Clontech Laboratories, Inc.

Clontech® PCR-Select™ Bacterial Genome Subtraction Kit User Manual

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Table of Contents

List of Figures

List of Tables

I. Introduction

A. Summary

Subtractive hybridization is a powerful technique that has been applied to research in many different fields. In studies of eukaryotic systems, the application of subtraction techniques typically focuses on differential gene expression—differences between two mRNA populations that have been converted into cDNA—rather than differences between genomes. This is because eukaryotic genomes are too complex for existing subtraction technologies. In contrast, bacterial genomes are considerably smaller, and are even less complex than many eukaryotic mRNA populations. Thus, subtraction methods can be used to identify sequences that are present in one bacterial genome, but are absent in another.

Although there are several different methods, the basic theory behind subtraction is simple. The genomic DNA sample that contains the sequences of interest is referred to as **tester** and the reference sample is referred to as **driver**. Tester and driver DNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized DNAs represent genes that are expressed in the tester yet absent from the driver DNA.

The **Clontech PCR-Select Bacterial Genome Subtraction Kit** (Cat. No. 673404) is based on a unique method of selective amplification of differentially expressed sequences which overcomes these and other technical limitations of traditional subtraction procedures. Like the **Clontech PCR-Select cDNA Subtraction Kit** (Cat. No. 637401), this kit is based on the suppression subtractive hybridization (SSH) method (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996). Figure 1 presents a brief overview of the PCR-Select procedure.

B. Molecular Basis of PCR-Select Bacterial Genome Subtraction

The following molecular events (Figure 1) occur during PCR-Select Bacterial Genome Subtraction:

1. **Bacterial genomic DNA isolation (Section IV.B)**
   The tester and driver genomic DNAs are isolated from the two strains of bacteria being compared.
2. **Restriction digestion to make genomic DNA ends blunt (Section IV.C)**
   The tester and driver genomic DNAs are each digested with an appropriate four-base-cutting restriction enzyme such as Rsa I (supplied with this kit) to yield shorter, blunt-ended molecules.
3. **Adaptor ligation (Section IV.D)**
   The tester DNA is then subdivided into two portions, and each is ligated with a different adaptor. The ends of the adaptors are unphosphorylated, so only one strand of each adaptor attaches to the 5’ ends of the DNA. The two adaptors have stretches of identical sequence, which allows annealing of the same PCR primer to both ends once the recessed ends have been filled in. (See Appendix B for detailed adaptor and primer sequences.)

4. **First hybridization (Section IV.E)**
   The first of two successive hybridizations is performed in order to equalize and enrich differentially expressed sequences:

   - An excess of driver is added to each adaptor-ligated tester sample. The samples are then heat denatured and allowed to anneal, generating the type **a**, **b**, **c**, and **d** molecules in each sample (Figure 1).
   - The single-stranded (ss) type **a** molecules are significantly enriched for tester-specific sequences, as DNA fragments that are not tester-specific form type c molecules with the driver. The concentrations of high- and low-abundance sequences are equalized among the type **a** molecules because reannealing is faster for the more abundant molecules due to the second-order kinetics of hybridization (see Nucleic Acid Hybridization, ed. by James & Higgins). At the same time, type **a** molecules are significantly enriched for differentially expressed sequences while DNAs that are not differentially expressed form type **c** molecules with the driver.

5. **Second hybridization (Section IV.F)**
   The two primary hybridization samples are mixed together without denaturing in order to generate PCR templates from differentially expressed sequences:

   - Only the remaining equalized and subtracted ss tester DNAs can reassociate and form new type **e** hybrids. These new hybrids are double-stranded (ds) tester molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R.
   - Fresh denatured driver DNA is added (again, without denaturing the subtraction mix) to further enrich fraction **e** for differentially expressed sequences. After filling in the ends by DNA polymerase, the type **e** molecules—the fraction that is greatly enriched for tester-specific sequences—have different primer annealing sites on their 5' and 3' ends.

6. **First PCR amplification (Section IV.G)**
   The entire population of molecules is then subjected to PCR to amplify the desired tester-specific sequences.

   - During this PCR, type **a** and **d** molecules are missing primer annealing sites, and thus cannot be amplified.
   - Due to the suppression PCR effect, most type **b** molecules form a panhandle-like structure that prevents their exponential amplification (see Appendix A for more details.)
   - Type **c** molecules have only one primer annealing site and amplify linearly.
   - **Only type e molecules**—the equalized, differentially expressed sequences with two different adaptors—**amplify exponentially**.

7. **Second PCR amplification (Section IV.G)**
   A secondary PCR amplification is performed using nested primers to further reduce any background PCR products and enrich for tester-specific sequences.

8. **Clone and confirm differentially expressed genes**
   The DNAs can then be cloned, allowing tester-specific DNA fragments to be identified by sequence and hybridization analysis. The **GenomeWalker™ Universal Kit** (Cat. No. 638904) is ideal for analyzing flanking genomic DNA sequences.

