacteria from the well (in this case, B4) were spread onto LB/ampicillin agar selection plates and 95 of the resulting colonies were subsequently screened by PCR, again using the same two gene-specific primers, to detect the Gene X cDNA insert. Agarose gel electrophoresis of the PCR products then revealed the bacterial colonies, which contained the library plasmid encoding Gene X (panel C).

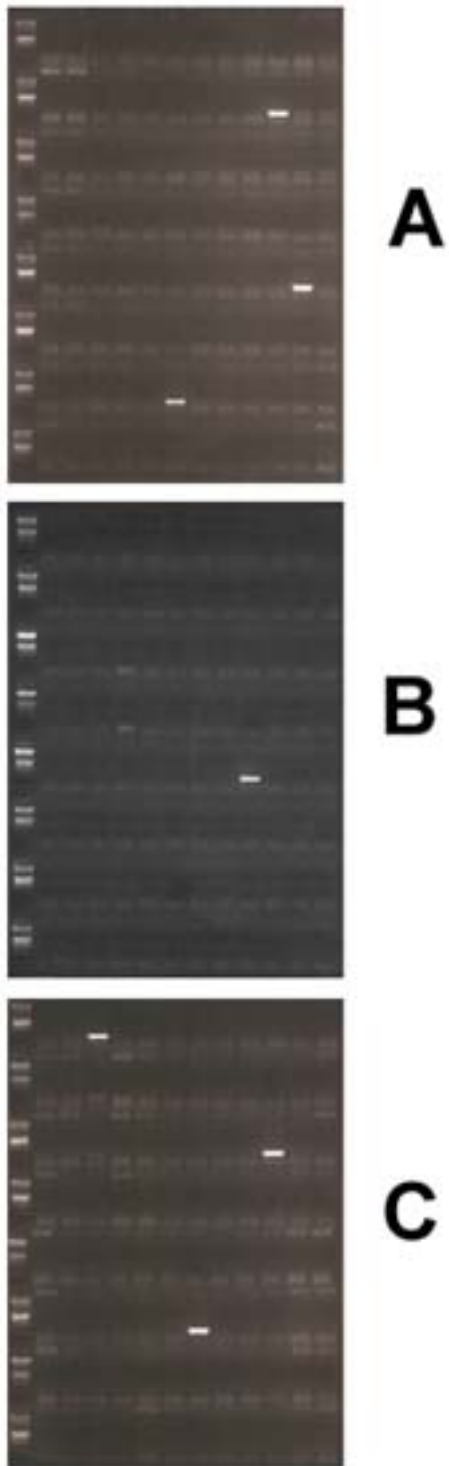


Fig. 3. Use of RapidScreen™ panels to screen for full-length cDNA.

Longest clone detection

If multiple positive clones are detected in the Master Plate, it is important to first determine which are the longest rather than to isolate all of the clones at once. This can be achieved by performing PCRs on DNA from only the positive Master Plate wells using either a 5' or 3' flanking vector primer plus a properly-oriented gene-specific primer. Panel A of Fig. 4 displays results in which three wells (5,000 clones/well) were found to be positive using gene-specific primers (forward and reverse).

To determine how far the known sequence lies from each end of the test gene, PCR with a 5' vector-specific primer (Vector Primer 3) and a reverse (3') gene-specific primer or PCR with a 3' vector-specific primer and a forward (5') gene-specific primer can be performed. Panel B of Fig. 4 shows results of PCR on the same wells used in Panel A using a 5' vector-specific and a reverse gene-specific primer. Of the three pools identified as positive in Panel A, it is clear that wells 1, 3 and 6 each contain a 5 kbp product. The positive clones in wells 1, 3 and 6 are probably "full-length" since they are large and redundant. Panel C shows the results of PCR on the same pools using a 3' vector-specific primer and a gene-specific primer. All three positives have the same-sized product (1.4 kbp) and therefore the same 3' end length. It can be concluded from the analysis that wells 1, 3 and 6 each contain a probable "full-length" clone of about 5.8 kbp.

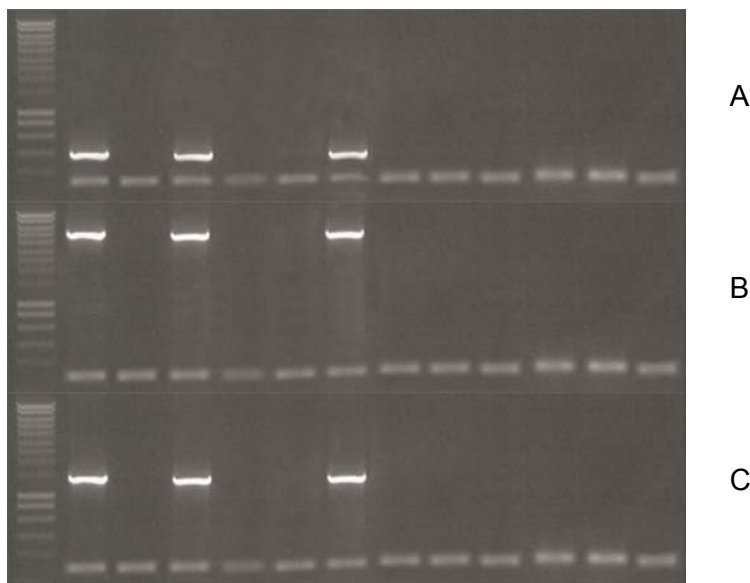


Fig. 4. Detection of longest clones with RapidScreen™ panels.

