RAPID-SCREEN™
cDNA LIBRARY PANELS

Applications Guide, version 2.1

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OriGene Technologies, Inc. is a research reagents company focused on developing products to help scientists clone genes with ease. As efforts in high-throughput gene sequencing and discovery intensify, novel genes will be identified at a rate of thousands per day.

We developed the Rapid-Screen™ cDNA Library Panels to serve this market. These panels are a break-through in cDNA library screening. In just three rounds of PCR*, you will complete the entire library screen, identify the cDNA clones for your gene(s) of interest and have plasmid DNA ready for transfection and expression studies in tissue culture cells. There is no lifting of filters, large platings of bacteria or hybridization needed for the library screen. The library screening method is simple and fast. The panels were generated with a bias towards longer cDNAs, so they are ideal for identifying longer cDNAs and the previously hard-to-clone 5' end of cDNAs. The library panels are each a series of 500,000 arrayed cDNA clones. We are currently offering 15 different panels from different tissues: human fetal brain, human adult brain, human heart, human kidney, human liver, human lung, human muscle, human peripheral blood leukocytes, human placenta, human small intestine, human spleen, human testis, rat brain, mouse brain, and fetal mouse. The master-plate for each library panel contains DNA from all 500,000 cDNA clones and is screened in one simple set of 96-well PCR. This first set of PCR reactions will identify the sub-plate(s) which contains your clone of interest. The sub-plates are *E. coli* glycerol stocks, contained in standard 96-well microtiter dishes. Each plate contains 50 cDNA clones per well, with approximately 5000 clones per plate. The sub-plates are screened with a single set of 96-well PCR to identify the positive well on the sub-plate. Then simply plate out the cells from that sub-plate well, screen the colonies by PCR and you will have your desired clone. It is that simple and that easy!

* PCR is covered by patents issued to Cetus Corporation and owned and licensed by Hoffmann LaRoche Molecular Systems, Inc. Purchase of any of OriGene's PCR-related products does not convey a license to use the PCR process, covered by these patents. Purchasers of these products must obtain a license to use the PCR process before performing PCR.

1. Package Contents and Storage Conditions

- **Master 96-well plate.** Each well of this plate contains 150 ng of dried plasmid DNA isolated from approximately 5000 individual clones in the library. The entire 96-well plate contains approximately 500,000 clones. This plate should be stored upright at room temperature and comes sealed with sealing tape. After resuspending the DNA, the plate should be stored at -20°C. A new piece of sealing tape should be used to seal the plate after use.
- **PCR Primer** for the cloning vectors (vector primer 3) is at a concentration of 10 pmoles/µl. The vector primer is located upstream of the 5' cloning site in the cloning vectors. For more detailed information about the sequence surrounding the polylinker site of cloning vectors, please refer to Appendix A of this manual. The primer should be stored at -20°C.
- **Positive control.** The positive control is a sample master plate well. The positive control primer mix amplifies a 483 bp PCR product from the positive control sample. The DNA concentration for the positive control well is 6 ng/ µl and 5 µl of this control should be used with 1 µl of the positive control primer mix in a 25 µl PCR reaction. Both the positive control and primer mix are shipped in individual tubes.
- **96-Well Sub-Plates** are not included with the order of the kit. Once you have screened the master DNA plate and have identified the positive wells, simply order the corresponding sub-plates and we will ship them to you on the next business day. For ordering details, please see pages 13 and 20.

Additional Reagents and Supplies Not Included

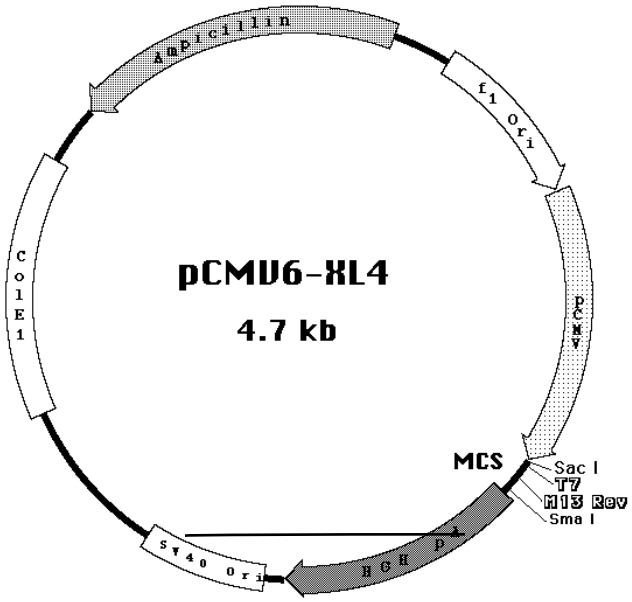
- Ampicillin
- LB agar + ampicillin plates
- LB broth 10g/L Bacto-tryptone
  5 g/L Bacto-yeast extract
  5 g/L NaCl
  Adjust pH to 7.0 with 1N NaOH and autoclave. Store at room temperature.
- PCR reagents, including Taq DNA polymerase, buffer, dNTPs
- Pipetmen used exclusively for PCR, to avoid inadvertent contamination
- Barrier pipet tips, to avoid aerosol contamination from the pipetmen
- PCR-grade water

2. Library Construction and Design

The Rapid-Screenä 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-Screenä cDNA Library Panels lies in the design and construction of the cDNA libraries themselves.

