# CHAPTER 3

# SUPPRESSION SUBTRACTIVE HYBRIDIZATION

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**Abstract**: Suppression subtractive hybridization (SSH) is a widely used method for separating DNA molecules that distinguish two closely related DNA samples. Two of the main SSH applications are cDNA subtraction and genomic DNA subtraction. To our knowledge, SSH is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. It is based primarily on a suppression polymerase chain reaction (PCR) technique (described narrowly in Chapter 3) and combines normalization and subtraction in a single procedure. The normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the populations being compared. This dramatically increases the probability of obtaining low-abundance differentially expressed cDNAs or genomic DNA fragments and simplifies analysis of the subtracted library. SSH technique is applicable to many comparative and functional genetic studies for the identification of disease, developmental, tissue-specific, or other differentially expressed genes, as well as for the recovery of genomic DNA fragments distinguishing the samples under comparison. This chapter provides an insight into SSH practical use and contains detailed protocol for generation of subtracted cDNAs (which is the most frequent SSH application) and differential screening of the resulting subtracted cDNA library. As shown in many examples, the SSH technique may result in over 1000-fold enrichment for rare sequences in a single round of subtractive hybridization. Finally, we discuss the characteristics of cDNA-subtracted libraries, the nature and level of background nondifferentially expressed clones in the libraries, as well as procedure for rapid identification of truly differentially expressed cDNA clones.

**Keywords**: Differentially regulated genes, suppression polymerase chain reaction (PCR) effect, enrichment, hybridization time, high complexity, false positive, mirror orientation selection, mirror-oriented selection (MOS), protocol, cap switch, tagging RNA 5′-ends, highly abundant cDNA, primer annealing site, adapter sequence, physical separation, mathematical model, background, restriction endonuclease, heat denaturation, reannealing, target sequence, technical comments, differential screening,



*A. Buzdin and S. Lukyanov (eds.), Nucleic Acids Hybridization,* 53–84.

efficiency of SSH, Northern blot, level of enrichment, random clones, removal of the adapter sequences, size of cDNA fragments, disadvantage, drawback, equalization.

**Abbreviations**: cDNA, complementary DNA; dNTP, deoxyribonucleotidetriphosphate; mRNA, messenger RNA; MOS, mirror orientation selection; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SSH, suppression subtractive hybridization.

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## 1. INTRODUCTION

Subtractive hybridization methods are valuable tools for identifying differentially regulated genes important for cellular growth and differentiation. Over the last decade, numerous subtractive hybridization techniques have been developed (many of which are described in this book, see Chapters 1–3, 6, and 7) and used to isolate significant genes in many systems (Sargent and Dawid 1983; Hedrick et al. 1984; Hara et al. 1991; Wang and Brown 1991; Hubank and Schatz 1994). However, while having some advantages many of them require either tedious, complicated procedures or large amounts of starting material, thereby reducing their overall utility. Hence, these can be greatly improved if the procedures can be streamlined and/or used with minute amounts of starting material. Suppression subtractive hybridization (SSH) is a widely used method for separating DNA molecules that distinguish two closely related DNA samples of either cDNA or genomic DNA nature (Luk'ianov et al. 1994; Diatchenko et al.