Long PCR

When performing vector primer plus gene-specific primer PCR, it is frequently not known what size PCR product to expect. The product could be several kilobase-pairs in size, requiring "long PCR" conditions to detect the product. The ability of two different *Taq* DNA polymerases to amplify four cDNAs, ranging in size from 1.0 to 6 kbp, from a Master Plate well (5,000 clones) was tested (Fig. 5). Enzyme 1 was used on lanes 1, 2, 5, 6, 9 and 10, while Enzyme 2 was used on lanes 3, 4, 7, 8, 11 and 12. Enzyme 1 was able to amplify all of the fragments. Enzyme 2 was able to amplify the 1.0- and 3.0-kbp fragments, but it could not successfully amplify the 6.0-kbp fragment. These experiments demonstrate the importance of choosing an efficient *Taq* DNA polymerase. If only small fragments are being amplified, then either of the two enzymes tested are suitable. To amplify longer fragments, a more efficient *Taq* DNA polymerase is required.

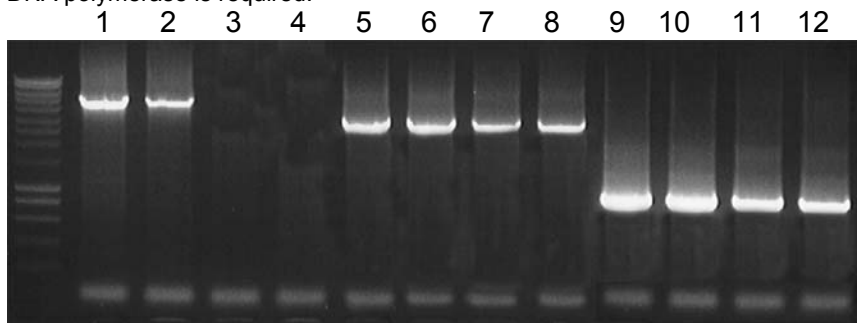


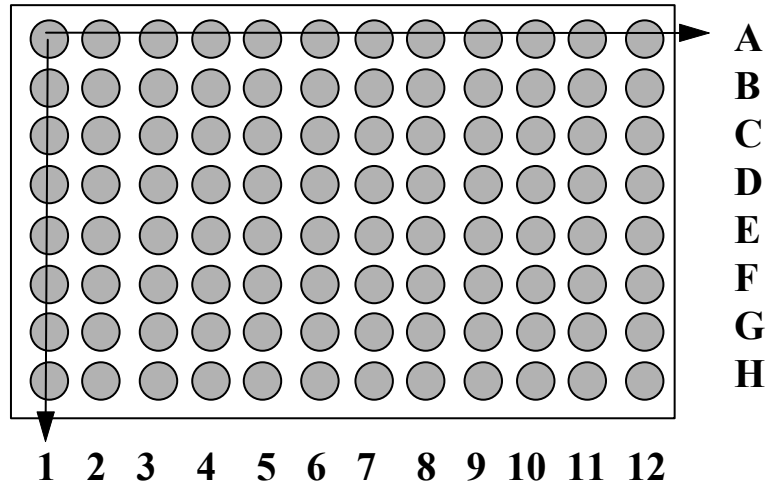
Fig. 5. RapidScreen™ analysis using different Taq DNA polymerases.

Pooling of wells (additional Protocols)

If it is not possible or desirable to perform 96 simultaneous PCRs, then pools of DNA from the wells in the Master Plate can be made. Using 5 µL of DNA from each well of the Master Plate, twelve pools (40,000 clones/pool) of DNA can be made from columns 1 through 12, and/or eight pools (60,000 clones/pool) of DNA can be made from rows A through H. These 20 pools can be analyzed by PCR using two gene-specific primers. As shown in Fig. 6, it is clear that column pool 9 and row pool E are positive, suggesting that there is a single clone in this library and that it is located in well E9 of the Master Plate. The one caveat to this approach is that since there are 40,000–60,000 independent clones per pool rather than only 5,000 from an individual well, both gene-specific primers must be highly specific. If only one of two primers is highly specific then they may work fine in PCR on an individual well but may not work on a pool. The pooling technique is most effective and informative when working with a low abundant versus a high abundant transcript.

Should the pooling techniques lead to the identification of many positives, it may be difficult to pinpoint a specific positive well. In that event, a second PCR may be needed to be carried out on individual wells of a positive row or column.

A



B

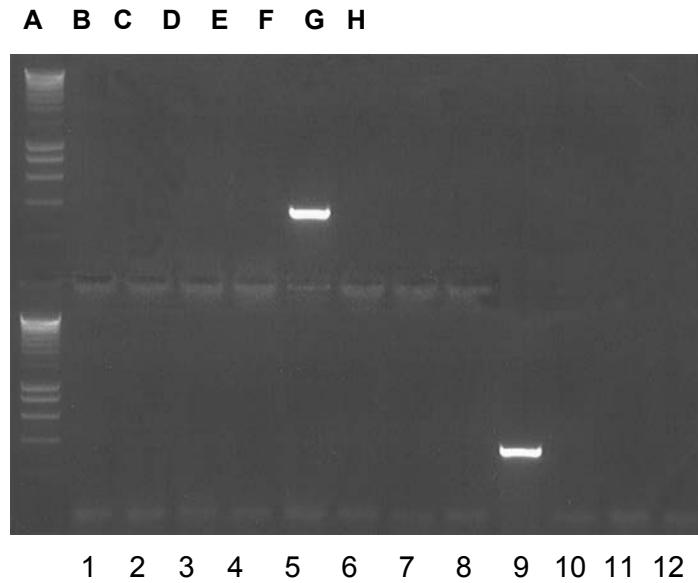


Fig. 6. Performing Rapid-Screen™ using pooled wells.

In this example, the single clone is found within well E9.