A. Vector

These libraries are constructed in the pCMV6-XL4 vector (see below) and the cDNA is cloned directionally into the *Eco* RI and *Sal* I sites**. Please note that the SalI site is destroyed in the cloning process.**



MCS (Multiple Cloning Sites)

pCMV6-XL3

PCMV → T7 promoter *Not* I *EcoR* I *Bgl* II *Kpn* I

```
AGAGCTCGTTTAGTGAACCGTCAGAATTTTGTAATACGACTCACTATAGGGCGGCCGCGAATTCAGATCTGGTACC
TCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGTGATATCCCGCCGGCGCTTAAGTCTAGACCATGG
```

EcoR V *Hind* III *Xba* I *Sal* I *Sfi* I T3 promoter *Sma* I

```
GATATCAAGCTTCTAGACTGCAGTCGACGGCCAAGTCGGCCTCCCTTTAGTGAGGGTTAATTTGTGATCCCGGGTGG
CTATAGTTCGAAGATCTGACGTCAGCTGCCGGTTCAGCCGGAGGGAAATCACTCCCAATTAAACACTAGGGCCCACC
```

pCMV6-XL4

PCMV → T7 promoter *Not* I *EcoR* I *Bgl* II *Kpn* I

```
AGAGCTCGTTTAGTGAACCGTCAGAATTTTGTAATACGACTCACTATAGGGCGGCCGCGAATTCAGATCTGGTACC
TCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGTGATATCCCGCCGGCGCTTAAGTCTAGACCATGG
```

*Eco*R V *Hind* III *Sal* I *Xba* I *Not I* *M13 Reverse* *Sma* I

```
GATATCAAGCTTGTCGACTCTAGATTGCGGCCGCGGTCATAGCTGTTTCCTGAACATGTGATCCCGGGTGG
CTATAGTTCGAACAGCTGAGATCTAACGCCGGCGCCAGTATCGACAAAGGACTTGTACACTAGGGCCCACC
```

Figure 1: Map of pCMV6-XL4 vectors

The vectors, pCMV6-XL4, can express cloned genes from the CMV promoter (shown as **pCMV** in the map). The plasmid size is 4.7 kb with the multiple cloning site as shown above. Downstream of the

cloning site is the **HGH poly A** (human growth hormone poly A) site. The **F1 origin** is the filamentous phage origin of replication which allows for recovery of single-stranded plasmid when *E. coli* are co-infected with helper phage. The **ColE1** origin is the origin of replication which is used in the absence of helper phage. The **SV40 Ori** is the origin of replication used in tissue culture cells. The **T7** and **M13 Reverse** promoters are useful for generating sense and antisense transcripts using *in vitro* transcription. They can also be used as primer annealing sites for sequencing cDNA clones. Not all M13 reverse primers anneal directly to the M13 reverse site at the 3' end of the cloning site. We recommend the use of the M13 Reverse sequencing primer with the sequence: 5' caggaaacagctatgacc 3' ****Note:** The SfiI site in pCMV6-XL3 in the polylinker is not unique. There is a second SfiI site 1 kb from the polylinker. pCMV6-XL3 was used to construct the human fetal brain, human spleen, and fetal mouse cDNA library panels. pCMV6-XL4 was used to construct all other cDNA library panels.

B. cDNA Synthesis and Fractionation

The cDNA used in making the library panels was synthesized from double purified mRNA using oligo(dT) primer and AMV reverse transcriptase. The cDNA was size fractionated on low melt agarose gels. The lanes were cut into multiple slices which were processed and ligated separately into the pCMV6-XL4 vector. The cDNA was directionally cloned into pCMV6-XL4 so that the CMV promoter will transcribe the cloned cDNAs. Each ligation was transformed and arrayed independently in the library panel. The libraries are arrayed so that larger sized cDNA clones are more highly represented in the arrayed panel.