1996; Gurskaya et al. 1996; Akopyants et al. 1998). In particular, the SSH protocol, which we describe here, normalizes (equalizes) sequence abundance among the target cDNA population, eliminates any intermediate step(s) for physical separation of single-stranded cDNAs and double-stranded cDNAs, requires only one round of subtractive hybridization, and can achieve greater than 1000-fold enrichment for differentially expressed cDNAs (Rebrikov et al. 2004). The SSH method is based on a suppression polymerase chain reaction (PCR) effect ((Lukyanov et al. 1995; Siebert et al. 1995), see Chapter 2) and combines normalization and subtraction in a single procedure (Gurskaya et al. 1996). The normalization step equalizes the abundance of DNA fragments within one round of subtraction (Diatchenko et al. 1996; Gurskaya et al. 1996; Jin et al. 1997). Nevertheless, in practice, not all differentially expressed genes are equally enriched by SSH. The level of enrichment of a particular cDNA depends on its original abundance, the ratio of its concentration in the samples being subtracted, and the number of other differentially expressed genes. Other factors, such as the complexity of a starting material, hybridization time, and ratio of two samples being subtracted, play a very important role in SSH's success in a given application. For instance, the high complexity of mammalian genomic DNA makes SSH application very difficult. Likewise, some cDNA subtractions are also challenging because of the nature of the starting samples. Subtracted libraries generated using complex samples may contain very high background. An especially challenging problem is the inclusion of "false positive" clones that generate a differential signal in a primary screening procedure, but are not confirmed by subsequent detailed analysis. To overcome this problem, a simple procedure called mirror orientation selection (MOS) can be used to substantially decrease the number of background clones (Rebrikov et al. 2000, 2004).

In this chapter, we describe the SSH technique for generating subtracted cDNA or genomic DNA libraries. Detailed protocols for cDNA synthesis, subtractive hybridization, PCR amplification, library generation, and differential screening analysis are provided. We also describe the MOS procedure that substantially decreases the number of background clones in SSH-generated libraries. Finally, we show an example of SSH- and MOS-subtracted library.

## 2. THE PRINCIPLE OF SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Figure 1 presents a brief overview of the SSH procedure (application to subtraction of cDNA samples). SSH includes several steps. First, cDNA is synthesized from the two types of tissues or cell populations being compared. The cDNA population in which specific transcripts are to be found is called tracer cDNA (or tester cDNA), and the reference cDNA population is called driver cDNA. For cDNA synthesis, the conventional method described by Gubler and Hoffman including poly(A) + RNA isolation (Gubler and

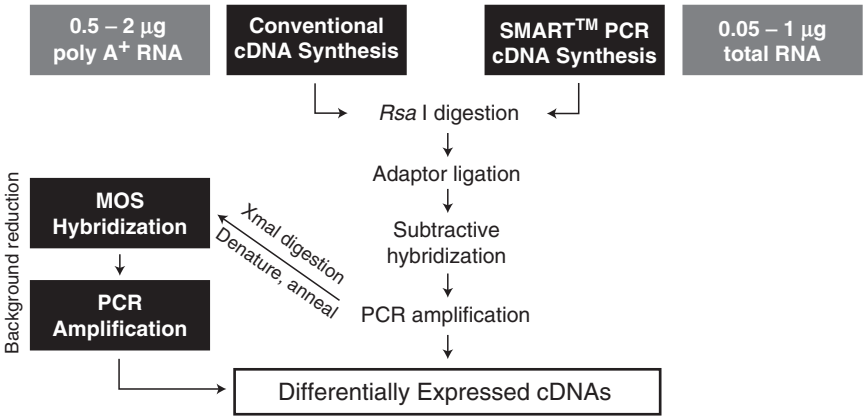


*Figure 1.* Brief overview of the suppression subtractive hybridization (SSH). For better results, mirror-oriented selection (MOS) procedure can be recruited as the option.

Hoffman 1983) can be used. If enough poly(A)+ RNA is not available, the "Cap Switch" amplification technology (SMART, BD Biosciences Clontech) or the FirstChoice RLM-RACE Kit (Ambion), exactly tagging RNA 5′-ends with synthetic oligonucleotides, can be used to preamplify high-quality cDNA from total RNA (Maruyama and Sugano 1994). At the second step, double-stranded cDNAs are synthesized independently from the tracer and driver, and are further digested with a four-base-cutting restriction enzyme that yields blunt ends, such as *Rsa* I or *Alu* I (Rebrikov et al. 2004). The tracer cDNA is then subdivided into two portions (1 and 2) and each is ligated to a different double-stranded adapter (adapters 1 [Ad1] and 2R [Ad2R]). The ends of the synthetic oligonucleotide-derived adapters are not phosphorylated, so only one strand of each adapter becomes covalently attached to the 5′-ends of the cDNAs. The molecular events that occur during subtractive hybridization and selective amplification of differentially expressed genes are illustrated in Figure 2. In the first hybridization, an excess of driver cDNA is added to each sample of tracer cDNA.