Alternatively-spliced transcripts

Rapid-Screen™ panels can be used to clone alternatively-spliced transcripts as well, even if the desired spliced variant is many times less abundant than its parent transcript. The detection of different-sized fragments by vector specific plus gene-specific primer PCR is sometimes attributable to alternative-splicing rather than to truncated transcripts (if the alternate splicing has already been characterized, then gene-specific primers can be designed that differentiate between the two spliced forms). As shown in Fig. 7, a 5' vector-specific primer plus a reverse gene-specific primer was used to detect two alternatively-spliced transcripts of a particular gene.

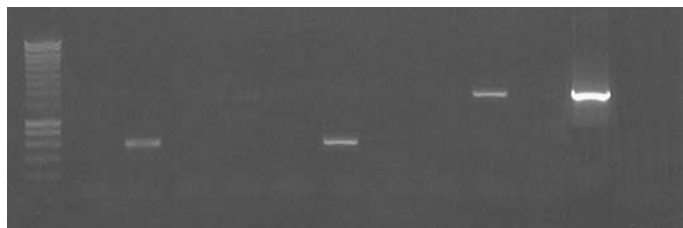


Fig. 7. Detection of alternatively-spliced transcripts with Rapid-Screen™.

Trouble-shooting Guide

A. No positive signal on Master Plate screen

1. Too few cycles. Up to 40 cycles can be used without over cycling problems, such as multiple or smeary bands.
2. Omitted PCR component. Be sure to use a written checklist when assembling reactions. Include a positive control with each reaction set.
3. Primer design. There is no ideal method for designing primers. To ensure that the primers will amplify the gene of interest, it is recommend that the primers be first tested on a known template.
4. Annealing temperature is not optimal. It is recommend that the annealing temperature be optimized using a known template before the library screen is performed. If no positive signal is observed, it is possible that the annealing temperature is simply too high for the primers.
5. Extension time is too short. The extension time should be 1 min for every 1 kbp of sequence being amplified.
6. Reagent concentrations are not optimal. The Mg⁺⁺ concentration can be varied from 1.0 to 2.5 mM. However, the standard 1.5 mM final Mg⁺⁺ concentration works well for most primer pairs. The concentration of buffers and dNTPs should also be carefully calculated.

B. Every well is positive

1. Contamination. Be sure to include the negative control of ddH₂O in place of a DNA sample in every PCR experiment to determine if the reagents, pipettors or other PCR-related equipment are contaminated with exogenous DNA. It is recommended that barrier tips be employed for all pipettors used in PCR to avoid aerosol contamination.
2. Annealing temperature not optimal. The annealing temperature may be too low. Increase it by increments of 2°C and test positive and negative control samples for optimization.
3. Primer design. The G/C content of the primers may be too low (<45%) resulting in the non-specific annealing of the primers to various templates.

4. Mg⁺⁺ concentration is too high. Adjust the Mg⁺⁺ concentration and repeat tests on positive and negative control DNA.

C. Smears on the gel

1. Too many cycles. Depending on the primers and the abundance of the cDNA of interest, as few as 25 cycles or as many as 40 cycles may be required to obtain specific product. Northern blot analysis can be used to determine the relative abundance of the transcript of interest in the corresponding tissue of the library panel.
2. See B1 and B2 above

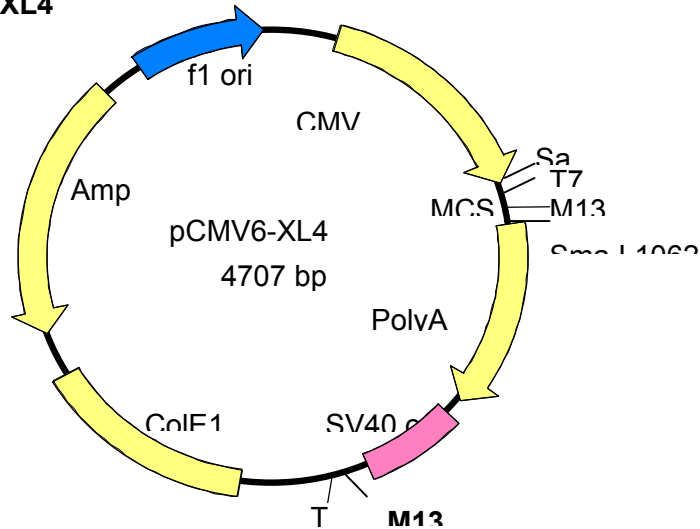
Published Papers Using Rapid-Screen™ Arrayed cDNA Library Panels

1. Sangoram, A. M., Saez, L., Anotch, M. P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E. M., Hotz Vitaterna, M., Shimomura, K., King, D. P., Young, M.W., Weitz, C. J., and Takahashi, J. S. (1998). Mammalian Circadian Autoregulatory Loop: A *Timeless* Ortholog and *mPer1* Interact and Negatively Regulate CLOCK-BMAL1-Induced Transcription. *Neuron* **21**, 1101-1103.
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Please check the OriGene website, www.origene.com, for the most current list of available references.

Appendices

Appendix A. Map of pCMV6-XL4



GenBank Accession Number: AF067196

The vector pCMV6-XL4 is 4.7 kbp in size and contains a polylinker (**Sac I** to **Sma I**). The cDNA library inserts are directionally cloned between the *EcoRI* and *SalI* sites. **Note:** The *SalI* site is destroyed in the cloning process. The **CMV promoter**, which can be used to express the cloned cDNAs, is followed by an hGH (human growth hormone) **polyA** region located downstream of the polylinker. The **ColE1 ori** is the bacterial origin of replication, the **SV40 ori** allows for replication in mammalian tissue culture cells and the **f1 ori** is the filamentous phage origin of replication that allows for recovery of single-stranded plasmid. Selection of the plasmid in *E. coli* is conferred by an ampicillin-resistance gene, **AmpR**.

Sequencing from the 5' end of cDNA inserts can be performed using Vector Primer 3 (provided). Its priming site is located approximately 60-bp upstream of the polylinker.