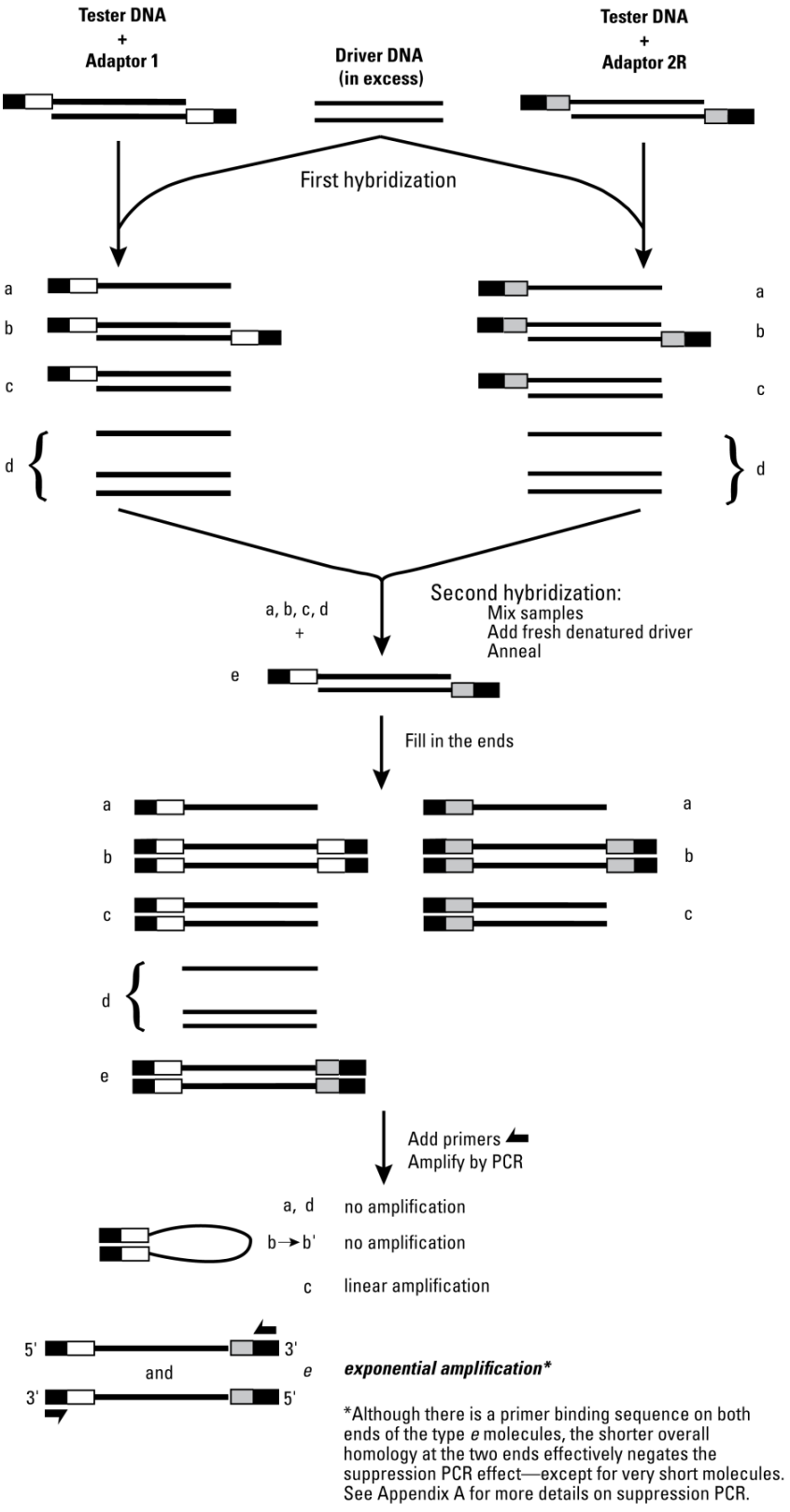

Figure 1. Schematic diagram of PCR-Select bacterial genome subtraction. Type e molecules are formed only if the sequence is present in the tester DNA, but absent in the driver DNA. Solid lines represent the Rsa I-digested DNAs. Solid boxes represent the outer part of the Adaptor 1 and 2R overhanging strands and corresponding PCR Primer 1 sequence. Clear boxes represent the inner part of Adaptor 1 and the corresponding Nested Primer 1 sequence. Shaded boxes represent the inner part of Adaptor 2R and the corresponding Nested Primer 2R sequence.

II. List of Components

Store all reagents at –20°C.

This kit includes enough reagents for one control and six complete subtractions (50 primary and 100 secondary PCRs). Refer to Appendix B for detailed adaptor and primer sequences.

Endonuclease Digestion

- 300 µl 10X Rsa I Restriction Buffer

  100 mM Bis Tris Propane-HCl (pH 7.0)
  100 mM $MgCl_2$
  1 mM DTT

- 12 µl Rsa I (10 units/µl)

Adaptor Ligation

- 21 µl T4 DNA Ligase (400 units/µl; contains 3 mM ATP)
- 200 µl 5X DNA Ligation Buffer

  250 mM Tris-HCl (pH 7.8)
  50 mM $MgCl_2$
  10 mM DTT
  0.25 mM BSA

- 30 µl Adaptor 1 (10 µM)
- 30 µl Adaptor 2R (10 µM)

Hybridization

- 200 µl 4X Hybridization Buffer

  200 mM HEPES-HCl (pH 8.0)
  2 mM NaCl
  0.8 mM EDTA (pH 8.0)

- 1.4 ml Dilution Buffer

  20 mM HEPES (pH 8.3)
  50 mM NaCl
  0.2 mM EDTA (pH 8.0)

PCR Amplification

- 50 µl PCR Primer 1 (10 µM)
- 100 µl Nested PCR Primer 1 (10 µM)
- 100 µl Nested PCR Primer 2R (10 µM)
- 10 µl PCR Control Subtracted Genomic DNA

Control Reagents

- 5 µl *E. coli* Genomic DNA (1 mg/ml)
- 10 µl Control DNA (3 ng/µl; Hae III-digested φX174 DNA)
- 50 µl 23S RNA Forward Primer (10 µM)*
- 50 µl 23S RNA Reverse Primer (10 µM)*

* These primers will amplify a 374 bp fragment of the *E. coli* 23S rRNA gene.

III. Additional Materials Required

The following reagents are required but not supplied.

- **Hae III digest of bacteriophage φX174**
  We recommend Takara Bio DNA size markers (Cat. Nos. 3405A & 3405B)
- **0.5 ml PCR reaction tubes**
- **80% ethanol & 95% ethanol**
- **Phenol:chloroform:isoamyl alcohol (25:24:1)**
- **Chloroform:isoamyl alcohol (24:1)**
- **Advantage® 2 Polymerase Mix (Cat. No. 639201); also provided in the Advantage 2 PCR Kits [Cat. Nos. 639206 & 639207]).**
- **10X PCR buffer**
  Use the 10X reaction buffer supplied with your DNA polymerase or mix (included with the Advantage 2 Polymerase Mix [Cat. No. 639201] and the Advantage 2 PCR Kit [Cat. Nos. 639206 & 639207]).
- **dNTP Mix for PCR** (10 mM each dATP, dCTP, dGTP, dTTP)
- **50X TAE electrophoresis buffer**

242 g	Tris base
57.1 ml	Glacial acetic acid
37.2 g	$Na_2EDTA•H_2O$

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

IV. PCR-Select Bacterial Genome Subtraction Protocols

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Considerations

- A hot start **MUST** be used to reduce nonspecific DNA synthesis during the setup of the PCR. We recommend using either TaqStart® Antibody (Kellogg *et al.*, 1994) or manual hot start (D'Aquila *et al.*, 1991). This protocol was optimized using our TaqStart Antibody (individually available as Cat. Nos. 639250 & 639251; also included in our Advantage 2 Polymerase Mix [Cat. No. 639201]).

B. Bacterial Genomic DNA Isolation

Most commonly used methods for bacterial genomic DNA isolation are appropriate for use with the Clontech PCR-Select Bacterial Genome Subtraction Kit. Nevertheless, the quality of genomic DNA is very important for successful subtraction. For best results, we recommend the protocol described by Ausubel *et al.* (2003). Alternatively, you may use any commercially available isolation kit.

C. Protocol: Restriction Enzyme Digestion

Perform the following procedure with each experimental tester and driver genomic DNA, as well as with the control *E. coli* Genomic DNA. This step generates shorter, blunt-ended DNA fragments which are optimal for subtraction and required for adaptor ligation in Section IV.D.

1. Add the following reagents into a 0.5 ml microcentrifuge tube:

Genomic DNA (2 µg)	x µl
10X Rsa I Restriction Buffer	5.0 µl
Rsa I (10 units/µl)	1.5 µl
Deionized H_2O	y µl
Total volume	50 µl

2. Mix by vortexing and briefly centrifuge.
3. Incubate at 37°C for 5–16 hr.
4. Set aside 5 µl of the digest mixture to analyze the efficiency of Rsa I digestion as described in Section V.A.
5. Add 2.5 µl of of 0.2 M EDTA to terminate the reaction.
6. Add 50 µl of phenol:chloroform:isoamyl alcohol (25:24:1).
7. Vortex thoroughly and centrifuge at 14,000 rpm for 10 min at room temperature to separate phases.
8. Carefully collect the top aqueous layer and place in a fresh 0.5 ml tube.
9. Add 50 µl of chloroform:isoamyl alcohol (24:1).
10. Repeat steps 7 and 8.
11. Add 0.5 volume of 4 M NH_4OAc and 2.5 volumes (of the total resulting volume) of 95% ethanol.
12. Repeat Step 7.
13. Centrifuge for 20 min at 14,000 rpm at room temperature.
14. Remove the supernatant.
15. Gently overlay the pellets with 200 µl of 80% ethanol.
16. Centrifuge at 14,000 rpm for 5 min.
17. Carefully remove the supernatant.
18. Air-dry the pellets for 5–10 min.
19. Dissolve the pellet in 6.5 µl of H_2O and store at –20°C.
    The final concentration of driver DNA should be ~300 ng/µl.

    These 6.5 µl samples of Rsa I-digested DNA will serve as your **experimental and control driver DNAs**. Proceed to Section IV.D to finish preparing your **experimental and control tester DNAs**.

D. Protocol: Adaptor Ligation

Figure 2 shows the experimental flowchart for preparing tester genomic DNA (Panel A). You should also perform a control subtraction (Panel B).

General Considerations

- **Experimental subtraction** (Figure 2, Panel A) is designed to enrich for differentially expressed sequences present in genomic DNA sample 1, but not genomic DNA sample 2—producing a subtracted DNA population with sequences specific to sample 1.
- **Control subtraction** (Figure 2, Panel B) should also be performed. In Step 2 (below), you will prepare tester DNA for this control subtraction by mixing the Rsa I-digested control *E. coli* genomic DNA with the Control DNA (φX174/Hae III digest).

