Takara Bio USA, Inc.

Clontech® PCR-Select™ Differential Screening Kit User Manual

Cat. No. 637403
(030218)

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Table of Contents

I. Introduction ... 3
II. List of Components ... 6
III. Additional Materials Required ... 7
IV. General Considerations ... 9
V. Secondary PCR of Subtracted cDNA ... 9
VI. Subtracted cDNA Library Construction ... 12
A. T/A Cloning ... 12
B. Site-Specific or Blunt-End Cloning ... 12
VII. Arraying Subtracted Clones ... 13
A. cDNA Array ... 13
B. Colony Array ... 14
VIII. Random Primer Labeling of cDNA Probes ... 15
IX. Hybridization with the Subtracted cDNA ... 16
X. Analysis of Results ... 17
XI. Troubleshooting Guide ... 20
XII. Differential Screening and Bacterial Genome Subtraction ... 23
A. Southern Blot Analysis ... 23
B. Differential Screening ... 23
XIII. References ... 26
Appendix: Adaptor and Primer Sequences ... 27

Table of Figures

Figure 1. Comparison of differential screening approaches. ... 4
Figure 2. Confirmation of putative differentially expressed scDNAs by Virtual Northern blot analysis. ... 5
Figure 3. Experimental set-up for PCR-Select cDNA subtraction and differential screening. ... 10
Figure 4. Sample differential screening results. ... 17
Figure 5. Experimental set-up for PCR-Select bacterial genome subtraction and differential screening. ... 24
Figure 6. Differential screening of a subtracted *Helicobacter pylori* DNA library. ... 25
Figure 7. Sequences of the adaptors and PCR primers. ... 27
Figure 8. Hypothetical forms of a subtracted cDNA. ... 27

Table of Tables

Table 1. Interpretation of Differential Screening Results ... 18

I. Introduction

A. Summary

The **Clontech PCR-Select Differential Screening Kit** (Cat. No. 637403) is a complete kit for differential screening of subtracted libraries obtained using the **Clontech PCR-Select cDNA Subtraction Kit** (Cat. No. 637401). The Clontech PCR-Select cDNA Subtraction Kit provides a powerful method for identifying differentially expressed cDNAs (Diatchenko *et al.*, 1996; Gurskaya *et al.,* 1996). In most cases, the PCR-Select method greatly enriches for differentially expressed sequences; nevertheless, the subtracted sample will still contain some cDNAs that are common to both the tester and driver samples. Although this background may depend somewhat on the quality of RNA purification and the performance of the particular subtraction, it mainly arises when very few mRNA species are differentially expressed in tester and driver. In general, fewer differentially expressed mRNAs and less quantitative difference in expression means higher background—even if you obtain a good enrichment of differentially expressed cDNAs. When background is high, picking random clones from the subtracted library for Northern blot analysis is time consuming and inefficient. Performing a differential screening step can help minimize background before you embark on Northern blot analysis.

B. Two complementary approaches for differential screening

There are two approaches for differentially screening the subtracted library. The first is to hybridize the subtracted library with ^{32}P-labeled probes synthesized as first-strand cDNA from tester and driver (Hedrick *et al.*, 1984; Sakaguchi *et al.*, 1986). Clones corresponding to differentially expressed mRNAs will hybridize only with the tester probe, and not with the driver probe. Although this approach is widely used, it has one disadvantage: only cDNA molecules corresponding to highly abundant mRNAs (i.e., mRNAs which constitute more than about 0.2% of the total cDNA in the probe) will produce detectable hybridization signals (see Nucleic Acid Hybridization, 1985; Wang & Brown, 1991). Clones corresponding to low-abundance differentially expressed mRNAs will not be detected by this screening procedure.

The second approach bypasses the problem of losing low-abundance sequences. In this method, the subtracted library is hybridized with forward- and reverse-subtracted cDNA probes. The forward-subtracted probe is made from the same subtracted cDNA used to construct the subtracted library. To make the reverse-subtracted probe, subtractive hybridization is performed with the original tester cDNA as a driver and the driver cDNA as a tester, as described in the Clontech PCR-Select cDNA Subtraction Kit User Manual (PT1117-1). Clones representing mRNAs that are truly differentially expressed will hybridize only with the forward-subtracted probe; clones that hybridize with the reverse-subtracted probe may be considered background (Diatchenko *et al.,* 1998; Wang & Brown, 1991; Lukyanov *et al.*, 1996). The one drawback of using subtracted probes is that they typically result in a slightly higher false-positive rate than the unsubtracted probes described above.

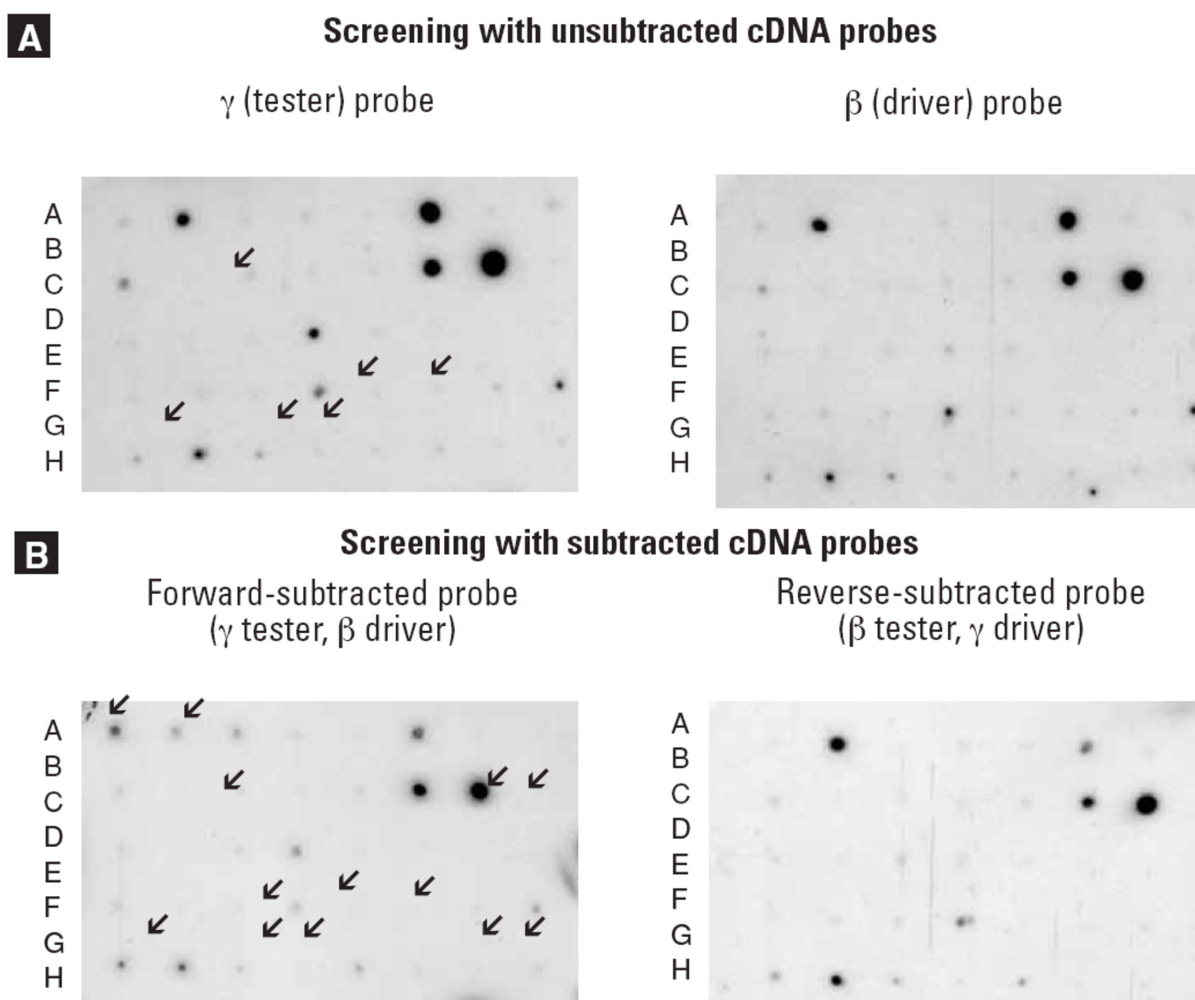

Figure 1. Comparison of differential screening approaches. Panel A. Dot blots hybridized with unsubtracted cDNA probes made from tester (γ-line) and driver (β-line) RNA. **Panel B.** Dot blots hybridized with cDNA probes made from forward-subtracted cDNA (γ-line tester, β-line driver) and reverse-subtracted cDNA (β-line tester, γ-line driver). On each membrane, the following clones correspond to controls. H10: γ-globin cDNA fragment (positive control); H11: Negative Hybridization Control (cDNA 1R); H12: Negative Hybridization Control (cDNA 2R).