Sequencing from the 3' end* of cDNA inserts can be performed using the XL39 vector reverse primer (provided). The priming site of XL39 is approximately 70bp downstream of the polylinker sequence.

T7 RNA polymerase can be used for generating transcripts of the cDNA insert by *in vitro* transcription. A **T7** promoter sequence is located 5' of the polylinker. Note: The cDNA insert fragment must be first isolated from the library vector before the reaction since a second T7 promoter is present in the vector. This can be accomplished by digesting the plasmid with *SacI* and *SmaI*.

*Please note: The M13 primer sequence is represented twice within the vector sequence with only a slight variation in bases between the two sites. Therefore, sequencing from the 3' end of cDNA inserts may **not** be performed using an M13 Reverse primer.

Appendix B. Polylinker Sequence of pCMV6-XL4

```

                                Sac I
G G T G G G A G G T C T A T A T A A G C A G A G C T C
C C A C C C T C C A G A T A T A T T C G T C T C G A G

Vector Primer 3
G T T T A G T G A A C C G T C A G A A T T T T G T A A
C A A A T C A C T T G G C A G T C T T A A A A C A T T

T7 Promoter Site
T A C G A C T C A C T A T A G G G C G G C C G C G A A
A T G C T G A G T G A T A T C C C G C C G G C G C T T

EcoRI      Bgl II      Kpn I      EcoRV      Hind III
T T C A G A T C T G G T A C C G A T A T C A A G C T T
A A G T C T A G A C C A T G G C T A T A G T T C G A A

G T C G A C T C T A G A T T G C G G C C G C G G T C A
C A G C T G A G A T C T A A C G C C G G C G C C A G T

Sal I      Xba I      Not I      Sma I
T A G C T G T T T C C T G A A C A T G T G A T C C C G
A T C G A C A A A G G A C T T G T A C A C T A G G G C

G G T G G C A T C C C T G T G A C C C C T C C C C A G
C C A C C G T A G G G A C A C T G G G G A G G G G T C

T G C C T C T C C T G G C C C T G G A A G T T G C C A
A C G G A G A G G A C C G G G A C C T T C A A C G G T

C T C C A G T G C C C A C C A G C C T T G T C C T A A
G A G G T C A C G G G T G G T C G G A A C A G G A T T
                                XL39 Reverse Vector Primer
T A A A A T T A A G T T G C A T C A T T T T G T C T G
A T T T T A A T T C A A C G T A G T A A A A C A G A C

A C T A G G T G T C C T T C T A T A A T A T T A T G G
T G A T C C A C A G G A A G A T A T T A T A A T A C C
Vector Primer 6
C C A C C T C C
G G T G G A G G

```

Vector Primer 3 5' GCAGAGCTCGTTT TAGTGAACC 3'
 Primer XL39 5' ATTAGGACAAGGCTGGTGGG 3'
 T7 Priming Site 5' AATACGACTCACTATAGGG 3'
 Vector Primer 6 5' TAGAAGGACACCTAGTCAGAC 3'

Appendix C. Primer Testing

PCR primers should be tested using a known template, such as a cDNA clone, before being used in a large-scale screen.

Dilute the cloned cDNA fragment in water to a concentration of 0.1 ng/μL.

Dilute each primer to a concentration of 10 pmol/μL.

Set up three PCRs using 0.1, 0.25 and 1.0 ng/μL of cDNA, respectively. Negative controls, including using each primer singly, should be performed.

Perform PCR following standard protocols, as in **Appendix D**.

Analyze the results by agarose gel electrophoresis. If a “clean” PCR product is not observed under these conditions, try altering the annealing temperature or Mg⁺⁺ concentration in the PCR. Determine the optimal conditions for PCR amplification before proceeding to the library screen.

Appendix D. Standard PCR Conditions

Prepare the PCR per sample as follows:

Stock Solution	Volume	Final
10× PCR Buffer	2.5 µL	1×
dNTPs (2 mM each)	2.5 µL	0.2 mM
Forward (5') Primer (10 pmol/µL)	0.5 µL	5 pmol
Reverse (3') Primer (10 pmol/µL)	0.5 µL	5 pmol
<i>Taq</i> DNA Polymerase	—	0.5 U
sterile ddH ₂ O	to 25 µL	—

The following cycling parameters are recommended:

Gene Specific Primers

Pre-Soak	94°C for 5 min	
Denaturation	94°C for 30 sec	
Annealing	60°C for 30 sec	30-35 cycles
Extension	72°C for 1.0 min (master plate) 72°C for 3.0 min (sub-plate)	
Final Extension	72°C for 5 min	
Hold	4°C	

Vector 3 and Reverse Primers (Touchdown Program)

Pre-Soak	94°C for 5 min	
Denaturation	94°C for 30 sec	
Annealing/Extension	68°C for 7 min	10 cycles
Denaturation	94°C for 30 sec	
Annealing	65°C for 30 sec	35 cycles
Extension	72°C for 7 min	
Final Extension	72°C for 7 min	
Hold	4°C	