C. Library Arraying and Preparation

The libraries are arrayed so that the master plate contains plasmid miniprep DNA from all of the clones in the array. Each well on the master plate contains DNA from approximately 5000 clones, with a total of 500,000 clones in each library. Each individual well on the master plate corresponds to a single sub-plate. Each sub-plate well contains glycerol stocks of*E. coli* amplified from an original 50 clones. Unlike standard cDNA libraries, where you would normally screen $1 - 5 \times 10^6$ cDNA clones to identify positive clones, you need screen only the 500,000 clones in a Rapid-Screenä cDNA Library Panel to get your gene of interest. Because the cDNA was subject to size selection prior to ligation, larger cDNAs were not selected against in the large ligations. These ligations were processed and transformed independently to assure maximum representation in the library panel. Additionally, we purposely over-represent the larger cDNAs in the arrayed panels relative to their expression levels in the cell. This allows you to clone larger cDNAs with ease.

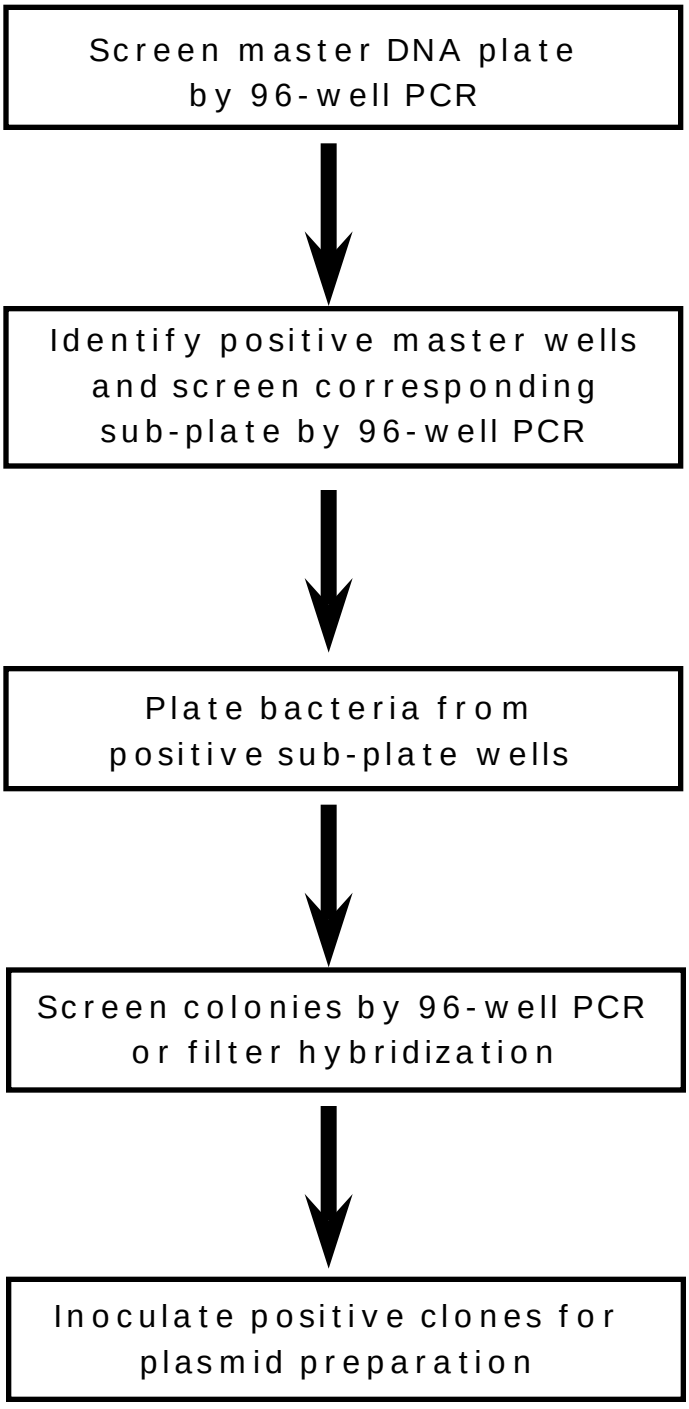
D. Quality Control Testing

The Rapid-Screenä cDNA Library Panels are subjected to a series of quality control checks, prior to distribution. We determine the insert size range and percent of clones which contain cDNA inserts for each size cut in each library panel. In all of the library panels, greater than 90% of the clones contain cDNA inserts. We ensure the "clonability" of each library panel by carrying out at least three library screens for known genes. Our current target genes are: transferrin receptor, HPRT, cyclophillin, neurofilament subunit NF-L and amyloid-beta protein (APP).

3. Screening Procedures

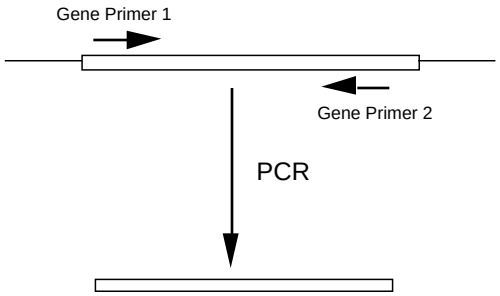
A clone of interest can be identified easily with just 3 sets of 96-well PCR reactions. An overview of the screening procedure is shown on the next page.