Figure 1 compares the results of both differential screening approaches. For this experiment, the SMART® PCR cDNA Synthesis Kit (Cat. No. 634902) was used to preamplify total RNA from a γ-globin-producing cell line (γ-line) and a β-globin-producing cell line (β-line). PCR-Select cDNA subtraction was performed using γ-line cDNA as tester and β-line cDNA as driver; a reverse subtraction was also performed using β-line cDNA as tester and γ-line cDNA as driver. The forward-subtracted cDNA was cloned, and 84 randomly selected clones were arrayed on four separate nylon membranes for screening. Although screening with unsubtracted probes easily identified abundant γ-line-specific sequences (Figure 1A), it missed sequences corresponding to rare messages in the tester population. The sensitivity of this procedure was increased by screening with subtracted probes (Figure 1B). Whereas only six clones showed a differential signal with the unsubtracted probes, 13 clones were identified using the subtracted probes (arrows).

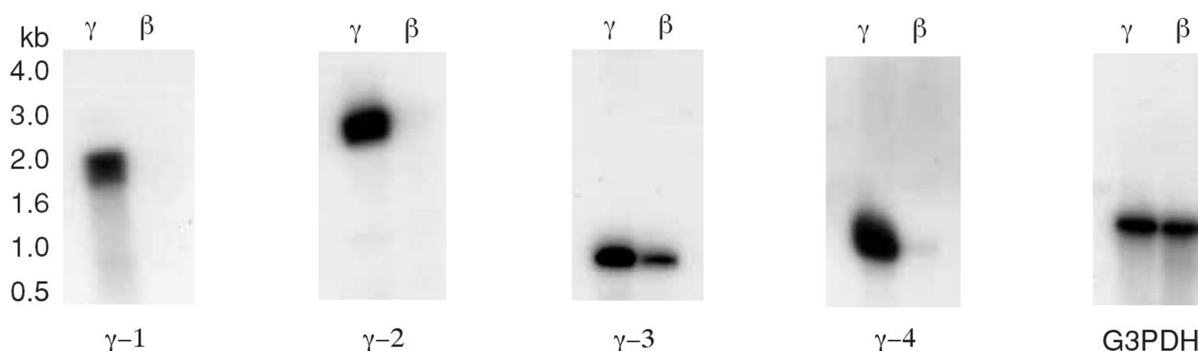

Figure 2. Confirmation of putative differentially expressed cDNAs by Virtual Northern blot analysis. Each lane contains 0.5 µg of SMART PCR-amplified cDNA from either γ- or β-globin-producing cell lines (as indicated). To produce each blot, SMART cDNA was electrophoresed on a 1.2% agarose gel and transferred to a nylon membrane. Each blot was hybridized to the indicated radioactively labeled cDNA clone. Exposure times were γ-1, γ-2, and γ-3: overnight; γ-4: 1 week; and G3PDH: 2 hr. G3PDH hybridization confirms that lanes were loaded equally.

Differential expression of those 13 cDNA clones was confirmed using "Virtual" Northern blots. Virtual Northern blots are made with SMART PCR-amplified cDNA instead of total or poly A^+ RNA, and they yield information similar to that provided by standard Northern blots (Diatchenko *et al.*, 1998; Chenchik *et al.*, 1998; Endege *et al.*, 1999). Four Virtual Northern blots are shown in Figure 2. All 13 clones represented genes expressed primarily or only in the γ-line, but not the β-line. Eleven clones were sequenced and nucleic acid homology searches were performed using the BLAST program. All 11 clones represented unique sequences and four represented novel genes.

In general, using subtracted probes for differential screening yields more sensitive results than unsubtracted probes, since rare sequences are retained. Thus, this method is recommended for high-throughput screening. However, we strongly recommend that you also screen in parallel with non-subtracted probes. Information from these hybridizations is very useful in confirming or disproving ambiguous results. The procedure in this User Manual can be used to make both subtracted and unsubtracted probes.

C. Overview of the PCR-Select differential screening procedure

Figure 3 (in Section V) shows the experimental set-up for PCR-Select subtraction and differential screening. This flow chart is based on the current version of the Clontech PCR-Select cDNA Subtraction Kit User Manual (PT1117-1). If you have an older version of the PCR-Select User Manual, you may have labeled your reaction tubes a little differently; compare your experimental set-up to Figure 3 to determine which reactions to use for the differential screening procedure.

In order to perform differential screening with subtracted probes, you must perform PCR-Select subtraction in both directions. Use the *primary* PCR product from your forward subtraction experiment (see Section IV.I of the PCR-Select User Manual in secondary PCR to make the subtracted library and use the forward-subtracted probe for the differential screening procedure; use the primary PCR product from the reverse subtraction in secondary PCR to make the reverse-subtracted probe. To make unsubtracted probes, you should use unamplified tester and driver cDNA if you have enough (50–100 ng single- or double-stranded cDNA). If necessary, first-strand cDNA can be prepared from poly A^+ RNA using the procedure described in the PCR-Select User Manual or your preferred protocol. If you do not have a sufficient quantity of tester and driver cDNA or RNA, you can amplify the Unsubtracted controls from the PCR-Select procedure, as described in Section V of this User Manual.

D. Differential screening and bacterial genome subtraction

Researchers who are using the PCR-Select Bacterial Genome Subtraction Kit (Cat. No. 637404) can also use differential screening to analyze their subtracted clones. Unlike eukaryotic PCR-Select cDNA libraries, prokaryotic libraries do not require using subtracted probes for differential screening. This is because bacterial genomes are less complex (only 1–5 x 10^6 bp) than eukaryotic mRNA populations. In addition, bacterial genomes generally contain only one copy of each gene, so rare genes will not be missed. Therefore, we recommend that you perform differential screening of your prokaryotic PCR-Select DNA library using unsubtracted probes.

This Differential Screening Kit is especially useful if you lack sufficient bacterial genomic DNA for Southern blot analysis and/or differential screening. For more information, see Section XII of this User Manual.

II. List of Components

Store all components at –20°C.

The kit contains enough components for making 10 cDNA probes for hybridization and 20 labeling reactions.

PCR-Select Differential Screening Kit (Cat. No. 637403):

- 100 µl Nested Primer 1
- 100 µl Nested Primer 2R
- 20 µl Negative Hybridization Control (cDNA 1R; 5 µg/ml)
- 20 µl Negative Hybridization Control (cDNA 2R; 5 µg/ml)
- 50 µl Random Primer Mix
  - 0.9 mg/ml Random nonamers
  - 50 mM Tris-HCl (pH 7.5)
  - 10 mM $MgCl_2$
  - 1 mM DTT
  - 50 µg/ml BSA
- 60 µl Reaction Buffer (–dCTP)
  - 333 mM Tris-HCl (pH 8.0)
  - 33.3 mM $MgCl_2$
  - 10 mM 2-mercaptoethanol
  - 170 mM dATP
  - 170 mM dGTP
  - 170 mM dTTP
- 60 µl Reaction Buffer (–dATP)
  - 333 mM Tris-HCl (pH 8.0)
  - 33.3 mM $MgCl_2$
  - 10 mM 2-mercaptoethanol
  - 170 mM dCTP
  - 170 mM dGTP
  - 170 mM dTTP
- 20 µl Klenow Enzyme (exo–; 2–5 units/µl)
- 100 µl Stop Solution (0.1 M EDTA; 500 µg/ml yeast tRNA)
- 1 ml Blocking Solution (for additional Blocking Solution, Cat. No. 637402)
  - 10 mg/ml Sheared salmon sperm DNA
  - 0.3 mg/ml Oligonucleotides corresponding to the Nested Primers and complementary sequences

III. Additional Materials Required

The following reagents are required but not supplied.

Secondary PCR of subtracted cDNA

- **50X PCR enzyme mix**
  We recommend Advantage® 2 Polymerase Mix (Cat. No. 639201; also provided in the Advantage 2 PCR Kit [Cat. Nos. 639206, 639207). The protocol was optimized using this mix, which was developed for long and accurate PCR (Barnes, 1994; Cheng *et al*., 1994). The Advantage 2 Polymerase Mix is comprised of Titanium® *Taq* DNA Polymerase—a nuclease-deficient N-terminal deletion of *Taq* DNA polymerase plus TaqStart® Antibody to provide automatic hot-start PCR (Kellogg *et al*., 1994)—and a minor amount of a proofreading polymerase. Alternatively, Titanium *Taq* DNA polymerase alone can be used, but 3–5 additional thermal cycles will be needed in both primary and secondary PCR, and the additional cycles will cause higher background.

  NOTE: If you choose not to use the Advantage 2 Mix or Titanium *Taq*, use TaqStart Antibody (Cat. Nos. 639250, 639251), manual hot start, or hot start with wax beads to reduce nonspecific DNA synthesis.

- **10X PCR buffer**
  Use the 10X PCR buffer supplied with your DNA polymerase or mix.

- **dNTP mix for PCR** (10 mM each dATP, dCTP, dGTP, dTTP)

Subtracted cDNA library construction

- **T/A cloning reagents**, or reagents for blunt-end or site-specific cloning

- **LB broth**

  10 g Bacto-tryptone
  5 g Bacto-yeast extract
  5 g NaCl

  Add H_2O to 900 ml. Adjust pH to 7.0 with 5 N NaOH, then bring up to a 1-L volume with H_2O. Autoclave.