Appendix E. pCMV6-XL4 Sequence

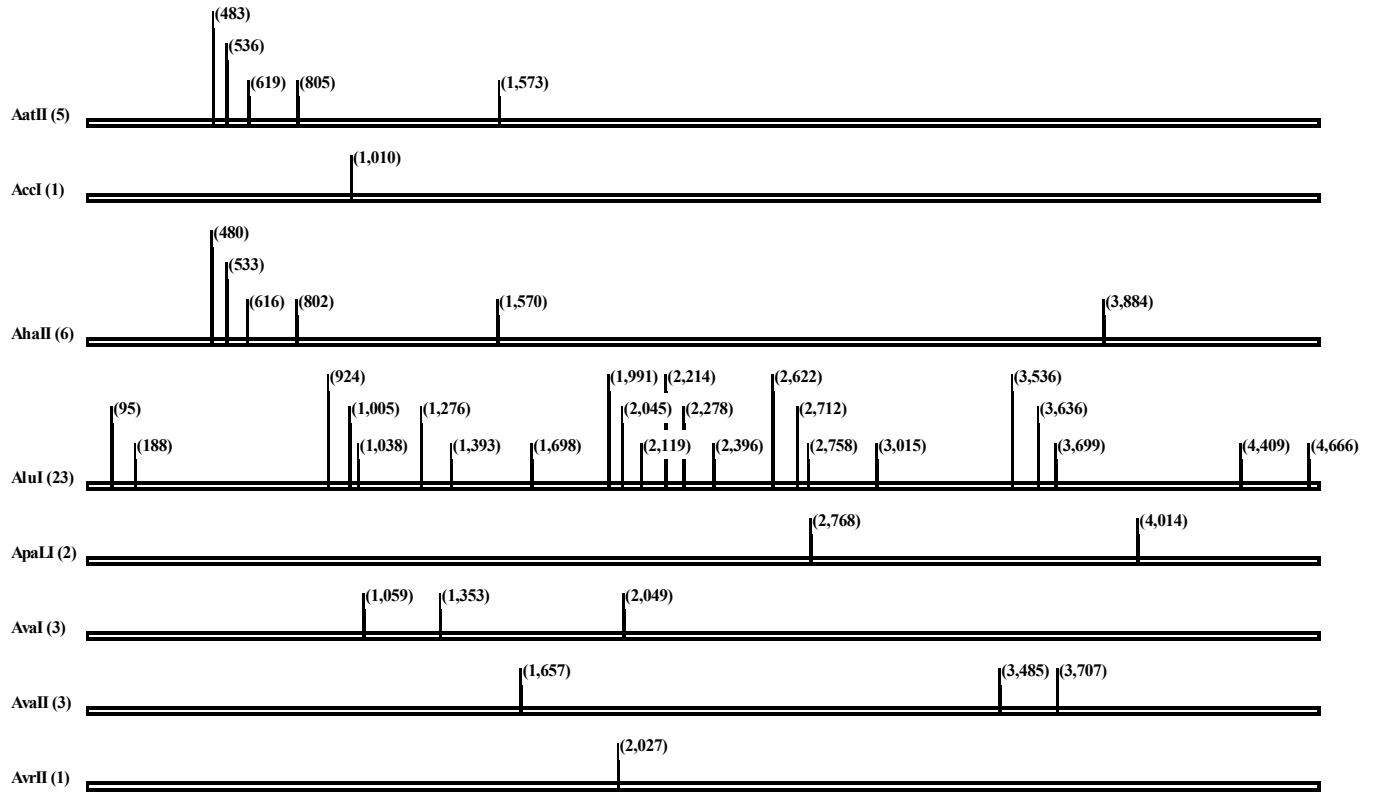
GenBank Accession Number: AF067196

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181 gtgccaagct gatctataca tgaatcaat attggcaatt agccatatta gtcattgggt
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361 gttattaata gtaatcaatt acggggatcat tagttcatag cccatatatg gagttccgag
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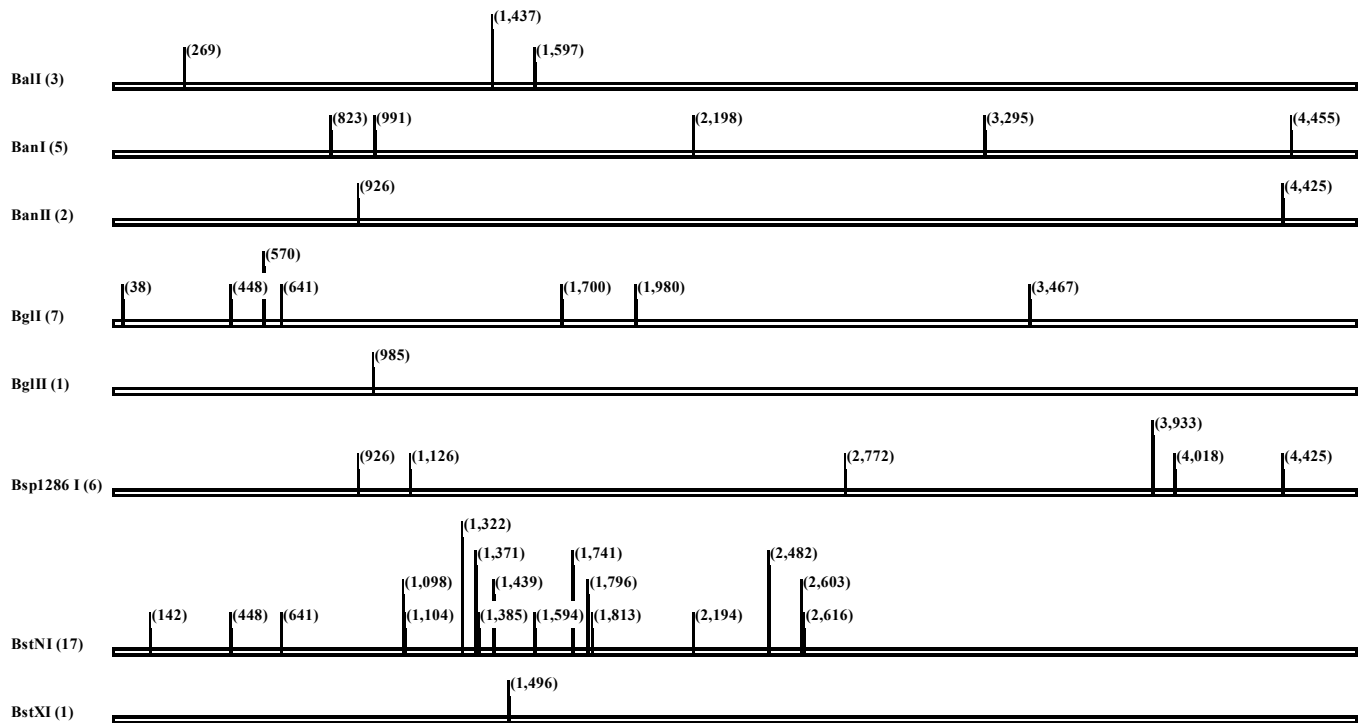
Appendix F. pCMV6-XL4 Restriction Map