The samples are then heat-denatured and allowed to anneal. Figure 2 shows the type A, B, C, and D molecules generated in each sample. During this first hybridization step the subset of single-stranded tracer molecules (fraction A) is normalized, which means that concentrations of high and low abundance cDNAs become roughly equal. Normalization occurs because the annealing process generating homohybrid (B) and heterohybrid (C) cDNAs is faster for more abundant molecules, due to the second order of hybridization kinetics, than annealing of the less abundant cDNAs that remain single-stranded (A). By controlling the extent of the hybridization, the single-stranded forms of highly abundant cDNAs can then be reduced to the same levels as those of less abundant ones, thereby normalizing the representation of tracer cDNA

population (Hames and Higgins 1985). At the same time, the population of type A molecules is significantly enriched for differentially expressed sequences, because common for tracer and driver samples nontarget cDNAs form type C molecules with the driver. During the second hybridization, the two samples from the first hybridization are combined and annealed further with additional freshly denatured driver. Under these conditions, only single-stranded type A tracer cDNAs are able to reassociate and form (B), (C), and new (E) hybrids (Figure 2). Type E hybrids are double-stranded tracer molecules with different single-stranded ends, one of which corresponds to Ad1 and another to Ad2R.

Freshly denatured driver is added to further enrich fraction E in differentially expressed sequences. The entire population of molecules is then subjected to two rounds of PCR to selectively amplify the differentially expressed sequences (Rebrikov et al. 2004). Prior to the first PCR, adapter ends are filled in, thus creating the complementary primer binding sites needed for amplification (Figure 2). Type A and D molecules lack primer annealing sites and cannot be amplified. Type B molecules form stem-loop pan handle-like structures that suppress amplification (Lukyanov et al. 1995; Siebert et al. 1995). Type C molecules have only one primer annealing site and can be amplified only at a linear rate. Only type E molecules, which have different adapter sequences at their ends and, thus, two different primer annealing sites, can be amplified exponentially.

Differentially expressed sequences are greatly enriched in type E fraction, and therefore in the subtracted cDNA pool. This method does not involve any physical separation of single-stranded molecules from double-stranded hybrids. The comprehensive mathematical model for the formation of fraction E molecules, as well as the rate of enrichment, has been described previously (Gurskaya et al. 1996). Although SSH technique in its standard form was shown to be very effective for many objects, in some cases, e.g. when the samples under comparison had relatively small number of differential sequences, there was a high background in the SSH-generated subtracted libraries, and another approach termed MOS could be helpful to significantly reduce the background.

## 3. THE PRINCIPLE OF MIRROR-ORIENTED SELECTION

The MOS technique is based on the rationale that, after PCR amplification during SSH, each kind of background molecule has only one orientation relative to the adapter sequences (Rebrikov et al. 2004). This directionality corresponds to the orientation of the progenitor molecule. On the contrary, the target DNA fragments are involved in PCR amplification owing to efficient enrichment in the SSH procedure. As a result, each specific sequence has many progenitors and is represented by both orientations relatively to adapter sequences (Rebrikov et al. 2000, 2004). The procedure includes removing adapter-derived primer NP1