- **Ampicillin** (50 mg/ml stock solution; store at –20°C)

cDNA array of subtracted clones

- PCR reagents (see above)
- 0.6 N NaOH
- 0.5 M Tris-HCl (pH 7.5)

Colony array of subtracted clones

- **Denaturing solution** (0.5 M NaOH, 1.5 M NaCl; make fresh each time)
- **Neutralizing solution** (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl)
- Nylon membrane

Preparing cDNA probes

- **Silica-based PCR purification reagents** (e.g., NucleoSpin Gel & PCR Clean-Up kit, Cat. No. 740609.50)
- **[α-^{32}P]dCTP or [α-^{32}P]dATP** (3,000 Ci/mmol)
- **Size-exclusion chromatography columns** (e.g., CHROMA SPIN™-100 Columns, Cat. Nos. 636072, 636073)

Hybridization with the subtracted cDNA

- **Hybridization solution**
  We strongly recommend that you use ExpressHyb™ Hybridization Solution (Cat Nos. 636831, 636832). This protocol is optimized for use with ExpressHyb. Alternatively, consult Sambrook *et al.* (2001) for a recipe to make your own solution.

- **20X SSC**
  175.3 g NaCl
  88.2 g $Na_3Citrate \bullet 2H_2O$

  Adjust pH to 7.0 with 1 M HCl. Add H_2O to 1 L. Store at room temperature.

- **20% SDS**
  200 g SDS

  Add H_2O to 1 L. Heat to 65°C to dissolve. Store at room temperature.

- **Low-stringency washing solution** (2X SSC, 0.5% SDS)

- **High-stringency washing solution** (0.2X SSC, 0.5% SDS)

General Reagents

- **Ethanol**

- **Sterile, deionized H_2O**

- **50X TAE electrophoresis buffer**
  242 g Tris base
  57.1 ml Glacial acetic acid
  37.2 g $Na_2EDTA \bullet 2H_2O$

  Add H_2O to 1 L. For 1X TAE buffer, dilute 50X stock solution 1:49 with H_2O.

IV. General Considerations

- The Negative Hybridization Controls are plasmids that contain one of two cDNA inserts. The cDNA 1R insert corresponds to 348 bp of a human homolog of a mouse mRNA for testis-specific protein (GenBank accession #X52128). The cDNA 2R insert corresponds to 182 bp of human semenogelin II mRNA (GenBank accession #AN M81652), which is specific to seminal vesicles. When hybridized to human multiple tissue Northern blots, cDNA 1R hybridizes only to the human testis poly A^+ RNA, and cDNA 2R hybridizes only to the human prostate poly A^+ RNA. (Prostate poly A^+ RNA is often contaminated by seminal vesicle-specific sequences because these two organs are in close anatomical proximity.) Negative Hybridization Control cDNAs 1R and 2R contain Nested Primer 1 and 2R sequences. Since at least one of these cDNAs will not be present in your subtracted cDNA library, they are good negative controls for hybridization.
- For differential screening of subtracted cDNA libraries, you should use both subtracted and unsubtracted probes. The procedure in this User Manual can be used for both. To make unsubtracted probes, use 50–100 ng of tester and driver ds or ss cDNA and follow the protocol in Section VIII. If you lack sufficient tester and driver cDNA, you can synthesize new aliquots of first-strand cDNA from 200 ng of poly A^+ RNA (preferred) or you can amplify your unsubtracted cDNA, as described in Section V. In Section VII, be sure to make four identical cDNA or colony arrays.
- For differential screening of subtracted prokaryotic DNA libraries, you should use unsubtracted probes. See Section XII for more information.
- All cycling parameters were optimized using a Perkin-Elmer DNA Thermal Cycler 480 or Perkin-Elmer GeneAmp PCR Systems 2400/9600. For other types of thermal cyclers, you should optimize the cycling parameters.

V. Secondary PCR of Subtracted cDNA

In this section, you will amplify the *primary* PCR products from the PCR-Select cDNA subtraction (from Section IV.I of the PCR-Select Protocol). To make subtracted probes, use the diluted products of the *primary* PCR from the forward- and reverse-subtractions (Figure 3). As shown in Figure 3, the secondary PCR product from the forward subtraction will be used to make the subtracted library (Section VI) ***and*** will be used as the forward-subtracted probe.

To make unsubtracted probes you should use single-stranded or double-stranded tester and driver cDNA if you have enough (at least 50 ng). Label the cDNA as described in Section VIII. Alternatively, use the following procedure to amplify your tester and driver cDNA. For the tester probe, use the *primary* PCR product of the **Unsubtracted tester control** (tube 1-c) from the forward subtraction as your template; for the driver probe, use the primary PCR product of the **Unsubtracted tester control** (tube 2-c) from the reverse subtraction as your template (Figure 3).

NOTES:

- All cycling parameters were optimized using a Perkin-Elmer DNA Thermal Cycler 480 and Perkin-Elmer GeneAmp PCR Systems 2400/9600. If a different type of thermal cycler is used, the cycling parameters must be optimized for that machine.
- If you do not use Advantage 2 Polymerase Mix, you can use Titanium *Taq* DNA polymerase alone; however, 3–5 more cycles will be needed. If you choose not to use the Advantage 2 Mix or Titanium *Taq*, you must use a hot start. TaqStart Antibody (Cat. Nos. 639250, 639251; included in the Advantage 2 and Titanium *Taq* Mixes) works best.

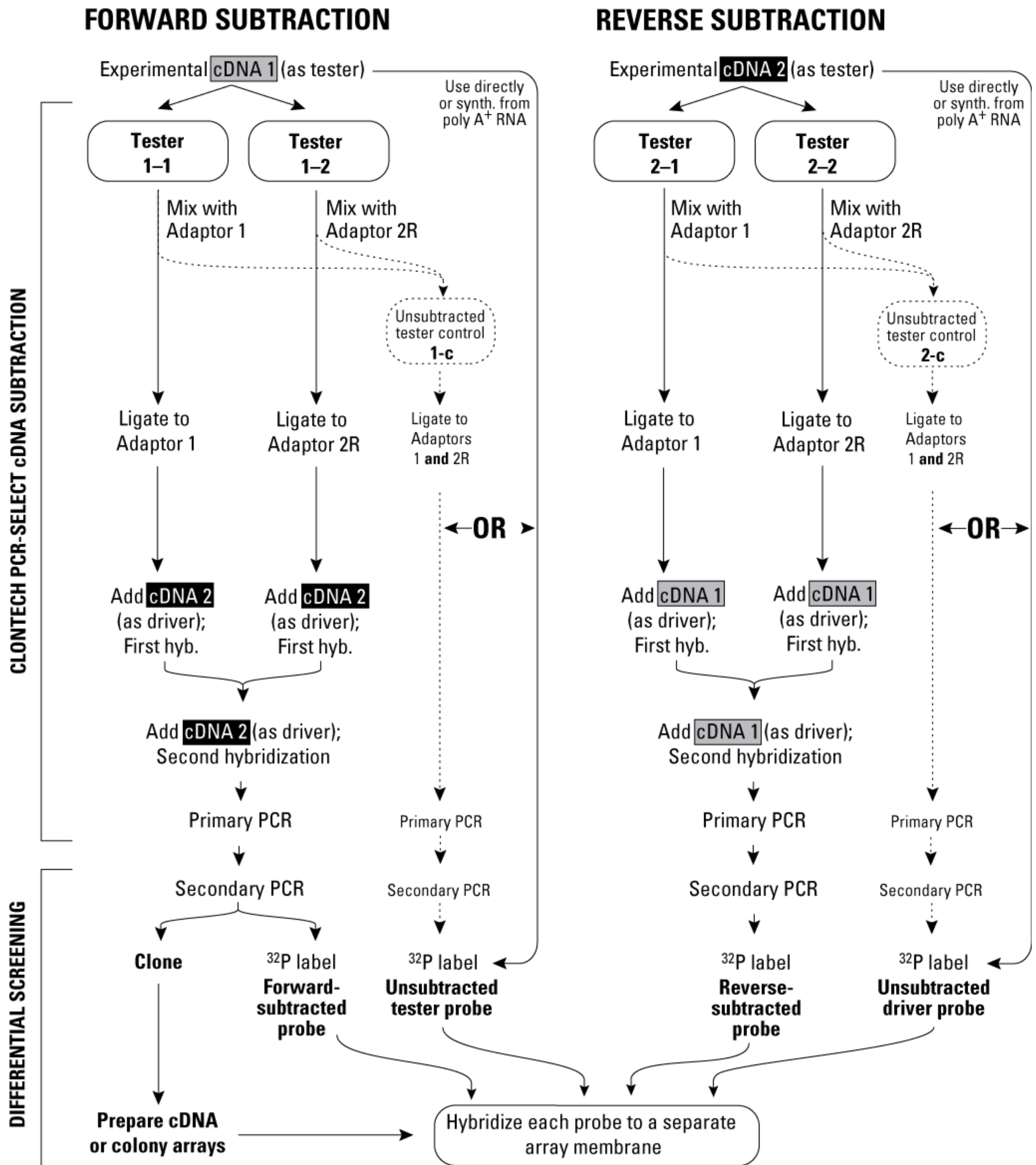

Figure 3. Experimental set-up for PCR-Select cDNA subtraction and differential screening. This flow chart shows how to prepare both subtracted and unsubtracted probes. In general, differential screening with subtracted probes is more sensitive. However, using both subtracted and unsubtracted probes is recommended. If you have sufficient material, you should label ss or ds tester and driver cDNA (non-adaptor-ligated). Alternatively, you can amplify the Primary PCR product of the Unsubtracted control cDNAs as shown (dotted lines).