GenBank Accession Number: AF067196



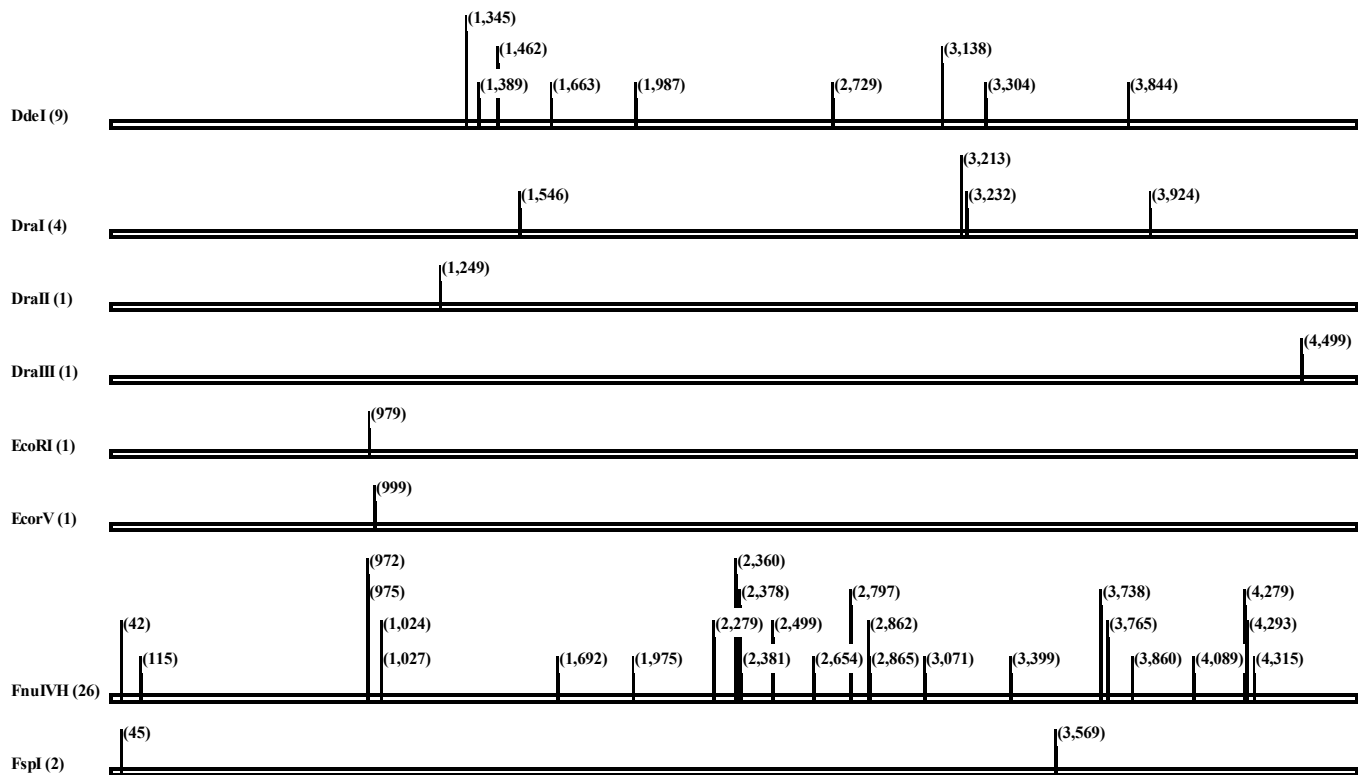
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Non-Cutters : Apal

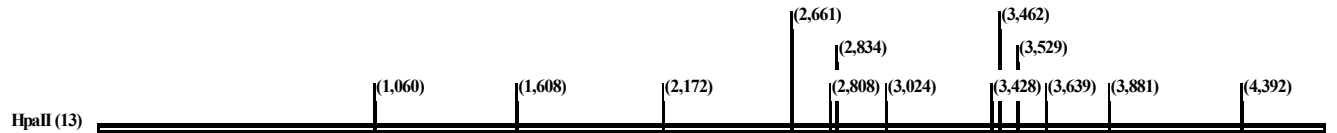
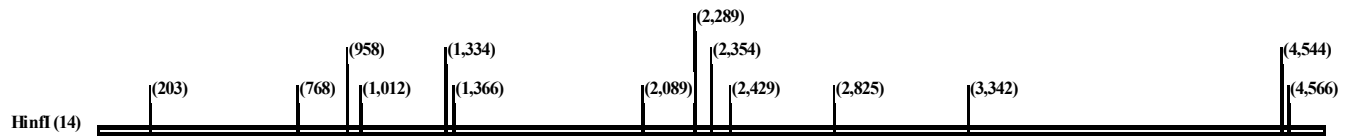
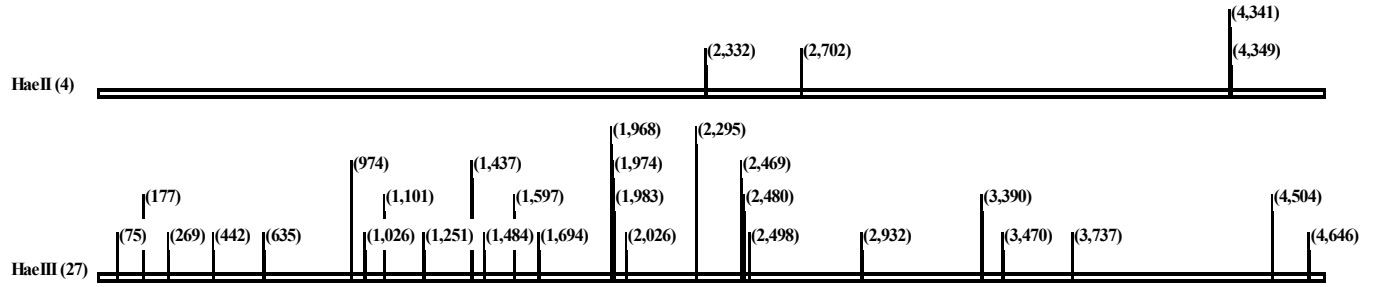