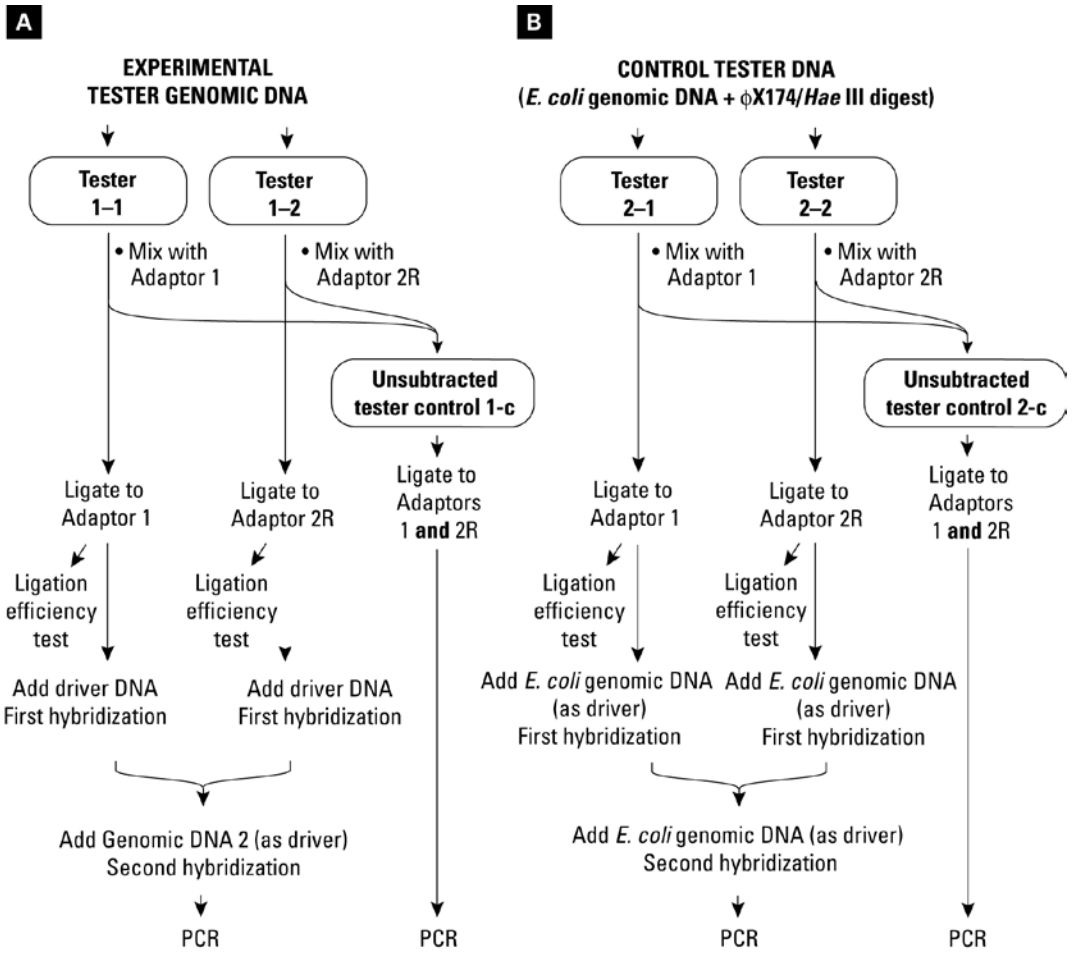

Figure 2. Preparing adaptor-ligated tester DNAs for hybridization and PCR. Each tester DNA (i.e., each different experimental genomic DNA and your control *E. coli* tester DNA) must be ligated to the appropriate adaptors as shown in the diagram above. **Panel A.** Your intended subtraction experiment. **Panel B.** Control subtraction. If you would like to use amplified tester and driver for Southern blotting or for differential screening of the subtracted DNA library (Section VI), you will need to perform a ligation of Adaptor 1 and Adaptor 2R to the driver sample.

Procedure for Adaptor Ligation

Three separate adaptor ligations (Figure 2) must be performed for each experimental tester genomic DNA, as well as for the control tester DNA.

General Ligation Set-up

- Each genomic DNA sample is aliquoted into two separate tubes: one aliquot is ligated with Adaptor 1 (Tester 1-1 and 2-1), and the second is ligated with Adaptor 2R (Tester 1-2 and 2-2).
- After the ligation reactions are set up, portions of each tester tube are combined so that the genomic DNA is ligated with both adaptors (unsubtracted tester control 1-c and 2-c).
- Each unsubtracted tester control DNA serves as a negative control for subtraction.

Considerations for Southern Blot Analysis

Ordinarily the adaptors are not ligated to the driver DNA. However, if you lack sufficient genomic DNA for Southern blot analysis of our subtracted DNA library, you can use amplified genomic DNA from the unsubtracted control to make Southern blots. In this case, you should generate unsubtracted controls from both tester and driver.

- To obtain unsubtracted controls for both tester and driver, be sure to ligate Adaptors 1 and 2R to both tester and driver DNA (this section), and amplify both unsubtracted controls (Section IV.G).
- For Southern blot analysis, electrophorese the amplified DNA on an agarose/EtBr gel, denature it in situ, and transfer it onto a nylon membrane.

Ligation Protocol

1. **Prepare your experimental tester genomic DNA 1 and genomic DNA 2:**

   a. Dilute 1.2 µl of each restriction enzyme-digested experimental DNA from Section IV.C.19 with 1.8 µl of sterile H_2O.

2. **Prepare your control tester genomic DNA:**

   a. Dilute your Control DNA(Hae III-digested φX174 DNA) to 0.2 ng/µl with sterile H_2O.

   b. Mix 1.2 µl of the Rsa I-digested control *E. coli* genomic DNA (Step IV.C.19) with 1.8 µl of the diluted φX174/Hae III DNA.

   This is your **control tester DNA**. It contains one copy of Hae III-digested φX174 DNA per *E. coli* genome (the *E. coli* genome is about 5 x 10^6 bp). After subtraction of the tester DNA against the driver DNA, the primary bands produced in the final PCR should correspond to these control fragments of the φX174/Hae III digest.

3. **Ligate your tester DNAs to the appropriate adaptors:**

   a. Prepare a ligation Master Mix by combining the following reagents in a 0.5 ml microcentrifuge tube. To ensure that you have sufficient Master Mix, prepare enough for all ligations plus one additional reaction.

	per rxn
Sterile H_2O	4 µl
5X Ligation Buffer	2 µl
T4 DNA Ligase (400 units/µl)	1 µl

b. For each experimental tester DNA and for the control tester DNA, combine the reagents in Table 1 in the order shown in 0.5 ml microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.

Table 1. Setting up the Ligation Reactions

(Repeat for each experimental tester DNA & the control tester DNA)

	Tube Number	
Component	1 **Tester 1-1***	2 **Tester 1-2***
Diluted tester DNA	1 µl	1 µl
Adaptor 1 (10 µM)	2 µl	—
Adaptor 2R (10 µM)	—	2 µl
Master Mix	7 µl	7 µl
Final volume	10 µl	10 µl

*Use the same setup for Tester 2-1 and 2-2.

c. In a fresh microcentrifuge tube, mix 1.5 µl of Tester 1-1 and 1.5 µl of Tester 1-2. After ligation is complete, this will be your unsubtracted tester control 1-c (see Figure 2). Do the same for the control tester DNA (tester 2-1 and 2-2). After ligation, approximately 1/3 of the DNA molecules in each unsubtracted tester control tube will bear two different adaptors.

d. Centrifuge briefly, and incubate at 16°C overnight.

e. Add 1 µl of 0.2 M EDTA to stop the ligation reaction.

f. Heat samples at 72°C for 5 min to inactivate the ligase.

g. Briefly centrifuge the tubes. Your experimental and control Adaptor-Ligated Tester DNAs 1-1, 2-1, 1-2, and 2-2 are now complete.

h. Remove 1 µl from each unsubtracted tester control (1-c and 2-c) and dilute into 1 ml of H_2O. These samples will be used for PCR amplification (Section IV.G.1).

i. Store samples at –20°C.