Procedure for Secondary PCR of Subtracted cDNA

1. Label sterile 0.5-ml reaction tubes for PCR. Prepare two secondary PCR tubes for each subtracted and/or unsubtracted probe, and one tube for each of the two Negative Controls (cDNAs 1R and 2R).
2. Denature an aliquot of each Negative Control:
   a. In a clean 0.5-ml microcentrifuge tube, mix 5 µl of each Negative Control with 3 µl of deionized H_2O.
   b. Heat tubes at 96°C for 5 min in a PCR thermal cycler. Alternatively, place tubes in boiling water for 5 min.
   c. Chill tubes on ice.
   d. Spin tubes briefly in a microcentrifuge to collect contents
3. Prepare a PCR Master Mix in a 1.5-ml microcentrifuge tube. To ensure that you have sufficient Master Mix for your experiment, calculate your volumes to include an extra reaction. Combine the following components in the order shown:

	per rxn
Sterile, deionized H_2O	18.5 µl
Nested Primer 1 (10 µM)	1.0 µl
Nested Primer 2R (10 µM)	1.0 µl
10X PCR buffer	2.5 µl
dNTP mix (10 mM)	0.5 µl
50X PCR enzyme mix	0.5 µl
Total volume	24.0 µl

4. Mix well by vortexing. Spin the tube briefly in a microcentrifuge to collect contents at the bottom.
5. Aliquot 24 µl of Master Mix into each reaction tube labeled in Step 1.
6. Into each tube, aliquot 1 µl of the appropriate template:
   a. For the negative controls, use the denatured mixture prepared in Step 2, above.
   b. For the forward- and reverse-subtracted probes, use the diluted products of primary PCR amplification from your PCR-Select cDNA subtraction.
   c. To amplify tester and driver cDNA to make unsubtracted probes:
      - For the tester probe, use the *primary* PCR product of the Unsubtracted tester control from the forward subtraction as your template.
      - For the driver probe, use the *primary* PCR product of the Unsubtracted tester control from the reverse subtraction as your template.
7. If you are not using a hot-lid thermal cycler, overlay each reaction with 1 drop of mineral oil.
8. Immediately commence thermal cycling:

Perkin-Elmer DNA Thermal Cycler 480:
11 cycles:
- 94°C for 30 sec
- 68°C for 30 sec
- 72°C for 1.5 min

One additional cycle:
- 72°C for 5 min

Perkin-Elmer GeneAmp PCR Systems 2400/9600:
11 cycles:
- 94°C for 10 sec
- 68°C for 30 sec
- 72°C for 1.5 min

One additional cycle:
72°C for 5 min

9. Electrophorese 8 µl from each reaction on a 2.0% agarose/EtBr gel in 1X TAE buffer. The PCR product should appear as a smear with or without several distinct bands (see Section V.D of the Clontech PCR-Select cDNA Subtraction Kit User Manual (PT1117-1). For the cDNA 1R control, you should observe a 340-bp band; for the cDNA 2R control, you should observe a 200-bp band.
10. Store reactions at –20°C or proceed directly to purify your secondary PCR product using a silica-based method such as the NucleoSpin Gel & PCR Clean-Up kit (Cat. No. 740609.50).

VI. Subtracted cDNA Library Construction

In this section, you will clone the *secondary* PCR product from the **forward subtraction** (from Section V) to make your subtracted cDNA library.

A. T/A Cloning

Clone 3 µl of the *secondary* PCR product from the forward subtraction (from Section V) using a T/A-based cloning system. Consult the manufacturer's protocol. For this application, it is important to optimize your cloning efficiency; a low cloning efficiency will result in a high background. To increase the efficiency of transformation, try electroporating into electrocompetent DH5α *E. coli* (#C2022-1). Using T/A cloning and electroporation, we typically obtain 10^4 independent clones from 1 µl of secondary PCR product.

If you plan to array your candidate subtracted clones for colony hybridization (Section VII.B) rather than cDNA hybridization (Section VII.A), you will also need to transform the Negative Hybridization Controls into *E. coli.* Use 10 pg of each plasmid for transformation. These clones will serve as negative controls for hybridization.

B. Site-Specific or Blunt-End Cloning

If you remove the adaptor sequences from your cDNAs for site-specific or blunt-end cloning, the inserts can no longer be amplified using the Nested Primers. In this case, if you want to make a cDNA array (Section VII.A), you will need to use primers specific for the vector you used for cloning for PCR amplification.

For site-specific cloning, digest the secondary PCR product from the forward subtraction (Section V) at the Eag I or at Eag I and Sma I (Xma I) sites in the adaptor and then ligate into an appropriate vector for site-specific ligation. (Eag I will also cut at Not I sites.)

For blunt-end cloning, cleave the Rsa I site in the adaptors and then ligate into an appropriate blunt-ended vector. Consult Sambrook *et al.* (2001) for an appropriate protocol, or use a commercially available cloning kit.

VII. Arraying Subtracted Clones

In this section, you will pick random clones from your subtracted cDNA library (from Section VI) and array them on nylon membranes. In Section IX, these arrays will be hybridized to the probes that you will prepare in Section VIII.

There are two ways to array putative subtracted clones for differential screening: cDNA dot blots and colony dot blots. Hybridization with colony arrays is less sensitive and produces higher background; however, it is faster and less expensive. Here we present both approaches.

A. cDNA Array

Amplify cDNA inserts by PCR:

For high-throughput screening, we recommend that you use a 96-well format for the following steps. For growing bacterial colonies, use a standard 96-well plate. For PCR, use a 96-well plate designed especially for PCR (e.g., MJ Research's Multiplate 96). If necessary, the steps can be performed in individual tubes, but this will be much less convenient.

1. Randomly pick 96 white bacterial colonies.
2. Grow each colony in 100 µl of LB-amp medium in a standard 96-well plate at 37°C for at least 2 hr with shaking.

   NOTE: If it is more convenient, this incubation step can proceed overnight.

3. Prepare a Master Mix for the clones you would like to amplify. To ensure that you have sufficient Master Mix for your experiment, calculate your volumes to include an extra reaction. Combine the following reagents in a clean microcentrifuge tube:

	per rxn
10X PCR reaction buffer	2.0 µl
Nested Primer 1*	0.6 µl
Nested Primer 2R*	0.6 µl
dNTP mix (10 mM)	0.4 µl
H_2O	15.2 µl
50X PCR enzyme mix	0.2 µl
Total volume	19.0 µl

   *If you removed the adaptor sequences from your cDNAs for site-specific or blunt-end cloning, the Nested Primers will not amplify the inserts. In this case, you will need to use PCR primers specific for the cloning vector.

4. Mix well by vortexing. Spin the tube briefly in a microcentrifuge.
5. Aliquot 19 µl of the Master Mix into each well of the reaction plate.
6. Transfer 1 µl of each bacterial culture (from Step 2, above) to each tube or well containing Master Mix.
7. Begin thermal cycling in a Perkin-Elmer GeneAmp PCR System 9600:
   - 94°C for 30 sec

   23 cycles:
   - 95°C for 10 sec*
   - 68°C for 3 min

   *If you are using a Perkin-Elmer DNA Thermal Cycler 480, the 95°C step should be increased to 30 sec.

8. Electrophorese 5 µl from each reaction on a 2.0% agarose/EtBr gel in 1X TAE buffer. Each PCR product should correspond to the cDNA insert.

Prepare cDNA dot blots of the PCR products:

9. For each PCR reaction, combine 5 µl of PCR product and 5 µl of 0.6 N NaOH (freshly made or at least freshly diluted from concentrated stock) in a 96-well plate. Include the PCR products from the Negative Hybridization Controls (from Section V). The NaOH will denature the DNA for hybridization.
10. Mix by briefly spinning the plate.
11. Using a micropipettor, transfer 1–2 µl of each mixture to a nylon membrane. Prepare four identical blots for hybridization with both subtracted and unsubtracted probes. For best results, array each cDNA in duplicate on each membrane.
12. Neutralize the blots for 2–4 min in 0.5 M Tris-HCl (pH 7.5) and wash in H_2O.
13. Cross-link the DNA to the membrane using a UV linking device (such as Stratagene's UV Stratalinker) under 120 mJ. Alternatively, bake the blots for 1–2 hours at 70°C in an oven.