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Non-Cutters : BamHI, BclI, Bsp106, BspMII, BssHII & BstEII

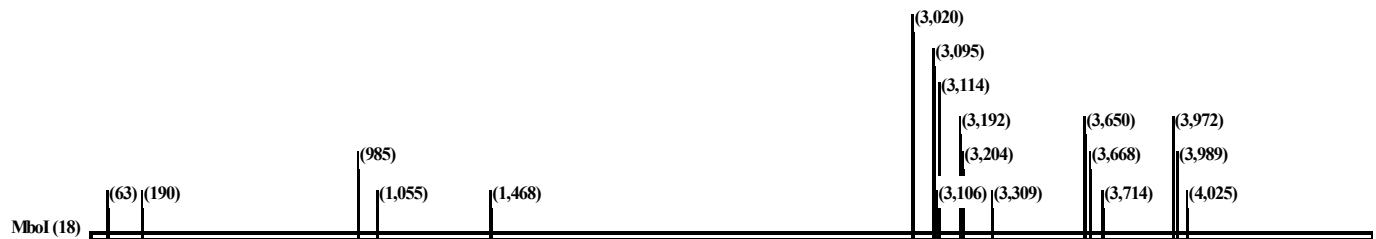


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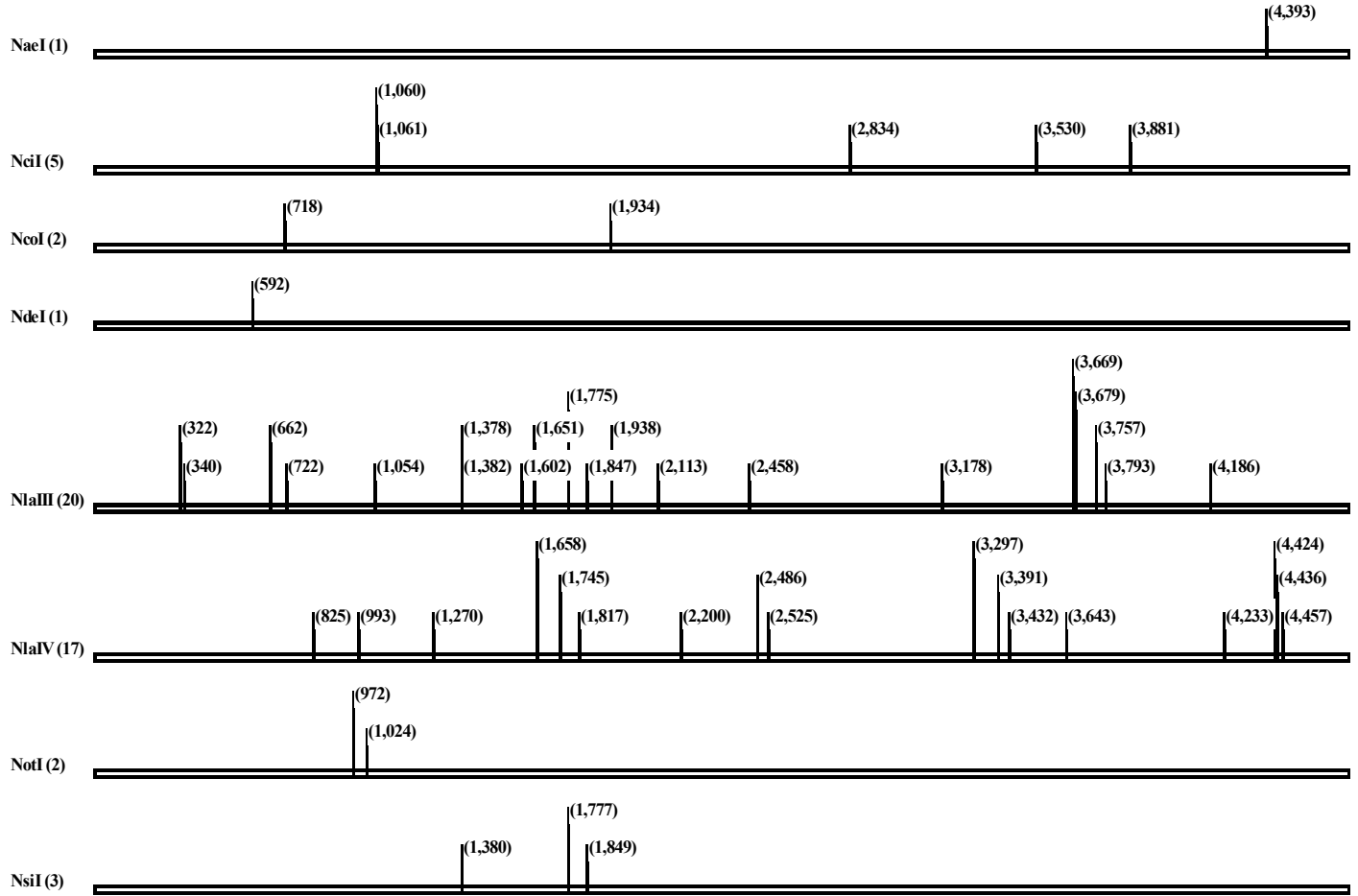
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Non-Cutters : HpaI