E. Protocol: First Hybridization

Perform the ligation efficiency analysis in Section V.B. **before proceeding with the hybridizations described below**. If your ligation was not efficient, repeat the ligation step before proceeding any further.

IMPORTANT: Before you begin the hybridization, **allow the 4X Hybridization Buffer to warm up to room temperature for at least 15–20 min**. Verify that there is no visible pellet or precipitate before using the buffer. If necessary, heat the buffer at 37°C for ~10 min to dissolve any precipitate.

1. For each of the experimental and control subtractions, combine the reagents in Table 2 in 0.5 ml tubes in the order shown.

Table 2. Setting Up the First Hybridization

(Repeat for each experimental tester DNA & the control tester DNA)

	Hybridization Sample	
Component	1 **Tester 1-1***	2 **Tester 1-2***
Rsa I-digested Driver DNA (IV.C.19)	2.0 µl	2.0 µl
Adaptor 1-ligated Tester 1-1* (IV.D.3.i)	1.0 µl	—
Adaptor 2R-ligated Tester 1-2 (IV.D.3.i)	—	1.0 µl
4X Hybridization Buffer	1.0 µl	1.0 µl
Final volume	4.0 µl	4.0 µl

*Use the same setup for Tester 2-1 and 2-2.

2. [Optional] Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples at 98°C for 1.5 min in a thermal cycler.
4. Incubate samples at 63°C* for 1.5 hr, then proceed immediately to the next section.

   * The hybridization temperature can be optimized for different bacteria, depending on the GC content of a particular genome. 63°C is the optimal temperature for genomic DNA with an average GC content (40–51%). Examples of bacteria with genomes that have an average GC content include *E. coli*, *Y. enterocolitica*, *Y. pestis*, *S. typhimurium*, and *D. nodosus* (reviewed in Hacker *et al.*, 1997). If the GC content of your genomic DNA is unusually low (e.g., the C. perfringens genome has a GC content of 26.5%), you may need to reduce the hybridization temperature.

F. Protocol: Second Hybridization

IMPORTANT: Do not denature the primary hybridization samples at this stage. Also, do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver.

Repeat the following steps for your experimental tester DNA and for the control tester DNA.

1. Dilute the 4X Hybridization Buffer with an equal volume of deionized H_2O to make 2X Hybridization Buffer.
2. Add the following reagents into a sterile tube:

	per rxn
Driver DNA (Step IV.C.19)	1 µl
2X Hybridization Buffer	1 µl

3. [Optional] Overlay with 1 drop of mineral oil.
4. Incubate at 98°C for 1.5 min in a thermal cycler.

5. Remove the tube of freshly denatured driver from the thermal cycler. Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (prepared in Section IV.E; see Table 2). This ensures that the two hybridization samples mix together **only** in the presence of freshly denatured driver.
    a. Set a micropipettor at 15 µl.
    b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
    c. Carefully draw the entire sample partially into the pipette tip.
    d. Remove the pipette tip from the tube, and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
    e. Repeat steps b–d with the tube containing the freshly denatured driver. The pipette tip should now contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket.
    f. Transfer the entire mixture to the tube containing hybridization sample 1.
    g. Mix by pipetting up and down.
6. Briefly centrifuge if necessary.
7. Incubate reaction at 63°C overnight.
8. Add 200 µl of dilution buffer and mix by pipetting.
9. Heat at 63°C for 7 min in a thermal cycler to eliminate nonspecific hybridization.
10. Store at –20°C.

G. Protocol: PCR Amplification

A minimum of five PCR reactions are recommended as described in Figure 2:

1) Subtracted experimental DNA
2) Unsubtracted tester control (1-c)
3) Subtracted control DNA from *E. coli*
4) Unsubtracted tester control for the control subtraction (2-c)
5) PCR Control Subtracted DNA (not shown in Figure 2)

The PCR Control Subtracted DNA provides a positive PCR control and contains a successfully subtracted mixture of Hae III-digested φX174 DNA. We recommend that you also perform a standard PCR control (i.e., the positive control template in the Advantage cDNA PCR Kit) to verify that your enzyme is performing efficiently.

PCR Amplification Procedure

1. Prepare the PCR templates:
    a. Aliquot 1 µl of each diluted DNA (i.e., each subtracted sample from Step IV.F.10 and the corresponding diluted unsubtracted tester control from Step IV.D.3.i) into an appropriately labeled tube.
    b. Aliquot 1 µl of the PCR Control Subtracted DNA into an appropriately labeled tube.
2. Prepare a Master Mix for all of the primary PCR tubes plus one additional tube. For each reaction planned, combine the reagents in Table 3 in the order shown:

Table 3. Preparation of the Primary PCR Master Mix

Reagent	Per Rxn	5-Rxn Mix*
Sterile H_2O	19.5 µl	117.0 µl
10X PCR reaction buffer	2.5 µl	15.0 µl
dNTP Mix (10 mM)	0.5 µl	3.0 µl
PCR Primer 1 (10 µM)	1.0 µl	6.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	3.0 µl
Total volume	24.0 µl	144.0 µl

* For each additional experimental DNA, prepare Master Mix for one additional reaction.

3. Mix well by vortexing, and briefly centrifuge the tube.
4. Aliquot 24 µl of Master Mix into each of the reaction tubes prepared in Step 1.
5. [Optional] Overlay with 50 µl of mineral oil.
6. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)

NOTE: This step "fills in" the missing strand of the adaptors (see Figure 1), thus creating binding sites for the PCR primers.

7. Immediately commence thermal cycling:

25 cycles:
- 94°C for 30 sec
- 66°C for 30 sec
- 72°C for 1.5 min

8. Analyze 7 µl from each tube on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section V.C for expected results.) Alternatively, you can set these 7 µl aliquots aside and run them on the same gel used to analyze the secondary PCR products (Step 16).
9. Dilute 3 µl of each primary PCR mixture in 39 µl of H_2O.
10. Aliquot 1 µl of each diluted primary PCR product mixture from Step 9 into an appropriately labeled tube.

11. Prepare Master Mix for the secondary PCR reactions plus one additional reaction by combining the reagents in Table 4 in the order shown:

Table 4. Preparation of the Secondary PCR Master Mix

Reagent	Per Rxn	5-Rxn Mix*
Sterile H_2O	18.5 µl	111.0 µl
10X PCR reaction buffer	2.5 µl	15.0 µl
Nested PCR primer 1 (10 µM)	1.0 µl	6.0 µl
Nested PCR primer 2R (10 µM)	1.0 µl	6.0 µl
dNTP Mix (10 mM)	0.5 µl	3.0 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	3.0 µl
Total volume	24.0 µl	144.0 µl

* For each additional experimental DNA, prepare Master Mix for one additional reaction.

12. Mix well by vortexing, and briefly centrifuge.
13. Aliquot 24 µl of Master Mix into each reaction from Step 12.
14. [Optional] Overlay with 1 drop of mineral oil.
15. Immediately commence thermal cycling:

    10–12 cycles:
    - 94°C for 30 sec
    - 68°C for 30 sec
    - 72°C for 1.5 min

16. Analyze 7 µl from each reaction on a 2.0% agarose/EtBr gel run in 1X TAE buffer. (See Section V.C for expected results.)
17. Store reaction products at –20°C.