B. Colony Array

1. Randomly pick 96 bacterial colonies. Include two colonies from each cloned Negative Hybridization Control cDNA (transformed into *E. coli* when you cloned your PCR product).
2. Grow each colony in 100 µl of LB-amp medium in a 96-well plate at 37°C for at least 2 hr with shaking (can go overnight).
3. Place each nylon membrane onto a LB/agar plate containing ampicillin or another appropriate selective antibiotic. Prepare four identical blots for hybridization with both subtracted and unsubtracted probes.
4. Transfer 1 µl of each bacterial culture onto each membrane. For easy transfer, we recommend the MULTI-PRINT applicator from V & P SCIENTIFIC. Alternatively, you can transfer bacterial culture using a multi-channel pipettor or a sterile pipette tip. In our experience, a single pipette tip dipped in a bacterial culture can be touched to up to four identical membranes. Be sure to include clones that correspond to the Negative Hybridization Control cDNAs. If you did not clone your PCR products from the Negative Hybridization Control cDNAs, you can apply the PCR products directly to the membrane; however, this will not provide an absolute control.
5. Incubate at 37°C overnight.
6. Denature colonies:
    a. Cut two pieces of Whatman 3MM Chr paper to the size of the membrane. Place each piece of paper in a separate glass or plastic tray. If you are using thinner Whatman paper, stack several pieces in each tray.
    b. Saturate the Whatman paper in the first tray with denaturing solution (0.5 M NaOH, 1.5 M NaCl; should be freshly made or at least freshly diluted from concentrated stock). Saturate the Whatman paper in the second tray with neutralizing solution (0.5 M Tris-HCl [pH 7.4], 1.5 M NaCl). Pour off any excess liquid from the trays.
    c. Set a third piece of Whatman paper (or a stack of thinner paper) on the bench next to the two trays; this paper does not need to be the exact size of the membrane.

d. Using blunt-ended forceps, place a membrane colony-side-up on the Whatman paper in the first tray (saturated with denaturing solution). Let sit for 4 min.

e. Transfer the membrane colony-side-up to the Whatman paper in the next tray (saturated by neutralizing solution). Let sit for 4 min.

f. Place the membrane colony-side-up on the dry Whatman paper from step c, above.

g. Repeat steps c–f for each additional membrane. Allow membranes to air dry for at least 30 min.

h. Sandwich each membrane between two sheets of dry Whatman paper. Fix the DNA to the filters by baking for 1–2 hours at 80°C in an oven.

VIII. Random Primer Labeling of cDNA Probes

In this section, you will prepare your probes for hybridization to the subtracted cDNA library (arrayed in Section VII).

1. In separate 0.5-ml microcentrifuge tubes, mix 3 µl (20–90 ng) of each of your forward- and reverse-subtracted cDNA (prepared in Section V) with 6 µl of H_2O.

   To label unsubtracted cDNA probes, use 50–100 ng of each ss or ds tester and driver cDNA. Alternatively, use 3 µl of the Unsubtracted tester control cDNAs if you amplified them in Section V. Adjust the volume to 9 µl with H_2O.

2. Denature by heating for 8 min at 95°C and then chill on ice.

3. Add the following to each tube from Step 2:

	volume
Reaction Buffer (–dCTP)*	3 µl
Random Primer Mix	2 µl
[α-^{32}P]dCTP* (50 µCi, 3000Ci/mmol, aqueous solution)	5 µl
Klenow Enzyme (exo-)	1 µl

   *If you are using [α-^{32}P]dATP for labeling, use the Reaction Buffer (–dATP).

4. Incubate tubes at 37°C for 30 min.

5. Terminate each reaction by adding 5 µl of Stop Solution.

6. Purify probe from unincorporated dNTPs using CHROMA SPIN-100 (Cat. Nos. 636072, 636073) or equivalent size exclusion chromatography columns.

7. Determine the specific activity of each probe. For example, use a QUICK-COUNT Benchtop Radioisotope Counter. Alternatively, perform a TCA precipitation (Sambrook *et al.*, 2001) and use a scintillation counter. You should obtain >10^7 cpm per probe.

IX. Hybridization with the Subtracted cDNA

In this section, you will hybridize your ^{32}P-labeled probes to the subtracted clones arrayed on nylon membranes.

For best results, we strongly recommend ExpressHyb Hybridization Solution (Cat. Nos. 636831, 636832). Alternatively, consult Sambrook *et al.* (2001) for a recipe to make your own hybridization solution. *If you use a hybridization solution that contains formamide, you must reduce the hybridization temperature to 42°C.*

Continuous agitation of the membranes is necessary during all prehybridization, hybridization, and washing steps. We usually use a hybridization incubator with rotating bottles. Alternatively, these steps may be performed in a sealed plastic bag or small plastic container with a tight-fitting lid, placed in a shaking incubator.

1. Make hybridization solution for each membrane prepared in Section VII:
    a. Combine 50 µl of 20 X SSC and 50 µl of Blocking Solution. Mix well.
    b. Boil for 5 min, then chill on ice.
    c. Combine with 5 ml of hybridization solution.
2. Place each membrane in a hybridization container and add the hybridization solution prepared in Step 1. Prehybridize for 40–60 min with continuous agitation at 72°C.
3. Prepare your hybridization probe:
    a. Mix 50 µl of 20X SSC, 50 µl of Blocking Solution, and your purified probe (at least 10^7 cpm per 100 ng of subtracted cDNA). Be sure to add the same number of cpm for each pair of probes.
    b. Boil for 5 min, then chill on ice.
4. To each hybridization container, add the mixture prepared in Step 3. Avoid adding the probe directly to the membrane.
5. Hybridize at 72°C overnight with continuous agitation.
6. Warm low-stringency (2X SSC/0.5% SDS) and high-stringency (0.2X SSC/0.5% SDS) washing solutions to 68°C. Keep buffers at 68°C during washing.
7. Wash membranes with low-stringency washing solution (4 x 20 min) at 68°C.
8. Wash membranes with high-stringency washing solution (2 x 20 min) at 68°C.
9. Expose the membrane to x-ray film overnight with an intensifying screen at –70°C. We recommend Kodak BioMax MR film. If the intensities of your hybridization signals vary considerably, try exposing the membranes to x-ray film for varying lengths of time (e.g., 3 hr, 6 hr, overnight, and 3 days), or use a phosphorimager. If the intensity of the signal is low, use a phosphorimager, or try Kodak BioMax MS film (with the corresponding intensifying screen). MS film is extremely sensitive but gives a higher background than MR film.
10. To reuse the membranes, remove the probes by stripping (100°C, 7 min in 0.5% SDS). Blots can typically be probed at least 5 times.

X. Analysis of Results

A. Interpretation of Hybridization Results

To best interpret the results of your differential screening experiment, try exposing the membranes to x-ray film for varying lengths of time (e.g., 3 hr, 6 hr, overnight, and 3 days). Alternatively, use a phosphorimager.

Table 1 (next page) provides guidelines for interpreting different combinations of hybridization for each of the four probes. Figure 4 below shows the same differential screening experiment presented in Figure 1 in the Introduction. Specific clones are highlighted in the array data that illustrate the hybridization combinations discussed in Table 1.