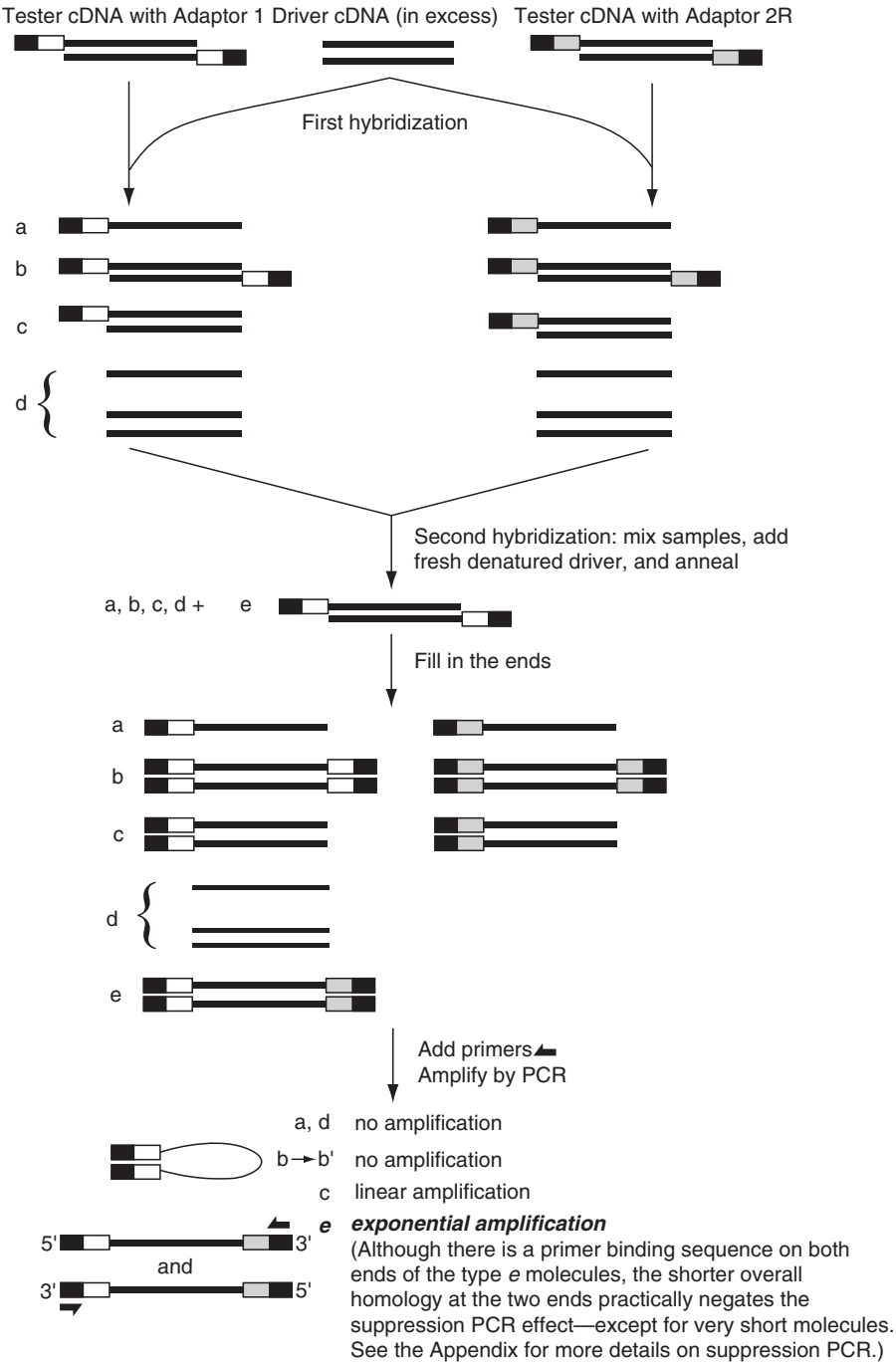


*Figure 2.*

(*Continued*)

in secondary PCR of SSH, (Figure 3) by restriction endonuclease (*Xma* I in this example), heat denaturation and reannealing of the SSH sample (Figure 3). Some of the newly formed hybrids from target DNAs bear adapter 2R (represented by adapter 2R-derived primer NP2R in secondary PCR of SSH, Figure 3) at both termini. These molecules are generated as a result of hybridization of molecules with "mirror" orientation of adapters 1 and 2. Thus, they can primarily (if not exclusively) be derived from a fraction of target DNA. Finally, the 3′-ends are filled in and PCR with primers corresponding to NP2R (also called NP2Rs or MOS PCR primer) is performed. In this reaction only molecules bearing NP2R at both termini can be amplified exponentially. Therefore, the final PCR products are enriched in target sequences.