Screening Overview

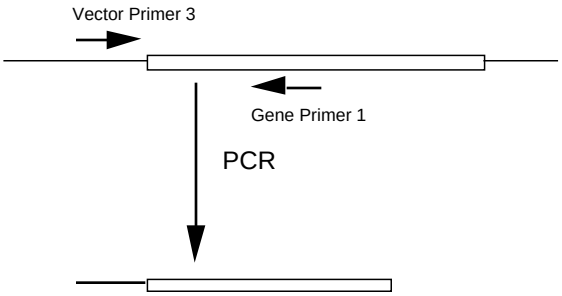


There are two basic ways to screen this library. The first is to use a specific pair of primers directed to your gene of interest to screen all of the wells in the panel. The second method of screening is to use vector primer PCR. This method uses a vector primer and a gene specific primer to identify which clones are the longest. The vector primer is located upstream of the T7 promoter in the cloning vector. Which method you use depends on the size of the mRNA of interest, location of the known sequence in the gene and the quality of sequence information. In the following sections, we will describe both screening methods and some general considerations for PCR.

Specific Primer PCR



Vector Primer PCR



A. Specific Primer PCR

This method is ideal for use with genes of lower abundance or when you do not know the location of the known sequence within a large gene (>3 kb.).

1) Primer Design

Primer design is probably the most important part of the cDNA cloning process. However, there is no simple method for the choice of primers; rather there are a set of guidelines which are reported to aid in the amplification of specific cDNA products. We typically design unique primers ranging in length from 19 to 25 nucleotides with a nearly equal A/T and G/C content. We avoid primer sequences which can form secondary structures which inhibit the PCR reaction. We generally choose primers with annealing temperatures of 55 °C. There are several commercially available computer programs for designing PCR primers.

2) Primer Testing

Testing of the primers to ensure proper amplification can be done using a known template, such as a cloned cDNA fragment.

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

2. Dissolve the primers to a concentration of 10 pmole/µl.

3. Set up 3 PCR reactions with 0.1, 0.25 and 1.0 ng/µl cDNA, respectively and with 0.5 µl of each primer. Negative controls with each primer by itself should also be included.

4. We routinely use the following PCR components at the concentrations given below in 25 µl PCR reactions:

Stock Solution	**Final Concentration**
10X PCR Reaction Buffer	1 X
dNTPs	0.2 mM each
Taq DNA Polymerase (5 units/µl)	0.5 units
dH_2O	to 25 µl final volume

5. The standard cycling conditions we use are: pre-soak at 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 90 seconds. Final extension at 72 °C for 5 minutes. The optimal PCR conditions for your primers may vary.

6. Analyze your results by agarose gel electrophoresis. If you do not observe a clean PCR product under these conditions, try altering the annealing temperature or Mg^{++} concentration in the PCR reactions. Repeat this testing until you determine the optimal conditions for PCR amplification with your chosen primer pair.

3) Specific Primer Screening of the Master Plate

1. Resuspend the DNA using 28 µl of sterile distilled water. Leave the plate at room temperature for 30 minutes before use. Remember to store the plate at -20°C after resuspending the DNA in water and to use a new piece of sealing tape before storing the plate.

2. Remove 5 µl from each well into a 96-well plate for PCR.

3. Place fresh sealing tape on the plate and freeze the master plate at -20 °C.

4. Add 20 µl of a mixture of the remaining PCR components to each well. We routinely use the following PCR components at the concentrations given below in 25 µl PCR reactions:

Stock Solution	**Final Concentration**
Plasmid DNA from master plate (30 ng)	5 µl
Primers (10 pmoles/µl)	0.5 µl of each primer
10X PCR Reaction Buffer	1 X
dNTPs	0.2 mM each
Taq DNA Polymerase (5 units/µl)	0.5 units

5. The standard cycling conditions we use are: pre-soak at 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 90 seconds. Final extension at 72 °C for 5 minutes. The optimal PCR conditions for your primers may vary.

6. Identify the positive wells by agarose gel electrophoresis of the PCR reactions.

B. Vector Primer PCR

This method is best used when you can design a PCR primer within 2 kb. of the 5' end of the cDNA of interest. This method will allow you to identify the longest cDNA clones.

1) Primer Design

For the vector PCR screening, we recommend designing a primer with annealing temperatures of 65°C. Please refer to section 3.A.1. (Primer Design) above for more details about primer design.

2) Primer Testing

Testing of the primer to ensure proper amplification can be done using a known template, such as a cloned cDNA fragment. You will need a 5' primer to use as a partner for the testing the PCR primer. This 5' primer can be complementary to either cloned cDNA sequence or to your cloning vector.

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

2. Dissolve the primers to a concentration of 10 pmole/µl.

3. Set up 3 PCR reactions with 0.1, 0.25 and 1.0 ng/µl cDNA, respectively and with 0.5 µl of each primer. Negative controls with each primer by itself should also be included.