- The PCR mixture is now enriched for tester-specific DNAs. In addition, tester-specific fragments that varied in abundance in the original genomic DNA due to different copy numbers should now be present in roughly equal proportions. Refer to Section V for Analysis of Results. Figure 5 shows the results of a successful control subtraction experiment with *E. coli* genomic DNA. We strongly recommend that you perform a simple subtraction efficiency test as shown in Figure 6.
- The uncloned subtracted mixture is an ideal hybridization probe for screening libraries of either genomic DNA or full-length cDNA (Diatchenko *et al.*, 1996). For all other applications, you should clone the products to make a subtracted DNA library. The DNAs can be directly inserted into a T/A cloning vector.
- For further analysis of your subtracted library, the following options are available:
  - **Differential screening**
    The Clontech PCR-Select Differential Screening Kit (Cat. No. 637403) contains the necessary reagents for differential screening, along with appropriate controls. For more information, see Section VI.B and the Clontech PCR-Select Differential Screening Kit User Manual at **www.clontech.com/manuals**
  - **Southern blot analysis**

V. Analysis of Results & Troubleshooting Guide

A. Analysis of Rsa I Digestion

Electrophorese 0.2 µg of undigested genomic DNA and 5 µl of Rsa I-digested genomic DNA (from Section IV.C) on a 1% agarose/EtBr gel in 1X TAE buffer, side-by-side.

Expected Results

The undigested genomic DNA should appear as a high-molecular weight band at the top of the gel. The Rsa I-digested DNA, in contrast, will be drastically decreased in size and should appear as a smear from 0.1 to 2 kb. Typical results are shown in Figure 3.

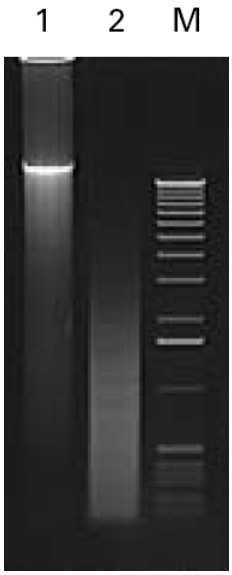

Figure 3. *E. coli* genomic DNA before (Lane 1) and after (Lane 2) Rsa I digestion. Lane M:DNA size markers.

Determining the Extent of Digestion

[Optional] To determine if a sample is completely digested, remove small samples at 60 and 120 min and compare on an agarose gel.

- If the DNA size distribution for both samples is identical, the digestion has progressed to completion.
- If the size distribution is not reduced after Rsa I digestion or is longer than 2 kb, then either your bacterial genomic DNA cannot be efficiently cut by:

  (i) any restriction enzyme due to the presence of an inhibitory impurity in the sample, or

  (ii) the Rsa I restriction enzyme because of the DNA's particular nucleotide sequence

Choosing the Appropriate Restriction Enzyme

- Choose the appropriate restriction enzyme based on its ability to digest a particular bacterial genomic DNA to produce fragments of optimal length. In addition to Rsa I, there are two other four-base-, blunt-cutting restriction enzymes: Hae III and Alu I.
- As an alternative, a combination of several six-base-, blunt-cutting restriction enzymes can also be used. In this case, perform several standard digestions with the different restriction enzymes.

Restriction Digestion Protocol

1. Add the following reagents into each reaction tube:

	per rxn
Genomic DNA (0.2 µg)	x µl
10X Restriction enzyme buffer	1.0 µl
Restriction enzyme (1 u)	1.0 µl
Deionized H_2O	y µl
Total volume	10 µl

2. Mix by vortexing and centrifuge briefly.
3. Incubate at 37°C for 3 hr.
4. Analyze the efficiency of digestion by agarose/EtBr gel electrophoresis.

   After you have chosen the appropriate restriction enzyme, perform the preparative digestion of your genomic DNA. If none of the restriction enzymes digest your experimental DNA, your sample contains inhibitory components. These can be removed by repeating the phenol/chloroform extraction and ethanol precipitation (Steps IV.C.6–19).

   NOTE: Incomplete digestion will significantly reduce the efficiency of subtraction.

B. Analysis of Ligation

We recommend that you perform the following PCR experiment to verify that at least 25% of the control tester DNA fragments have adaptors on both ends. This experiment is designed to amplify fragments that span the adaptor/DNA junctions of Testers 2-1 and 2-2 (see Section IV.D).

General Considerations

- This experiment is designed for the control *E. coli* Genomic DNA included in the kit. The primers amplify a 270 bp region of the 23S rRNA gene of *E. coli*.
- To analyze the ligation of your experimental DNA, you should use primers specific for a gene present in your bacterial DNA, if available. Keep in mind that the primers should amplify a fragment that does not contain a Rsa I site (or other restriction site if you used a different enzyme for digestion).
- Do not assume that a successful adaptor ligation with the control *E. coli* Genomic DNA guarantees a successful ligation with your experimental DNA—impurities in your genomic DNA preparation may inhibit the ligase.

Ligation Analysis Procedure

1. Dilute 1 µl of each *E. coli* control-ligated DNA from Section IV.D (e.g., Testers 2-1 and 2-2) into 200 µl of H_2O.
2. Combine the reagents in Table 5 in four separate tubes:

Table 5. Setting Up the Ligation Analysis

Component	Tube (µl)			
	1	2	3	4
Tester 2-1* (ligated to Adaptor 1)	1	1	—	—
Tester 2-2* (ligated to Adaptor 2R)	—	—	1	1
23S RNA Forward Primer (10 µM)	1	1	1	1
23S RNA Reverse Primer (10 µM)	—	1	—	1
PCR Primer 1 (10 µM)	1	—	1	—
Total volume	3	3	3	3

* Use the same set-up for your own experimental Tester 1-1 and 1-2, but use your own gene-specific primers.

3. Prepare a Master Mix for all of the reaction tubes plus one additional tube. For each reaction planned, combine the reagents in Table 6 in the order shown:

Table 6. Preparation of the Ligation Analysis PCR Master Mix

Component	Per Rxn	4-Rxn Mix
Sterile H_2O	18.5 µl	92.5 µl
10X PCR reaction buffer	2.5 µl	12.5 µl
dNTP Mix (10 mM)	0.5 µl	2.5 µl
50X Advantage cDNA Polymerase Mix	0.5 µl	2.5 µl
Total volume	22.0 µl	110.0 µl

4. Mix well by vortexing and briefly centrifuging.
5. Aliquot 22 µl of Master Mix into each of the reactions from Step 2.
6. Mix well by vortexing and briefly centrifuging.
7. Overlay with 50 µl of mineral oil.
8. Incubate the reaction mix at 72°C for 2 min in a thermal cycler to extend the adaptors. (Do not remove the samples from the thermal cycler.)
9. Immediately commence thermal cycling:

   23 cycles:
   - 94°C for 30 sec
   - 65°C for 30 sec
   - 68°C for 1 min

10. Examine the products by electrophoresis on a 2.0 % agarose/EtBr gel. Typical results are shown in Figure 4.

    - If you cannot see a product after 23 cycles, perform 4 additional cycles, and analyze by gel electrophoresis. As shown in Figure 4, the PCR product generated using one gene-specific primer (23S RNA Forward Primer) and PCR Primer 1 should be about the same intensity as the PCR product amplified using two gene-specific primers (23S RNA Forward and Reverse Primers). If the band intensity for these PCR products differs by more than 4-fold, your ligation was less than 25% complete, and will significantly reduce your subtraction efficiency. We recommend that you repeat the ligation reaction (Section IV.D).
    - If the PCR product amplified using the 23S RNA Forward Primer and PCR Primer 1 contains a 574 bp band in addition to the expected 374 bp band, this indicates that your DNA was not fully digested. If there is a significant amount of this 574 bp product, we recommend that you repeat the Rsa I digestion (Section IV.C)

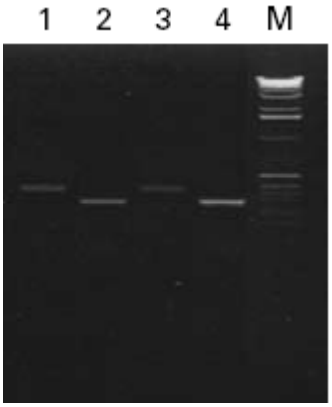

Figure 4. Typical results of ligation efficiency analysis. The results shown here are for the control *E. coli* Genomic DNA. Lane 1: PCR products obtained using Tester 1-1 (Adaptor 1-ligated) as template with 23S RNA Forward Primer and PCR Primer 1. Lane 2: PCR products obtained using Tester 1-1 (Adaptor 1-ligated) as template with 23S RNA Reverse and Forward primers. Lane 3: PCR products obtained using Tester 1-2 (Adaptor 2R-ligated) as template with 23S RNA Forward Primer and PCR Primer 1. Lane 4: PCR products obtained using Tester 1-2 (Adaptor 2R-ligated) as template with 23S RNA Reverse and Forward Primers. Lane M: 1kb DNA size markers. 2.0% agarose/EtBr gel.