## 4. TECHNICAL COMMENTS AND CONSIDERATIONS

### 4.1 Normalization Step and the Efficiency of SSH

The SSH technique has been demonstrated to be efficient for generating cDNAs highly enriched for differentially expressed genes of both high and low abundance. The high level of enrichment of cDNAs for rare transcripts has been achieved by the inclusion of a normalization step in the subtraction procedure, as evidenced by Northern blot analysis of random cDNA clones from many subtracted cDNA libraries (Siebert et al. 1995).

Using the mathematical model of the subtraction procedure (Ermolaeva et al. 1996; Gurskaya et al. 1996) and SUBTRACT program (Ermolaeva and Wagner 1995), it was calculated that the rare specific transcripts can be enriched by greater than 1000 during one round of subtraction. This conclusion was supported in a model experiment with artificial targets added to cDNA libraries (Diatchenko et al. 1996). In practice, the level of enrichment for a particular

*Figure 2.* cont'd. Overall scheme for suppression subtractive hybridization. There are six steps in this procedure. (1) double-stranded cDNAs are synthesized from tracer and driver mRNAs and digested with *Rsa* I to generate optimal fragments for hybridization reactions. (2) adapters Ad1 and Ad2R are ligated to two separate populations of the tracer cDNAs which are then (3) mixed with 30X excess driver cDNAs. The mixtures are processed in the first hybridization to normalize and enrich differentially expressed sequences among ss tracer molecules. (4) The reactions from the first hybridization are mixed and processed for a second hybridization in the presence of additional driver ss cDNAs, resulting in combination of different hybrid types. (5) The ends of the respective hybrids are filed in to generate cDNA fragments from differentially expressed genes that can be preferentially amplified by PCR using appropriate primers. (6) Two rounds of PCR are performed to preferentially amplify differentially expressed genes. Solid lines represent the *Rsa* I-digested tracer or driver cDNA. Solid boxes represent the outer part of the adapter Ad1 and Ad2 longer strands and corresponding PCR primer P1 sequence. Clear boxes represent the inner part of the adapters Ad1 longer strand and corresponding nested PCR primers NP1. Shaded boxes represent the inner part of the adapter Ad2R longer strand and corresponding nested PCR primer NP2R. Note that after filling in the recessed 3′-ends with DNA polymerase, type a, b, and c molecules having adapter 2 are also present, but are not shown.