4. We routinely use the following PCR components at the concentrations given below in 25 µl PCR reactions:

Stock Solution	**Final Concentration**
10X PCR Reaction Buffer	1 X
dNTPs	0.2 mM each
Taq DNA Polymerase (5 units/µl)	0.5 units
dH_2O	to 25 µl final volume

5. The standard cycling conditions we use are: pre-soak at 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 65 °C for 1 minute and extension at 72 °C for 90 seconds. Final extension at 72 °C for 5 minutes.

6. Analyze your results by agarose gel electrophoresis. If you do not observe a clean PCR product under these conditions, try altering the annealing temperature or Mg^{++} concentration in the PCR reactions. Repeat this testing until you determine the optimal conditions for PCR amplification with your chosen primer pair.

3) Vector primer screening of the master plate

1. Resuspend the DNA using 28 µl of sterile distilled water. Leave the plate at room temperature for 30 minutes before use. Remember to store the plate at -20°C after resuspending the DNA in water and to use a new piece of sealing tape before storing the plate.

2. Remove 5 µl from each well into a 96-well plate for PCR.

3. Place fresh sealing tape on the plate and freeze the master plate at -20 °C.

4. Add 20 µl of a mixture of the remaining PCR components to each well. We routinely use the following PCR components at the concentrations given below in 25 µl PCR reactions:

Stock Solution	**Final Concentration**
Plasmid DNA from master plate (30 ng)	5 µl
Primers (10 pmoles/µl)	0.5 µl of each primer
10X PCR Reaction Buffer	1 X
dNTPs	0.2 mM each
Taq DNA Polymerase (5 units/µl)	0.5 units

5. The standard cycling conditions we use are: pre-soak at 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 65 °C for 1 minute and extension at 72 °C for 90 seconds. Final extension at 72 °C for 5 minutes.

6. Identify the positive wells by agarose gel electrophoresis of the PCR reactions.

C. Positive and Negative Controls

1) Positive Control

If you observe no positive signal in your screen, we recommend that you check your PCR reagents in a test reaction with the included positive control DNA and primer mix.

1. Thaw PCR reagents, positive control DNA and primer mix.

2. Set up the following reaction:

5 µl positive control DNA

1 µl primer mix

2.5 µl 10X PCR reaction buffer

0.2 mM of each dNTP
0.5 units of Taq DNA polymerase
dH_2O to 25 µl.

3. Use the following cycling conditions: pre-soak at 94 °C for three minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute and extension at 72 °C for 90 seconds. Final extension at 72 °C for 5 minutes.

4. Analyze your results by agarose gel electrophoresis. You should observe a 483 bp. band. If not, it is likely that one of your PCR reagents has gone bad and all of your reagents should be replaced.

2) Negative Control

We recommend testing each of your primers by itself in a PCR reaction with cloned cDNA template to ensure that you do not get amplification of a DNA fragment other than the one that you want.

D. Ordering of sub-plates

The sub-plate number corresponds to the column number and row letter of the positive well on the master plate. For example, if well 10F is positive on the master plate, the corresponding sub-plate is number 10F. Once you identify the positive well(s) on the master plate, simply fax us a filled-out copy of the **Sub-plate Order Form**, found at the end of this manual. We will ship you the sub-plate(s) requested on the next business day.

E. Screening of sub-plates

The sub-plates contain 30 µl glycerol stocks of *E. coli* cultures in each well.

1µl of each stock is more than sufficient as template for a 25 µl PCR reaction. The same PCR conditions should be used for the master plate, sub-plate and colony screens. The sub-plates can be thawed and refrozen at -80 °C many times.

Remove the sub-plate from the -80 °C freezer. The best method for handling this plate is to spin the sealed frozen plate for 2 minutes at low speed in a refrigerated tabletop centrifuge to remove any condensation from the tape. The sealing tape should then be removed from the plate, while the samples are still frozen. Care should be taken when removing the sealing tape on the sub-plate, not to contaminate one well with another.

F. Colony screening

Once positive well(s) on a sub-plate are identified, you will want to screen individual colonies to identify the clone of interest. There are two recommended methods for this screening: PCR and filter hybridization. There are advantages and disadvantages to both methods.

1) Colony screening by PCR

1. Remove the sub-plate from the -20°C freezer. Care should be taken when removing the sealing tape on the master plate, not to contaminate one well with another. The best method for handling this plate is to spin the frozen plate for 2 minutes at low speed in a refrigerated tabletop centrifuge to remove any condensation from the tape. The sealing tape should then be removed from the plate, while the samples are still frozen. Thaw the sub-plate. Stir your well of interest with your pipet tip and remove 1 µl. Prepare a 1:1000 dilution in LB Broth.