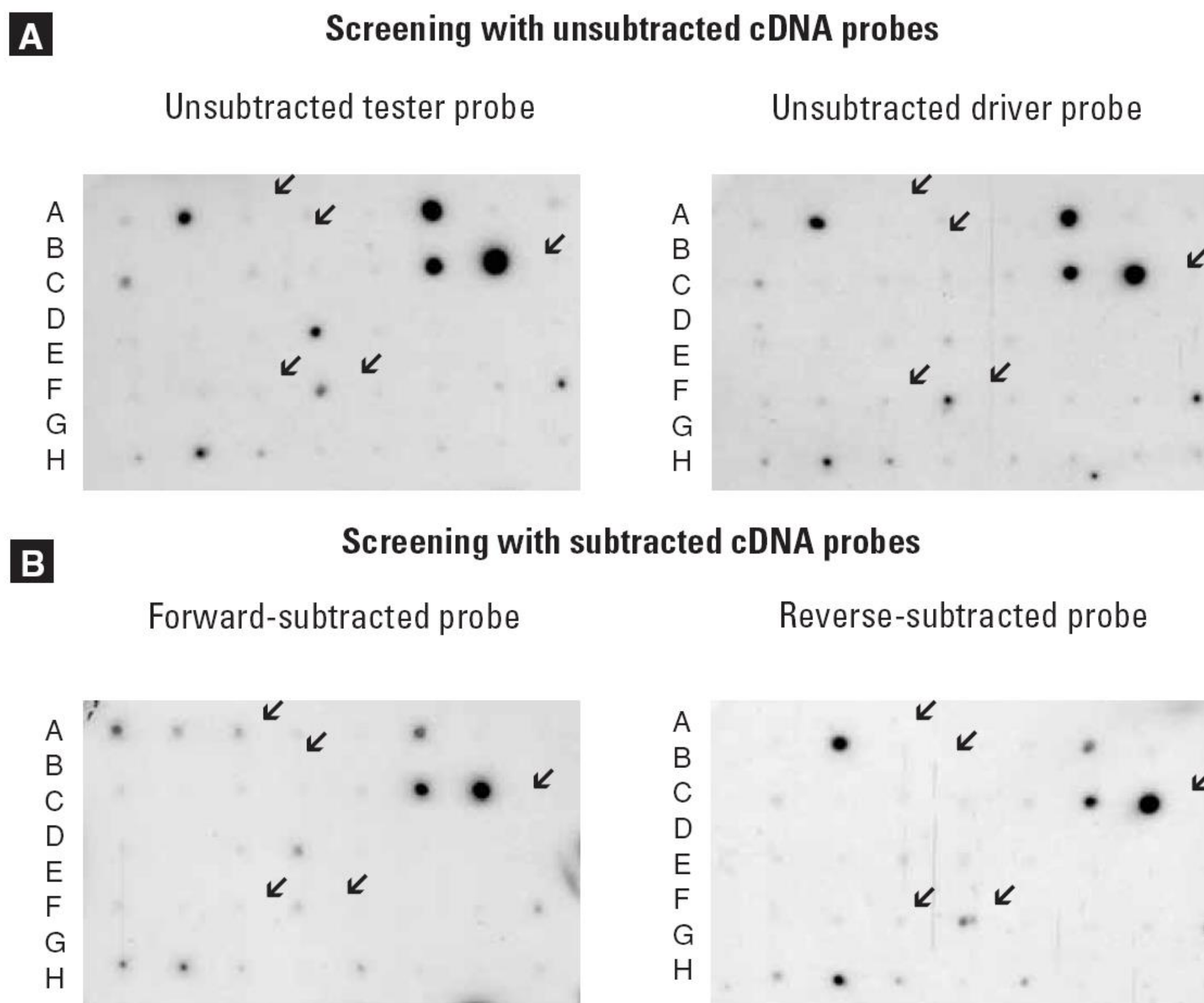

Figure 4. Sample differential screening results. Panel A. Dot blots hybridized with unsubtracted cDNA probes made from tester (γ-line) and driver (β-line) RNA. **Panel B.** Dot blots hybridized with cDNA probes made from forward-subtracted cDNA (γ-line tester, β-line driver) and reverse-subtracted cDNA (β-line tester, γ-line driver). On each membrane, the following clones correspond to controls. H10: γ-globin cDNA fragment (positive control); H11: Negative Hybridization Control (cDNA 1R); H12: Negative Hybridization Control (cDNA 2R).

Table 1. Interpretation of Differential Screening Results

Type of Probe				
Forward-subtr.	**Reverse-subtr.**	**Unsubtr. tester**	**Unsubtr. driver**	**Interpretation**
+	–	+	–	Clones that hybridize to the forward-subtracted and unsubtracted tester probes but not to the reverse-subtracted or unsubtracted driver probes almost always (95% probability) correspond to differentially expressed genes. (Clone F7)
+	–	–	–	Clones that hybridize only to the forward-subtracted probe are strong candidates for differential expression. (Clone F5) These clones typically correspond to low-abundance transcripts which were enriched during the subtraction. In most cases enrichment is a result of differential expression, but it can occasionally be an artifact.
+	–	+	+	Clones that hybridize to the forward-subtracted probe and both unsubtracted probes but not the reverse-subtracted probe can be difficult to interpret. Many of these cDNAs are only artifactually enriched. In some cases, Northern analysis shows that the clone hybridizes to both the tester and driver RNA, but either (1) the transcripts have different sizes and are probably alternatively spliced forms, or (2) several different transcripts are identified with one or more bands specific to the tester RNA. The latter is often the case for genes that are members of multi-gene families.
++	+	+	+ or –	Clones that hybridize to both subtracted probes, but with different intensities. When the difference in signal intensity is >5-fold (e.g., C12), a clone probably corresponds to a differentially expressed gene. However, when the difference in signal intensity is <3-fold (e.g., C9), the explanation is most likely a random fluctuation in the efficiency of the forward and reverse subtractions. Generally, you can use the results of the unsubtracted probes to corroborate or disprove the subtracted probe results.
+	+	+	+	Clones that hybridize equally to both subtracted probes and to both unsubtracted probes are almost never differentially expressed. (Clone B6)
–	–	–	–	Clones that do not have a detectable hybridization signal for either of the subtracted probes usually represent non-differentially expressed cDNAs. Some non-differentially expressed cDNA fragments are randomly present in the subtracted library as single copies. Because these molecules are present at such low levels in the subtracted probe, they do not hybridize to the corresponding clone. Clone A5)

+ = Signal is observed (++ = strong signal)

– = No signal is observed

B. Confirmation of Differential Screening Results

In our hands, approximately 1–2% of the clones identified by differential screening with subtracted probes turn out to be false positives. Thus, if only 5% of the clones in your subtracted library showed a differential signal by differential screening, half of your putative clones may not be confirmed.

There are several ways to confirm differential screening results:

1. If you have enough poly A^+ RNA, Northern blot analysis is the most direct way to confirm differential gene expression.

   Drawback: Each Northern blot requires at least 2 µg of poly A^+ RNA.

2. If you lack sufficient poly A^+ RNA for standard Northern blot analysis, you can create Virtual Northern blots, which yield similar information. To make a Virtual Northern blot, use the SMART PCR cDNA Synthesis Kit (Cat. No. 634902) to generate cDNA from your total or poly A^+ RNA sample. Then, electrophorese the SMART cDNA on an agarose/EtBr gel, denature it *in situ*, and transfer it onto a nylon membrane. Figure 2 (in the Introduction) shows several examples of Virtual Northern blots. For more information on SMART cDNA synthesis technology and Virtual Northern blots, see Diatchenko *et al*., 1998, Chenchik *et al.*, 1998, and Endege *et al*., 1999.

   Drawback: Not all the mRNA appears on the blot as a single band; sometimes several bands are present (especially for long mRNAs). Even so, a differential signal will be clearly detectable.

3. Another alternative to Northern blot analysis is to use a poly A^+ RNA dot blot. Since the sensitivity of dot blot analysis is about 10-fold higher then Northern blot analysis (Nucleic Acid Hybridization, 1985), only 200 ng of poly A^+ RNA is required for each dot.

   Drawback: Dot blots provide no information about differential expression of alternatively spliced transcripts and transcripts belonging to multi-gene families (see Table 1, above).

4. RT-PCR analysis is extremely sensitive, but is merely semiquantitative. You can sequence your candidate clones and design specific PCR primers. Using your initial RNA sample as a template, set up RT-PCRs and run 22 thermal cycles; then, compare the products on an agarose/EtBr gel. If you cannot see PCR products after 22 cycles, return the PCR tubes to the thermal cycler and run five more cycles; check again by agarose/EtBr gel electrophoresis. Be sure to keep your reactions within the linear phase of amplification.

   Drawbacks: (1) Suitable primers must be designed. (2) Obtaining quantitative data is challenging because these types of PCR reactions are often over-cycled into the plateau phase of amplification, eliminating differences in samples.

XI. Troubleshooting Guide

A. Agarose Gel Analysis of Secondary PCR Products (Section V)

The secondary PCR products from your subtracted cDNA should appear on an agarose/EtBr gel as a smear with or without a number of distinct bands. Amplification of Negative Hybridization Control cDNA 1R by the Nested Primers should yield a 348-bp cDNA fragment. Amplification of Negative Hybridization Control cDNA 2R by the Nested Primers should yield a 182-bp cDNA fragment. Occasionally, you may observe a minor band of ~4 kb that corresponds to the vector.

1. If you did not observe 348- and 182-bp products after amplification of the Negative Hybridization Controls:

   a. You may have made an error during the procedure, such as using the wrong cycling conditions or omitting an essential component. Carefully check the protocol and repeat the PCR.

   b. Make sure that your thermostable DNA polymerase is working using a positive control. We recommend our Advantage 2 Polymerase Mix (Cat. No. 639201), also available in the Advantage 2 PCR Kit (Cat. Nos. 639206, 639207).

   c. If you are using a thermal cycler other than the Perkin-Elmer DNA Thermal Cycler 480 or Perkin-Elmer GeneAmp Systems 2400/9600, you may need to optimize the PCR conditions for your machine.

2. If amplification of the Negative Hybridization Controls yielded 348- and 182-bp products, but you did not observe a smear for your subtracted cDNA after 11 cycles:

   You may need to use more PCR cycles. Return the tube containing your subtracted cDNA to the thermal cycler and run three more cycles. Analyze the PCR product on a 2% agarose/EtBr gel. Remember that too many cycles may result in high background. If you still cannot see a PCR product from the subtracted cDNA, even after additional cycles, you should determine whether there is a problem with your thermostable DNA polymerase or thermal cycler.

B. Subtracted cDNA Library Construction (Section VI)

If the efficiency of cloning is too low:

1. For troubleshooting T/A cloning, please refer to the protocol accompanying your T/A cloning kit. For this application, it is important to optimize your cloning efficiency; a low cloning efficiency will result in a high background. One way to increase the efficiency of transformation is to electroporate into electrocompetent DH5α E. coli (#C2022-1). Using T/A cloning and electroporation, we typically obtain 104 independent clones from 1 µl of secondary PCR product.

2. For blunt-end or site-specific cloning, consult Sambrook *et al.* (2001). If you used a kit, consult the manufacturer's instructions.