C. Analysis of PCR Products

1. Agarose/EtBr Gel Electrophoresis of Primary PCR

Perform your primary PCR side-by-side with the PCR Control Subtracted DNA which will have major bands that appear after 25 cycles and should correspond to the φX174/Hae III fragments. This result should look similar to the control subtraction you performed; however, the correct φX174/Hae III bands may appear only after secondary PCR. The experimental primary PCR subtraction products usually appear as a smear from 0.2–2 kb, with some distinct bands.

a. **If you cannot see any products after 25 cycles**, use 3 additional cycles, and analyze by gel electrophoresis.

b. **If you cannot detect PCR products in the subtracted or unsubtracted (tester control 1-c) samples or the PCR Control Subtracted DNA**, you must verify that your polymerase is working. If the problem is not with your polymerase mix, try optimizing the PCR cycling parameters (Steps IV.G.7–8) by decreasing the annealing and extension temperature in **small**

increments—each degree lower can dramatically increase background. As a starting point, reduce the annealing temperature from 66°C to 64°C and the extension temperature from 72°C to 71°C.

c. **If you can detect PCR products in the unsubtracted (tester control 1-c) samples, but not in the subtracted sample**, dilute your primary PCR sample and perform additional cycles of secondary PCR.

2. Agarose/EtBr Gel Analysis of Secondary PCR

The patterns of secondary PCR products from the PCR Control Subtracted DNA and from the control subtraction should resemble Figure 5. A few additional bands may appear. The subtracted samples usually appear as smears with a number of distinct bands.

a. **If you obtain the expected φX174/Hae III bands in the PCR Control Subtracted DNA, but not in the control subtraction (e.g., a smear or random size bands)**, it is likely that your subtraction was not optimal. One of the most likely explanations is poor ligation efficiency. For troubleshooting, see Section V.C.

b. **If the ligation efficiency is high**, verify your protocol and repeat the subtraction. Problems during hybridization are uncommon.

c. **If you cannot see a product after 12 cycles**, use 3 additional cycles, and analyze by gel electrophoresis. Add cycles sparingly as too many cycles can increase background.

d. **If you do not observe φX174/Hae III bands in the PCR Control Subtracted DNA**, you must optimize the PCR conditions.

e. **In a successful subtraction**, the banding pattern of your unsubtracted DNA ligated with both adaptors (unsubtracted tester control 1-c) should be different from the banding pattern of your subtracted DNA samples (Figure 5).

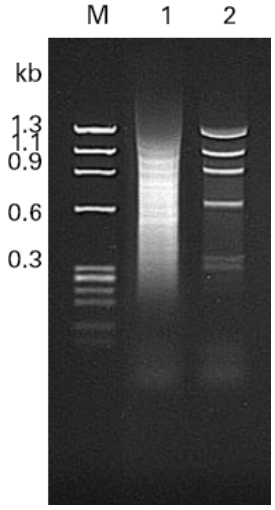

Figure 5. Typical results of control *E. coli* genomic DNA subtraction. The secondary PCR product contains mostly DNA fragments corresponding to the φX174/Hae III digest. The adaptor sequences on both ends of DNA fragments produces a mobility shift of these PCR products in comparison to the original, Hae III-digested φX174 DNA. Lane M: φX174/Hae III DNA size markers. Lane 1: Secondary PCR products of unsubtracted *E. coli* genomic DNA containing 1 copy of Hae III-digested φX174 DNA per *E. coli* genome. Lane 2: Secondary PCR products of subtracted tester *E. coli* genomic DNA ligated with both Adaptors 1 and 2R (generated in Section IV.G) and containing 1 copy of Hae III-digested φX174 DNA per *E. coli* genome. 2.0% agarose/EtBr gel.

D. Analysis of Subtraction Efficiency

Either hybridization analysis or PCR can be used to estimate the efficiency of subtraction. In both cases, this is done by comparing the abundance of known genes before and after subtraction. Ideally, analysis should be performed with a gene present in both tester and driver genomic DNAs, and with a gene known to be tester specific.

1. Hybridization Analysis

- **Generate Southern blots** of subtracted and unsubtracted (tester control 1-c) secondary PCR products.
- **Use different genes as probes** to determine the success of your subtraction.

2. PCR Analysis

General Considerations

- **PCR provides a faster test than hybridization analysis**. The sample PCR analysis described below uses primers for the *E. coli* 23S rRNA gene (provided with the kit). We do not recommend that you carry out this exact test because, in the control subtraction using the *E. coli* Genomic DNA, the banding pattern of the φX174/Hae III digest (Figure 5, Lane 2) has already indicated whether or not subtraction was successful.
- **This test will help you confirm the reduced relative abundance of the 23S rRNA gene** following the PCR-Select procedure. For your experimental subtraction, you should use primers specific for a gene present in your bacterial DNA. Keep in mind that this fragment should have no Rsa I restriction site (or other restriction site if you used a different enzyme for digestion).
- **Not all genes are subtracted evenly**. In certain instances a particular gene is present at different levels in tester and driver genomic DNA due to different copy numbers. If the copy number of your common gene is even 2-fold higher in the tester sample, this gene will not be efficiently subtracted out. This also may be true for the 23S rRNA gene, depending on your bacterial strain. If this is the case, choose another housekeeping gene as a control for subtraction efficiency.

Procedure for PCR Analysis of Subtraction Efficiency

1. Dilute the subtracted and unsubtracted (tester control 1-c) secondary PCR products 10-fold in H_2O. The concentration of subtracted and unsubtracted product should be roughly equal.
2. Combine the following reagents in 0.5 ml microcentrifuge tubes in the order shown:

	Rxn 1	Rxn 2
Diluted subtracted DNA (2° PCR product)	1.0 µl	—
Diluted unsubtracted tester control 1-c (2° PCR product)	—	1.0 µl
23S RNA Forward Primer (10 µM)	1.2 µl	1.2 µl
23S RNA Reverse Primer (10 µM)	1.2 µl	1.2 µl
sterile H_2O	22.4 µl	22.4 µl
10X PCR reaction buffer	3.0 µl	3.0 µl
dNTP Mix (10 mM each)	0.6 µl	0.6 µl
50X Advantage cDNA Polymerase Mix	0.6 µl	0.6 µl
Total volume	30.0 µl	30.0 µl

3. Mix by vortexing and briefly centrifuging.
4. Overlay with one drop of mineral oil.

5. Use the following thermal cycling program:

   18 cycles:
   - 94°C 30 sec
   - 60°C 30 sec
   - 68°C 2 min

6. Remove 5 µl from each reaction and place it in a clean tube. Return the remainder of each reaction to the thermal cycler for 3 additional cycles.

7. Repeat Step 6 twice (i.e., remove 5 µl after 24 and 37 cycles).

8. Examine the 5 µl samples (i.e., the aliquots that were removed from each reaction after 18, 21, 24, and 27 cycles) on a 2.0% agarose/EtBr gel.

Analysis of Subtraction Efficiency Results

Figure 6 shows an example of 23S rRNA gene reduction in a successfully subtracted control *E. coli* genomic DNA sample. For your unsubtracted DNA, you should see a PCR product after 18–23 cycles, depending on the abundance of your gene in a given sample. With your subtracted samples, you should detect a product ~6–9 cycles later.