2. Plate 1 µl, 5 µl and 10 µl of the dilution onto two separate LB/amp plates.

3. Incubate overnight at 37°C. If your desired clone is large, incubate at 30°C overnight. This will help to avoid the selective growing of clones with shorter insert sizes.

4. The following morning, prepare a PCR mix for a 96-well plate with the desired primer pair and aliquot 25 µl of the mix to the wells of the PCR tray.

5. Pick 95 individual colonies onto a gridded plate and into each of the individual PCR wells. Incubate the plate at 37°C overnight.

6. Put 1 µl of the positive glycerol stock into well number 96 as a positive control.

7. Run the PCR reactions as before.

8. Identify the positive clones by agarose gel electrophoresis of the PCR reactions.

9. Go back to the gridded master plate and pick your positive clone(s) and inoculate for plasmid preparation the following day. This method is rapid, but can be tedious for screening multiple positive wells from the sub-plates.

2) Colony screening by hybridization

When you have many positive sub-plate wells to screen, it may be preferable to screen by colony hybridization. This procedure will take several days longer than the simple PCR approach, but many samples can be processed in parallel. The following is a suggested procedure for colony screening by hybridization (Adapted from Molecular Cloning: A Laboratory Manual, Sambrook, Fritsch, Maniatis).

1. Cut four pieces of Whatman 3MM paper and place them at the bottom of four trays. Saturate each of the pieces of 3MM paper with one of the following solutions: 10% SDS, Denaturing Solution (0.5N NaOH, 1.5 M NaCl), Neutralizing Solution (1.5M NaCl, 0.5M Tris, pH 7.4), 2X SSC. Pour off any excess liquid.

2. Chill the LB/amp agar plates with colonies at 4°C for 30 minutes.

3. Number nitrocellulose filters and place the filter on the surface of the LB agar plate, in contact with the colonies, so that the filter is completely wet. Mark the filter asymmetrically with a 16-gauge needle.

4. Use blunt-end forceps to peel off the filter.

5. Place the bacterial plate back at 37°C to grow for 5 hours to regenerate the colonies.

6. Place the filter, colony side up, on the SDS-soaked paper for 3 minutes.

7. Transfer the filter to the 3MM paper with the denaturing solution for 5 minutes.

8. Transfer the filter to the 3MM paper with the neutralizing solution for 5 minutes.

9. Transfer the filter to the 3MM paper with the 2XSSC for 5 minutes.

10. Dry the filters, colony side up on a dry sheet of Whatman 3MM paper at room temperature for 30 minutes.

11. Fix the DNA to the filters by either baking for 1-2 hours at 80°C in a vacuum oven or by UV crosslinking.

12. Float the filters on the surface of a tray of 2X SSC until they are thoroughly wet and submerge for 5 minutes.

13. Incubate the filters for 30 minutes at 50°C in prewashing solution (5X SSC, 0.5% SDS, 1mM EDTA). **Do not allow the filters to dry out during any of the prewashing, prehybridization or hybridization steps.**

14. Transfer the filters to the prehybridization solution (6X SSC, 0.05X BLOTTO) and incubate at 68°C for 1 hour. 1X BLOTTO is 5% nonfat dried milk dissolved in water containing 0.02% sodium azide. Occasionally, high background will result from the use of this hybridization buffer in combination with the brand of membrane. An alternative buffer is 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.1% SDS and 100μg/ml of salmon sperm DNA.

15. Denature ^{32}P-labeled double-stranded DNA probes by boiling for 5 minutes. Chill in ice water for 1 minute. Add the probe to the prehybridization solution, at a concentration of 1 X 10^6 cpm/ml of prehybridization solution. Incubate overnight at 68°C. If you are using the alternative formamide buffer, the hybridization temperature should be reduced to 42°C, but can be increased up to 55°C if high background signal is a problem. The hybridization time can be reduced to as little as 2-3 hours, since the signal will be very stong.

16. Remove the hybridization solution and immediately immerse the filters in 2XSSC and 0.1% SDS at room temperature. Agitate the filters gently. After 5 minutes, transfer to fresh washing buffer and repeat this wash two more times. **Do not allow the filters to dry during any of the washes.**

17. Wash the filters twice for 30 minutes in 1XSSC, 0.1% SDS at 68°C. If the background is still high, wash in 0.2XSSC, 0.1% SDS at 68°C for additional time.

18. Dry the filters at room temperature on Whatman 3MM paper. Tape the filters to a fresh piece of Whatman 3MM paper, mark the paper asymmetrically with fluorescent ink and cover with Saran Wrap. Expose the filters to X-ray film overnight at -70°C with an intensifying screen.

19. Develop the film, identify the positive colonies and inoculate the colonies into LB/ampicillin media for growth and plasmid preparation.