C. cDNA Insert Amplification (Section VII.A)

Upon agarose/EtBr gel analysis of your PCR products:

1. If some of your tubes do not contain any PCR product, the corresponding clones probably do not contain inserts. There are usually some clones present that lack inserts; however, if more than 50% of your clones lack inserts, we recommend that you repeat the cloning procedure. For troubleshooting T/A cloning, please refer to the protocol accompanying your T/A cloning kit. For blunt-end or site-specific cloning, consult Sambrook *et al.* (2001). If you used a kit, consult the manufacturer's instructions.
2. If bands are present but faint, return the reaction tube to the thermal cycler for four more cycles (same parameters as for the previous 24 cycles).
3. If you do not observe any PCR products, repeat the PCR using the Negative Hybridization Controls as templates.
    a. If you do not observe a band corresponding to either control cDNA, you may have made an error during the procedure, such as using the wrong cycling conditions or omitting an essential component. Carefully check the protocol and repeat the PCR.
    b. If bands are present for the control cDNAs, but not for your inserts:
        i. Your cloning procedure failed. For troubleshooting T/A cloning, please refer to the protocol accompanying your T/A cloning kit. For blunt-end or site-specific cloning, consult Sambrook *et al.* (2001). If you used a kit, consult the manufacturer's instructions.
        ii. You used the wrong primers. Remember: if you removed the adaptors, the Nested Primers will not work. In this case, repeat the PCR using vector-specific primers.

D. Random Primer Labeling of cDNA Probes (Section VIII.B)

If you obtain very low incorporation of [α-^{32}P]dCTP or [α-^{32}P]dATP:

1. Your [α-^{32}P]dCTP or [α-^{32}P]dATP may be too old (>two weeks).
2. You may have lost your DNA during purification. Check the quantity of DNA in your sample by spectrophotometry. The concentration should be 8–30 ng/µl. If you have less DNA, adjust the volume of the cDNA used in the labeling procedure and concentrate your cDNA by ethanol precipitation.

E. Hybridization with the Subtracted cDNA (Section IX)

1. High background

   One of the problems often encountered in differential screening is high background. High background may be caused by the high complexity of cDNA probes and the presence of adaptor sequences in the arrayed clones and subtracted probes. Hybridizations that have acceptable levels of background meet the following two criteria: (1) on the array membrane, cDNA fragments or colonies corresponding to the Negative Hybridization Controls should not hybridize to the cDNA probes; and (2) many of your clones should not hybridize to either the forward- or reverse-subtracted cDNA probes.

   a. General considerations:

      i. Using high temperatures for hybridization (72°C) and washing (68°C) is important. Do not be afraid that these temperatures will wash out your hybridization signal—unless you are using a hybridization solution that contains formamide. If your hybridization solution contains formamide, do not exceed 42°C for the hybridization.

      ii. Do not add hybridization probes directly to the membranes.

      iii. Keep your washing solutions at 68°C throughout the washing procedure.

      iv. When preparing cDNA arrays, it is very important to grow colonies in LB medium before amplification.

   b. High background sometimes results from the presence of adaptor sequences in the probe. To eliminate this type of background, high concentrations of the adaptor sequences are included in the Blocking Solution. However, the Blocking Solution is occasionally not sufficient to overcome hybridization to the adaptor sequences. In these instances, the adaptors can be removed by digesting cDNAs with Rsa I, Eag I, and Sma I restriction enzymes prior to probe labeling.

2. Hybridization signal absent

   a. Use at least 5 x 10^6 cpm of probe per ml of hybridization mixture.

   b. If your purified labeled cDNA probe is seriously contaminated by unincorporated [α-^{32}P]dCTP or [α-^{32}P]dATP, you may not have realized that your probe was not labeled. Use CHROMA SPIN-100 (Cat. Nos. 636072, 636073) or equivalent size-exclusion chromatography columns after the labeling reaction.

   c. Check the concentration of your purified cDNA (from Section VIII.A). If the concentration of your cDNA is too high, the probe specific activity will be too low. Use less cDNA for labeling.

XII. Differential Screening and Bacterial Genome Subtraction

For researchers who are using the PCR-Select Bacterial Genome Subtraction Kit (Cat. No. 637404), Figure 5 shows the experimental set-up.

A. Southern Blot Analysis

If you lack sufficient genomic DNA for standard Southern blot analysis, you can use amplified genomic DNA from the unsubtracted controls. In this case, you should generate unsubtracted controls for both tester and driver. Electrophorese the amplified tester and driver DNAs on an agarose/EtBr gel, denature in situ, and transfer onto a nylon membrane. When performing the hybridization, be sure to use the Blocking Solution as described in Section IX.

B. Differential Screening

Unlike eukaryotic PCR-Select cDNA libraries, prokaryotic libraries do not require using subtracted probes for differential screening. This is because bacterial genomes are less complex (only 1–5 x 10^6 bp) than eukaryotic mRNA populations. In addition, bacterial genomes generally contain only one copy of each gene, so rare genes will not be missed.

We recommend an approach by which the subtracted library is hybridized with the original tester and driver genomic DNAs. If you have enough of each genomic DNA sample to make hybridization probes (~100 ng), you may array DNA inserts from the subtracted library on a nylon membrane and hybridize with ^{32}P-labeled tester and driver probes (e.g., Figure 6). Clones representing the DNA fragments that are truly tester specific will hybridize only with the tester probe; clones that hybridize with both probes may be considered background.

NOTE: The hybridization temperature and washing conditions may depend on the GC content of your particular bacterial genomic DNA.

If you lack sufficient genomic DNA for differential screening, you can use the amplified Unsubtracted controls for both tester and driver instead of the initial restriction enzyme-digested genomic DNA. In this case, you should generate unsubtracted controls for both tester and driver.

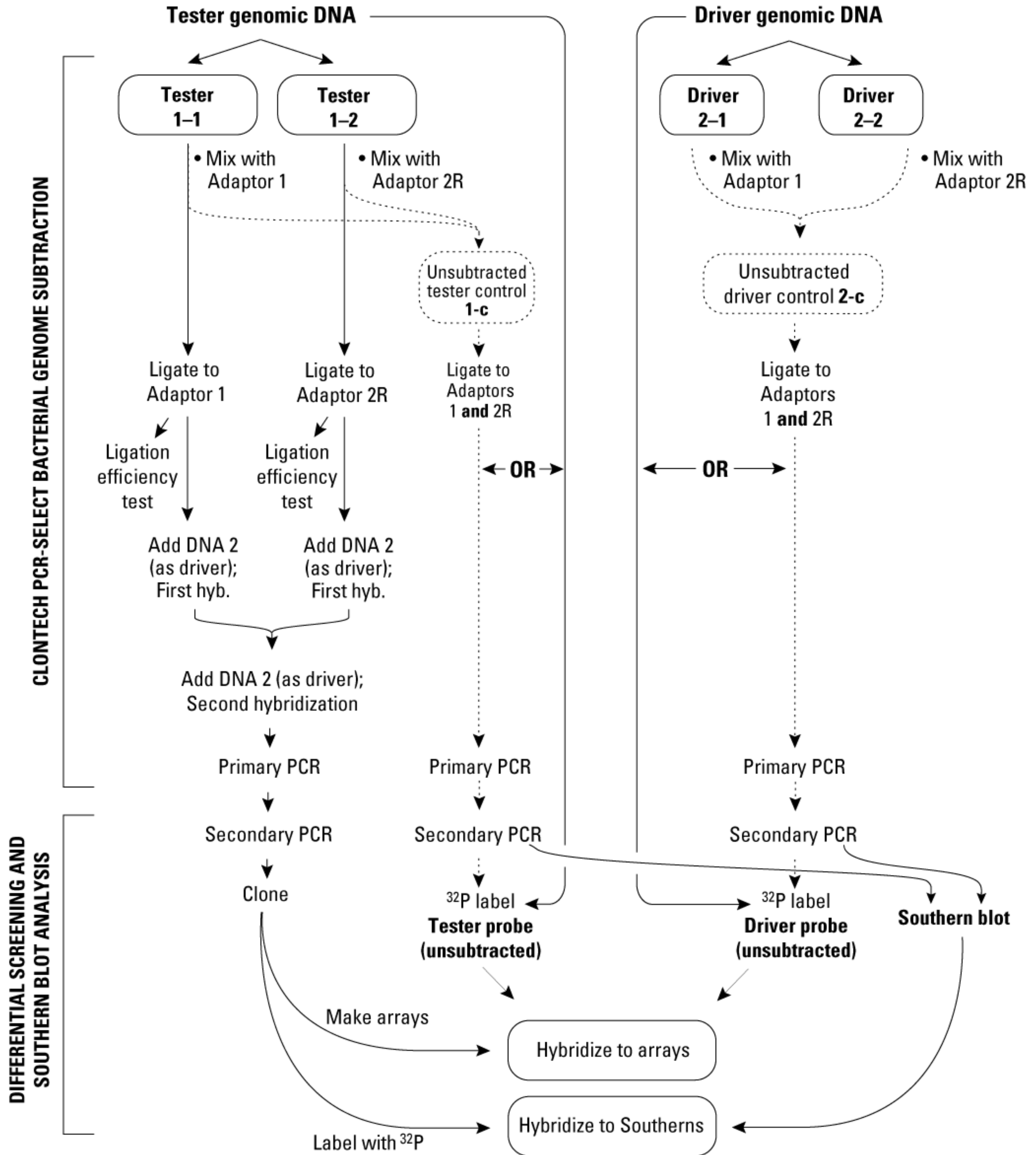

Figure 5. Experimental set-up for PCR-Select bacterial genome subtraction and differential screening. If you have sufficient digested tester and driver genomic DNA, you can use them directly in probe synthesis instead of amplifying the Unsubtracted controls.