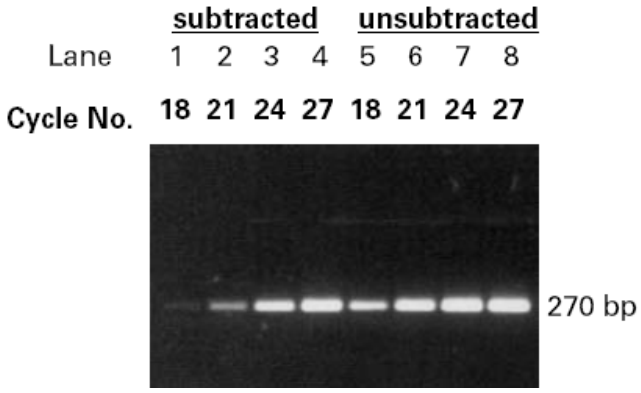

Figure 6. Reduction of 23S rRNA gene abundance by PCR-Select subtraction. PCR was performed on subtracted (Lanes 1–4) or unsubtracted (Lanes 5–8) secondary PCR products of the control *E. coli* Genomic DNA with the 23S RNA Reverse and Forward Primers (included in the kit). Lanes 1 & 5: 18 cycles; Lanes 2 & 6: 21 cycles; Lanes 3 & 7: 24 cycles; Lanes 4 & 8: 27 cycles.

1. **If you did not observe a decrease in abundance of genes common to both tester and driver,** repeat the PCR amplification (Section IV.G). Perform two independent primary and secondary PCRs and check the abundance of the gene common to both tester and driver in both sets.

   - During the first hybridization step of the subtraction procedure, equalization of the ss tester molecules takes place. As a result, a very low concentration of DNA molecules with different adaptors on each end forms during the second hybridization (see Introduction & Figure 1); only about 10,000 of these target molecules are present in 1 ml of diluted subtracted DNA. If one such molecule representing the common gene happens to be present, your gene abundance will not appear to decrease.

   - If you see reduction of your gene abundance in the independent PCR-amplified subtracted DNA, it indicates that this random event did occur.

2. **As a positive control for the enrichment of differentially expressed genes**, repeat the procedure above using PCR primers for a gene known to be expressed in the tester DNA, but not in the driver DNA.
    - This gene should become enriched during subtraction. The difference in the number of cycles required for equal amplification of the corresponding PCR product in subtracted and unsubtracted samples indicates the efficiency of your subtraction. Three cycles corresponds roughly to a 6-fold enrichment.
    - Because of the equalization that occurs during subtraction, the level of enrichment will depend on the initial abundance of each tester-specific gene, as well as the difference in abundance for each gene in the tester and driver. Tester-specific genes that are present as unique copies in the tester genomic DNA will be enriched more than genes that are present at high copy number.

NOTE: Do not use PCR primers that amplify a DNA fragment that contains an Rsa I restriction site between the PCR priming sites (or another site if you used a different restriction enzyme for digestion).

VI. Confirming Tester-Specific Sequences

When you have generated your subtracted DNA library, you may use Southern blot analysis to confirm the distribution of the DNA fragments derived from individual clones in the initial tester and driver DNA. If high background makes it difficult to pick random clones from the subtracted library, differential screening can help eliminate false positives prior to Southern blot analysis.

A. Southern Blot Analysis

- In our experience, the percentage of clones in the subtracted library that corresponds to tester-specific DNA is typically over 50%. We recommend that you randomly pick 10–20 clones from the subtracted library for use as probes on Southern blots.
- If you lack sufficient genomic DNA for standard Southern blot analysis, you can use amplified genomic DNA from the unsubtracted control. In this case, you should generate unsubtracted controls for both tester and driver. You will need to ligate Adaptors 1 and 2R to both tester and driver DNA samples (Section IV.D); then, amplify both unsubtracted controls (Section IV.G). Electrophorese the amplified DNAs on an agarose/EtBr gel, denature in situ, and transfer onto a nylon membrane for Southern blot analysis.
- Use of the amplified unsubstracted controls as tester and driver for Southern blotting requires one additional step: removal of the adaptor sequences from the ends of the DNA molecules generated by the subtraction procedure. Despite their small size, these adaptor sequences produce a very high background when hybridized with the inserts amplified from randomly picked clones.

B. Differential Screening

1. **In most cases, the Clontech PCR-Select Bacterial Genome Subtraction Kit method greatly enriches for tester-specific genes; nevertheless, the subtracted sample will still contain some DNA fragments common to both the tester and driver samples.**

    - This background may depend on the quality of DNA preparation, restriction enzyme digestion, and the performance of the subtraction.
    - With high background, picking random clones from the subtracted library for Southern blot analysis is extremely time-consuming and inefficient. If less than 25% of the clones are confirmed as tester-specific genes, you should perform differential screening to eliminate false positives.
    - The **Clontech PCR-Select Differential Screening Kit** (Cat. No. 637403) contains everything needed to generate subtracted and unsubtracted probes to screen your subtracted DNA library. This ensures a good ratio of signal to background in your Southern analyses. For more details, please see the Clontech PCR-Select Differential Screening Kit User Manual and our website at **www.clontech.com/manuals**

2. **The following approach is recommended for differentially screening the subtracted library:**

    - Hybridize the subtracted library with the original tester and driver genomic DNAs. If you have enough of each genomic DNA sample to make hybridization probes, you may array DNA inserts from the subtracted library on a nylon membrane and hybridize with labeled tester and driver probes. An example performed with a *Helicobacter pylori* DNA library is shown in Figure 7. Clones hybridizing with tester but not driver probe are tester-specific DNA fragments; clones hybridizing to both probes are considered background.

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

    - If you lack sufficient genomic DNA for differential screening, you can use the amplified unsubtracted controls for both tester and driver (Section IV.G) instead of the initial restriction enzyme-digested genomic DNA. In this case, you should generate unsubtracted controls for both tester and driver. You will need to ligate Adaptors 1 and 2R to both tester and driver DNA samples (Section IV.D); then, amplify both unsubtracted controls for use as probes (Section IV.G). You must remove the adaptor sequences from the ends of these amplified unsubtracted control fragments before using them as probes. This step is required because the inserts from randomly picked clones that are arrayed on the membrane also contain the adaptor sequences. Despite their small size, these adaptor sequences produce very high background during hybridization.

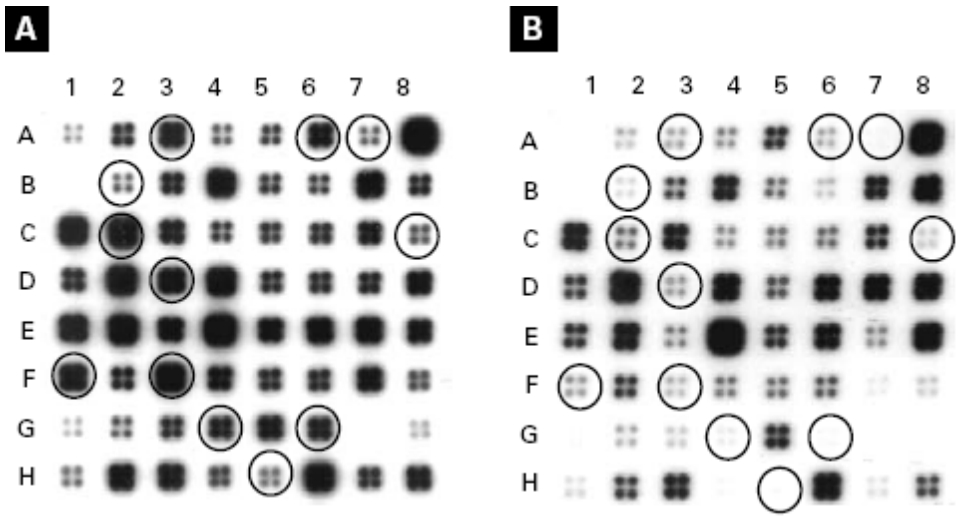

Figure 7. Differential screening of a subtracted *Helicobacter pylori* DNA library. PCR-Select subtraction was performed using *H. pylori* strain J166 as tester and *H. pylori* strain 26695 as driver. The subtracted genomic DNA was T/A cloned. Eighty-four clones were randomly picked and their inserts were amplified and arrayed in quadruplicate on nylon membranes. Membranes were 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 at 65°C for 15 min in solution containing 6X SSC, 5X Denhardt's solution, 1% SDS, and 100 µg/ml calf thymus DNA before overnight hybridization with probes in the same solution. 2 x 15-min washes were performed in a solution containing 2X SSC and 0.5% SDS. Circles indicate true tester-specific clones. (Data were kindly provided by Natalia S. Akopyants and Dr. Douglas Berg, Washington University School of Medicine, St. Louis, MO.)