4. Sample Library Screens

A. **Example 1:** *You have a small sequence of cDNA (600bp.), but the location of the sequence within the mRNA is unknown. You have shown by Northern blot analysis, using this small fragment as a probe, that the mRNA is 6.5 kb and the message is expressed in human spleen.*

Screening:

1. Sythesie a pair of primers, one at either end of your 600 bp sequence. A single primer for use with the vector primers can also be designed. However, it is possible that the small sequence may be near the 3' end of the 6.5 kb. cDNA. If this is the case, you will not get any informative PCR products by vector primer PCR. Therefore, it is more prudent to try the gene specific PCR and determine how many positive sub-plates there are.

2. Perform 96-well PCR on the spleen master plate using this pair of primers.

3. Run the samples on an agarose gel for analysis.

4. Since your cDNA is predicted to be 6.5 kb., we recommend that you order sub-plates which were generated from the largest size cut ligations. For this human spleen library, these sub-plates correspond to the master plate wells found in columns 1,2 and 3.

5. When you receive your sub-plate(s), screen them by PCR. If you observe multiple positive wells within a single sub-plate, these are each independent clones.

B. **Example 2:** *You have obtained the EST clones for your gene of interest. You have shown by Northern blot analysis that the corresponding mRNA is 4.0 kb. and is expressed at relatively high levels in fetal brain. However, after sequencing your EST clones, you determine that you are missing 1.0 kb. at the 5' end of the 4 kb. cDNA.*

Screening:

1. Synthesize three primers. One of the antisense primers will be used for vector primer PCR and should be located between 500 bp. and 1 kb from

the 5' end of the EST sequence. The estimated melting temperature for the outer antisense primer should be 65 °C, while the other antisense primer should have an estimated melting temperature of 55 °C. The third primer is a sense primer and can be used in conjunction with the smaller one of the antisense primers for gene specific PCR.

2. Since you know that your gene of interest is abundant in fetal brain and that you have sequence information within 2 kb of the 5' end of the cDNA, we recommend that your first attempt at screening the fetal brain library should be using vector primer PCR. This approach will allow you to identify the longest of the clones for this gene in this library.

3. Run the samples on an agarose gel for analysis. Since this mRNA is well-expressed in fetal brain, you may observe more than 50 positive wells. The vector primer PCR approach will allow you to differentiate amongst these wells for the longest clones.

4. Order sub-plates which correspond to master-plate wells which contain the longest cDNA clones.

5. We recommend that the PCR screen of the sub-plate(s) be done by gene specific PCR. If you observe multiple positive wells within a single sub-plate, these are each independent clones.

5. Tips on excising your insert

For pCMV6-XL4, we recommend excising the insert with NotI and XbaI to get a directionality to your cDNA clone. When using the pCMV6-XL3 vector, at the 5' end of the cloned cDNA are two restriction enzyme sites, NotI and EcoRI, which can be used to separate the vector sequence from the insert sequence. Similarly, at the 3' end of the cloned cDNA are two restriction enzyme sites, SfiI and SmaI, which can be used to separate the vector sequence from the insert sequence. We recommend that you use one enzyme from either side of the insert to excise your cDNA clone from pCMV6-XL3. Our preference is to use NotI and SmaI. EcoRI cuts more frequently than NotI and the SfiI site in the multiple cloning site of this vector is not unique. The second SfiI site is 1kb. downstream from the first SfiI site. This vector-derived 1kb. band in the restriction digest can present some diffiuclties in sub-cloning your cDNA of interest if your clone is of a similar size.

A note about sequencing clones in pCMV6-XL4, not all M13 reverse primers anneal directly to the M13 reverse site at the 3' end of the cloning site. We recommend the use of the M13 Reverse sequencing primer with the sequence: 5' caggaaacagctatgacc 3'

6. Trouble-shooting Guide

A. No positive signal on master-plate screen.

1) Too few cycles You can use up to 40 cycles with our running into overcycling problems, such as multiple or smeary bands.

2) Omitted PCR component Be sure to use a written checklist when assembling reactions. It is best to include a positive control with each reaction set.

3) Primer design There is no ideal method for designing primers. However, secondary structure within the primer can inhibit PCR. To ensure that your primers will amplify your gene of interest, we recommend that you check your primers on a known template before using them to screen the library panel.

4) Annealing temperature is not optimal We recommend that you optimize the annealing temperature on a known template before you screen the library. If you do not observe any positive signal, it is possible that the annealing temperature is simply to high for your primers.

5) Extension time is too short The extension time should be 1 min. for every 1 kb. you are trying to amplify.

6) Reagent concentrations are not optimal The Mg^{++} concentration can be varied from 1.0 to 2.5 mM. However we have found that a 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 dH_2O in place of a DNA sample in every PCR experiment to determine if the PCR reagents, pipettors or other PCR-related equipment are contaminated with exogenous DNA. We recommend the use of barrier tips for all pipetman used for PCR to avoid aerosol contamination.