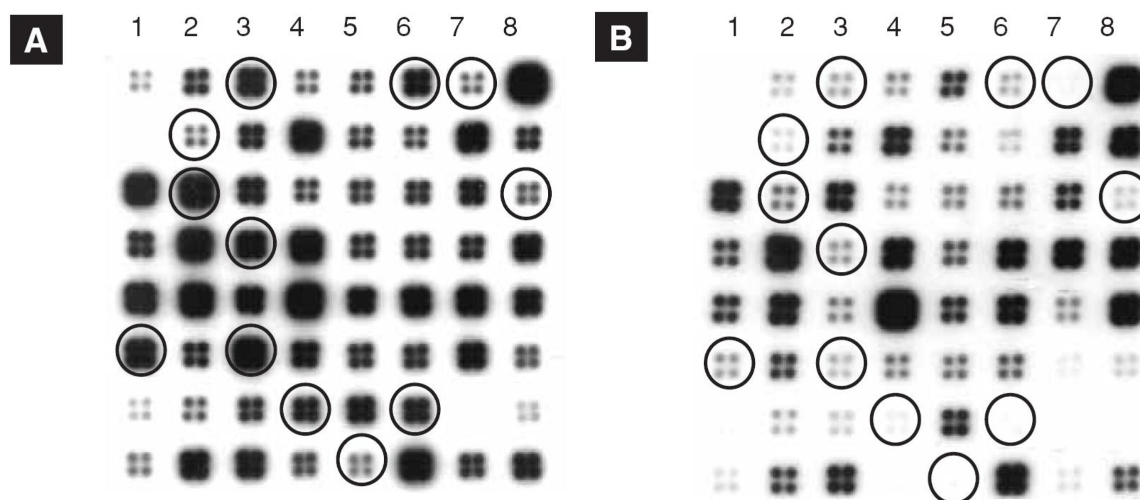

Figure 6. Differential screening of a subtracted *Helicobacter pylori* DNA library. PCR-Select subtraction was performed using *H. pylori* strain J166 as tester and strain 26695 as driver. The subtracted DNA was cloned using T/A cloning. Eighty-four clones were randomly picked; the inserts were amplified and arrayed in quadruplets on nylon membranes. The membranes were then screened by hybridization with genomic DNA from tester (**Panel A**) and driver (**Panel B**). 500 ng of each genomic DNA sample was labeled by random priming. Membranes were prehybridized in solution containing 6X SSC, 5X Denhardt's solution, 1% SDS, and 100 µg/ml calf thymus DNA at 65°C for 15 min. The membranes were hybridized with the probes overnight in the same solution. Two 15-min washes were performed in a solution containing 2X SSC, 0.5% SDS. Circles indicate tester-specific clones. (Data were kindly provided by Natalia S. Akopyants and Dr. Douglas Berg, Washington University School of Medicine, St. Louis, MO.)

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Appendix: Adaptor and Primer Sequences

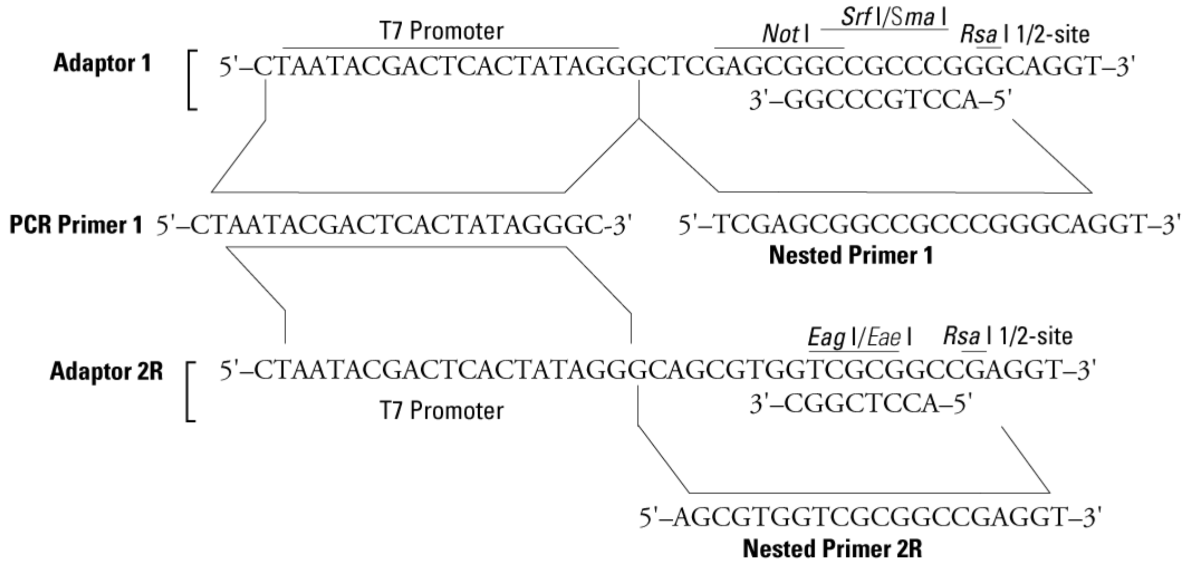

Figure 7. Sequences of the adaptors and PCR primers.

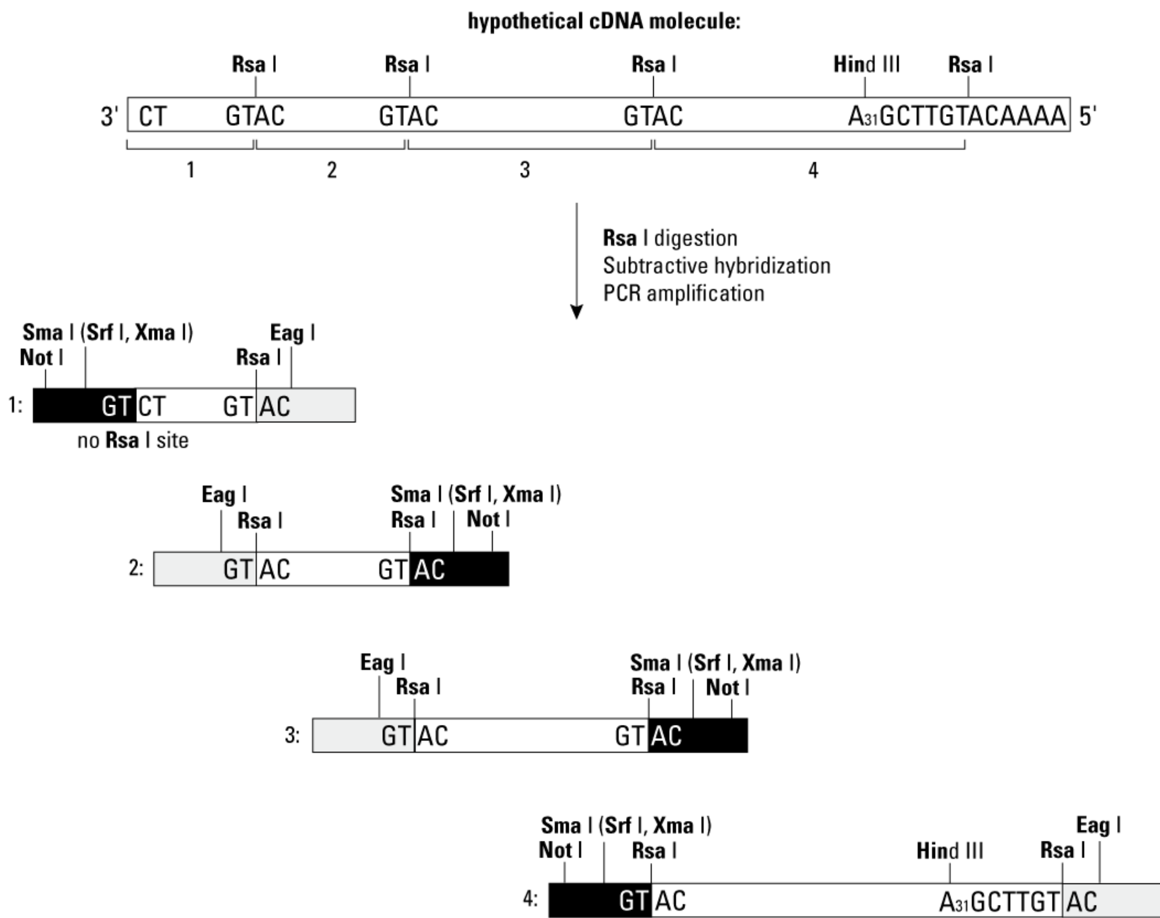

Figure 8. Hypothetical forms of a subtracted cDNA. Solid boxes represent the Nester Primer 1 sequence; shaded boxes represent the Nested Primer 2R sequence. Note that the distribution of the Nested Primer sequences on the ends of the molecules is random.

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