VII. References

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Specific References for PCR-Select Technology

Suppression subtractive hybridization—the technology that all of our PCR-Select kits are built upon—was originally described by Diatchenko *et al.* (1996). The following selected articles—found within the above references list—contain published experimental data obtained using PCR-Select technology.

Cao *et al.* (2004)

Jeong *et al.* (2004)

Knaup *et al.* (2004)

Lian *et al.* (2004)

Norton *et al.* (2004)

Pan *et al.* (2004)

Appendix A: Suppression PCR

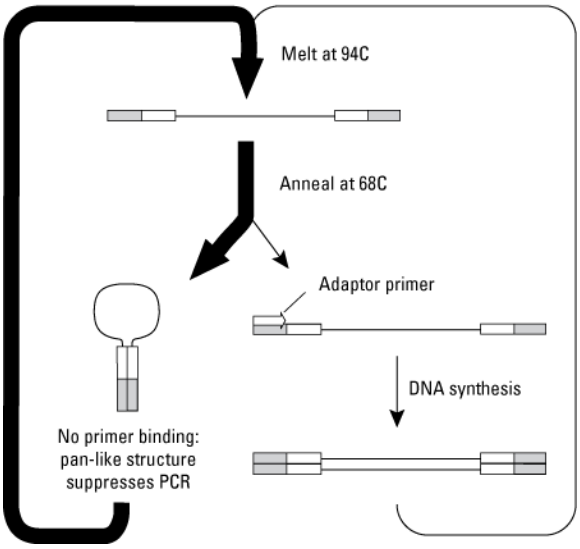

Figure 8. Suppression PCR.

The PCR-Select adaptors are engineered to prevent undesirable amplification during PCR by means of a method called **suppression PCR** (U.S. Patent No. 5,565,340; Siebert *et al.*, 1995).

- Suppression occurs when complementary sequences are present on each end of a single-stranded DNA fragment. During each primer annealing step, the hybridization kinetics strongly favor the formation of a pan-like secondary structure that prevents primer annealing.
- Occasionally when a primer anneals and is extended, the newly synthesized strand will also contain the inverted terminal repeats and form another pan-like structure. Thus, during PCR, nonspecific amplification is efficiently suppressed, and specific amplification of DNA molecules with different adaptors at both ends can proceed normally.
- The 5’ ends of Adaptors 1 and 2R have an identical stretch of 22 nucleotides (nt). Primary PCR therefore requires only one primer for amplification, eliminating the problem of primer dimerization (Lukyanov *et al.*, 1995).
- Furthermore, the identical sequences on the 3’ and 5’ ends of the differentially expressed molecules introduces a slight suppression PCR effect. Since these identical sequences are the same length as PCR Primer 1, the suppression effect becomes significant only for very short DNAs (under 200 nt), because the formation of pan structures for shorter molecules is more efficient (Lukyanov *et al.*, 1995).
- Thus, longer molecules are preferentially enriched, balancing the inherent tendency of the subtraction procedure to favor short DNA fragments. These shorter fragments are more efficiently hybridized, amplified, and cloned than longer fragments.

Appendix B: Adaptor and Primer Sequences

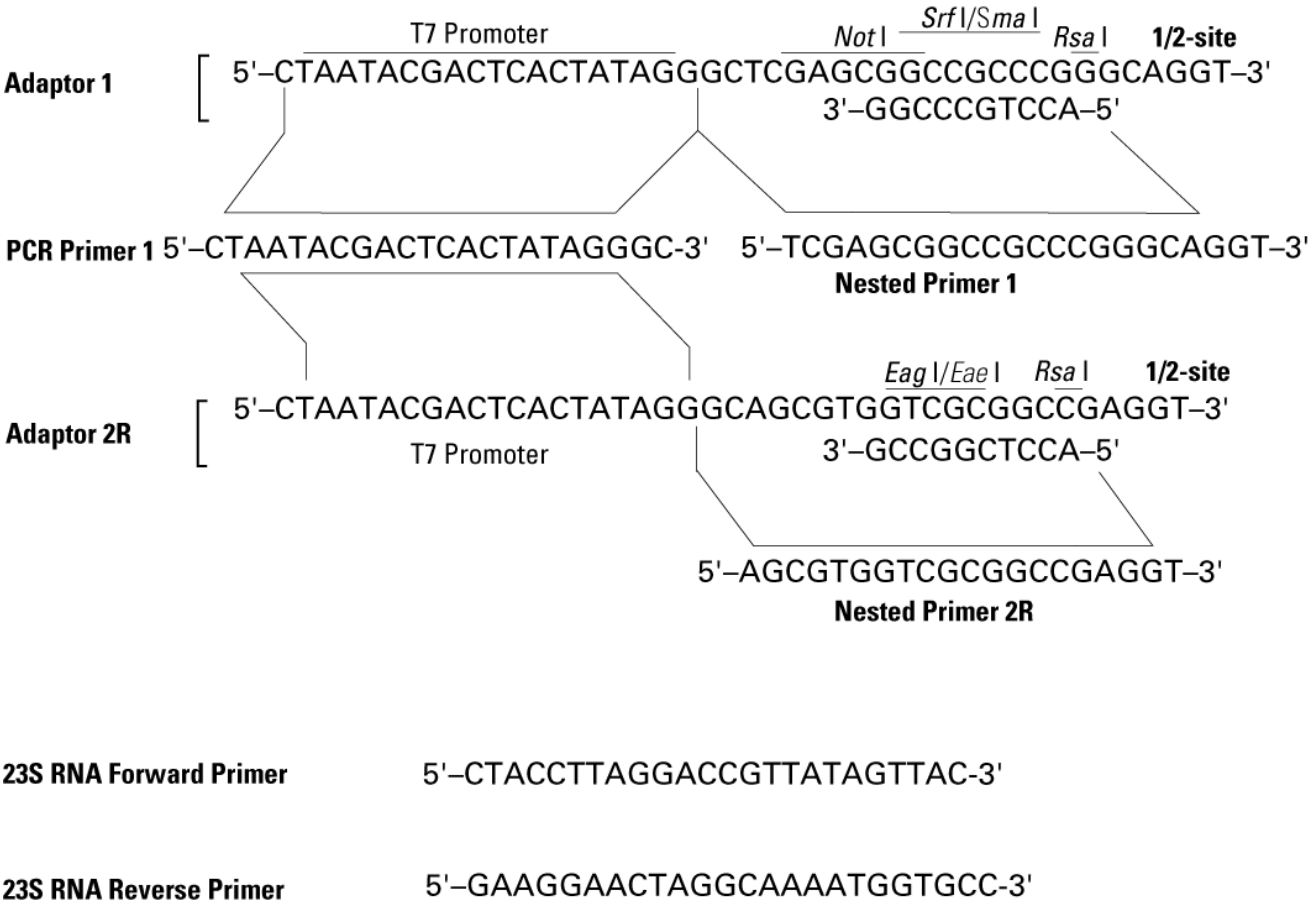

Figure 9. Sequences of the PCR-Select adaptors, PCR primer, and Control Primers. When Adaptors 1 and 2R are ligated to Rsa I-digested DNA, the Rsa I site is restored.

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