2) Annealing temperature not optimal The annealing temperature may be too low. Increase it by increments of 2 °C on test positive and negative control samples for optimization.

3) Primer design The GC content of your primers may be low (<45%) and you are getting some unspecific 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 DNAs.

C. Smears on the gel.

1) Too many cycles Depending on your primers and the abundance of your gene of interest, you many need as few as 25 cycles or as many as 40 cycles to see your specific product. You can use Northern blot analysis to determine the relative abundance of your gene of interest in the corresponding tissue to the library panel. Too many PCR cycles can result in multiple PCR products or smeariness of the PCR products.

2) See 2a and 2b above

If you have any questions about this product, please contact Technical Support at 1-888-267-4436 or 1-301-340-3188. You can also reach us by e-mail at custsupport@origene.com

7. References

Molecular Biology Protocols

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., ed., Current Protocols in Molecular Biology, Canada: John Wiley and Sons, Inc., 1994.

Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd Edition, New York: Cold Spring Harbor Laboratory Press, 1989.

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Eckert, K.A. and Kunkel, T.A., (1990) High fidelity DNA synthesis by the Thermus aquaticus DNA polymerase, *Nucleic Acids Research*, 18:3739-3744.

Erlich, H.A., ed., PCR Technology: Principles and Applications for DNA Amplification, New York: Stockton Press, 1989.

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8. Related Products

Multiple Choice™ Northern Blots
Multiple Choice™ cDNAs
DUP-LexA ™ Yeast Two-Hybrid System
Rapid-Load ™

9. Published Papers using Rapid-Screening cDNA Library Panel

1. **"Mammalian Circadian Autoregulatory Loop: A *Timeless* Ortholog and *mPer*1 Interact and Negatively Regaulate CLOCK-BMAL1-Induced Transcription**." 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 J. S. Takahashi. ***Neuron***, 21:1101-1103. November, 1998.

2. **"A gene related to *Caenorhabditis elegans* spermatogenesis factor *fer*-1 is mutated in limb-girdle muscular dystrophy type 2B"** Bashir, R., Britton, S., Strachan, T., Kerrs, S., Vafiadaki, E., Lako, M., Richard, I., Marchand, S., Bourg, N., Argov, Z., Sadeh, M., Mahjneh, I., Marconi G., Passos-Bueno, M. R., de S Moreira, E., Zatz, M., Beckman, J. S., and K. Bushby. ***Nature Genetics***, 20:37-42. 20 Septmeber 1998.

Appendix A: Sequence information around the polylinker site of pCMV6-XL3

```
ACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGG
TGAAAGGTTTTACAGCATTATTGGGGCGGGGCAACTGCGTTTACCCGCCATCCGCACATGCC

                                Vector Primer 3                    T7 Promoter
TGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACC   GTCAGAATTTTGTAATACGACTCAC
ACCCTCCAGATATATTCGTCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGTG

          Not I      EcoR I          Kpn I  EcoRV  Hind III      Xba I      Pst I
TATAGGG CGGCCGCGAATTCAGATCTGGTACCGATATCAAGCTTAAGCTTCTAGACTGCAGT
ATATCCC GCCGGCGCTTAAGTCTAGACCATGGCTATAGTTCGAATTCGAAGATCTGACGTCA

    Sal I      Sfi I                T3 Promoter              Sma I
CGACGGCCAAGTCGGCCTCCCTTTAGTGAGGGTTAATT    TGTGATCCCGGGTGGC
GCTGCCGGTTCAGCCGGAGGGAAATCACTCCCAATTAA    ACACTAGGGCCCACCG
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Sequence information around the polylinker site of pCMV6-XL4

```
ACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGG
TGAAAGGTTTTACAGCATTATTGGGGCGGGGCAACTGCGTTTACCCGCCATCCGCACATGCC

                                Vector Primer 3                    T7 Promoter
TGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAACC   GTCAGAATTTTGTAATACGACTCAC
ACCCTCCAGATATATTCGTCTCGAGCAAATCACTTGGCAGTCTTAAAACATTATGCTGAGTG

          Not I      EcoR I          Kpn I  EcoRV  Hind III  Sal I   Xba I     NotI
TATAGGG CGGCCGCGAATTCAGATCTGGTACCGATATCAAGCTTGTCGACTCTAGATTGCGG
ATATCCC GCCGGCGCTTAAGTCTAGACCATGGCTATAGTTCGAACAGCTGAGATCTAACGCC

          M13 Reverse                    SmaI
CCGCGGTCATAGCTGTTTCC   TGAACATGTGATCCCGGGTGGC
GGCGCCAGTATCGACAAAGG   ACTTGTACACTAGGGCCCACCG
```

Appendix B: Accession Numbers from Gene Bank

pCMV-XL3 - #AF067197

pCMV-XL4 - #AF067196