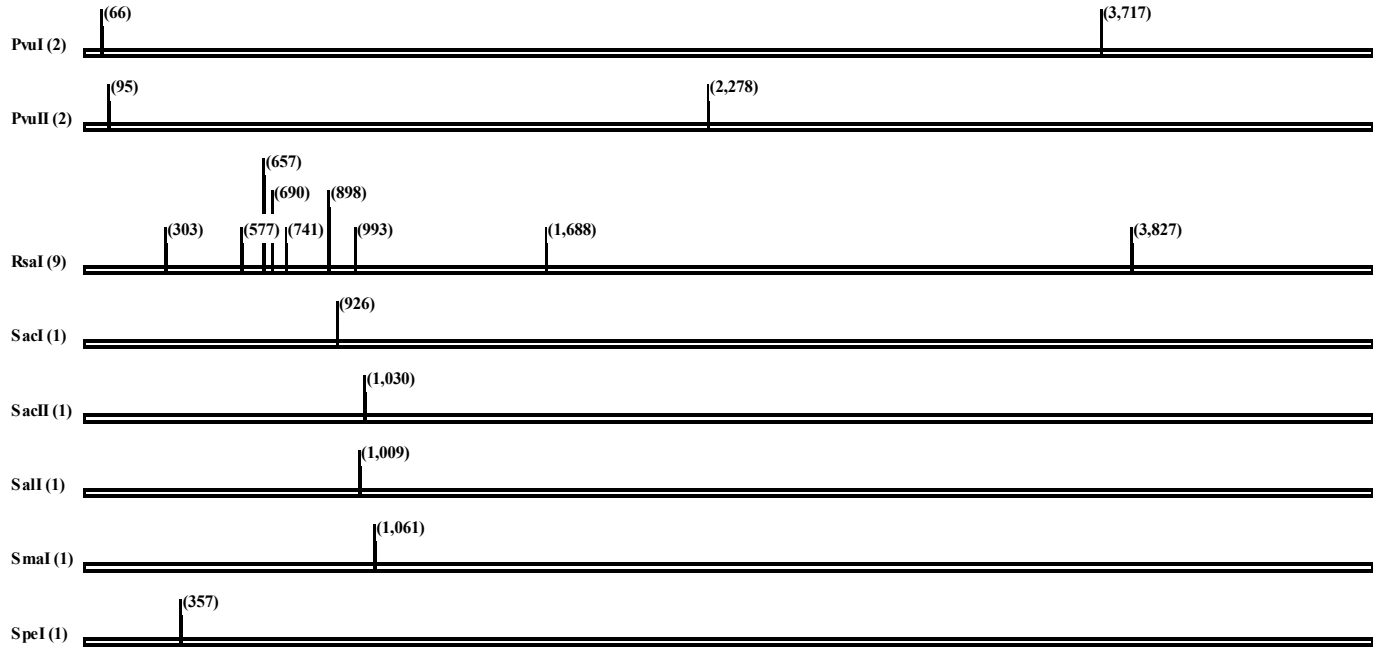
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Non-Cutters : MluI & MstII



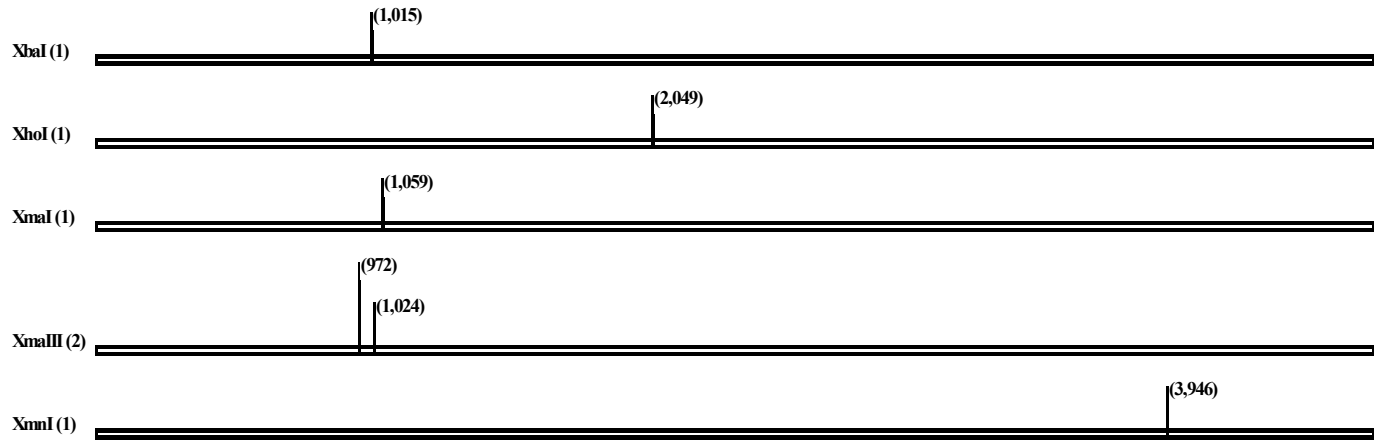
Mapping all cutsites.

Non-Cutters : NarI, NheI & NruI



Mapping all cutsites.

Non-Cutters : PpuMI, PstI & RsrII



Mapping all cutsites.

Appendix G. Limited License Agreement

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