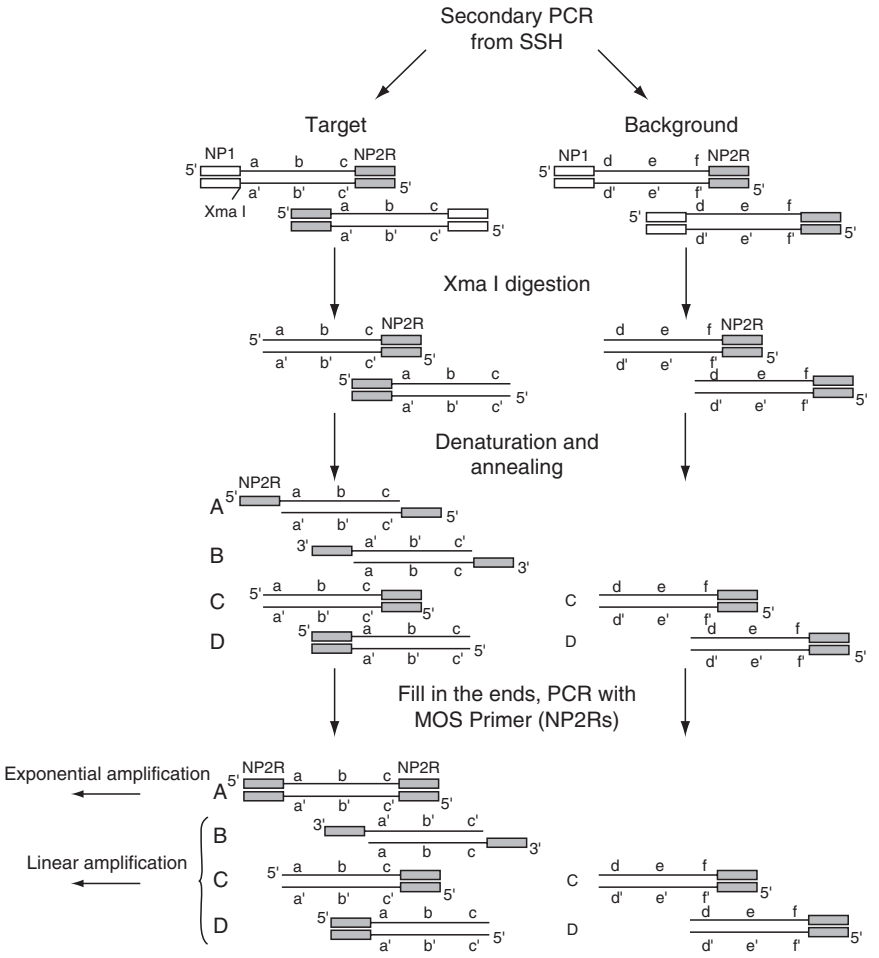


*Figure 3.* Schematic diagram of the mirror-oriented selection (MOS) procedure. The method is based on the assumption that each background molecule has only one orientation with respect to the Ad1 (represented by NP1) and Ad2R (represented by NP2R) adapters used in suppression subtractive hybridization (SSH), whereas truly differentially expressed target cDNA fragments are represented by both sequence orientations. MOS PCR primer is a shortened variant of the NP2R primer used in SSH.

gene depends greatly on its original abundance, the ratio of its concentrations in driver and tracer, and a number of other differentially expressed genes. With the incorporation of a normalization step in subtraction procedure, the highest enrichment level can be obtained for differentially expressed mRNAs exhibiting low abundance and/or large differences in expression levels in the tracer and driver RNA populations. However, as with other subtractive hybridization techniques, the efficiency of SSH is lower in experiments designed to detect mRNAs that show only moderate (e.g. twofold to fourfold) differences between the tracer and driver populations. Nevertheless, these types of differentially expressed

genes can still be identified by the extensive screening of subtracted libraries (Rebrikov et al. 2004; Gurskaya et al. 1996).

### 4.2 Differential Screening of the Subtracted Libraries

Although SSH method greatly enriches for differentially expressed genes, and MOS approach application significantly reduces SSH background, the subtracted sample will still contain some cDNAs that correspond to mRNAs common to both the tracer and driver samples, depending somewhat on the quality of RNA purification and the performance of the particular subtraction. However, it mainly arises when very few mRNA species are differentially expressed in tracer and driver. In general, fewer differentially expressed mRNAs and less quantitative difference in expression lead to higher background – even if one obtains a good enrichment for differentially expressed cDNAs. When background is expected to be high, identification of differentially expressed cDNAs by picking random clones from the subtracted library for Northern blot analysis can be time consuming. To solve this problem, the incorporation of a differential screening step is an efficient and desirable means to minimize background before embarking on Northern blot analysis (Rebrikov et al. 2004).

There are two approaches for differentially screening the subtracted library. The first is to hybridize the subtracted library with [$\alpha^{32}$P]dCTP-labeled cDNA probes synthesized directly from the tracer and driver mRNAs (Hedrick et al. 1984). Clones corresponding to differentially expressed mRNAs will hybridize only with the tracer probe, and not with the driver probe. Although this approach is widely used, it has one major disadvantage: only cDNA molecules corresponding to highly abundant mRNAs (i.e. mRNAs which constitute more than ~0.2% of the total cDNA in the probe) can hybridize with the clones (Hames and Higgins 1985; Wang and Brown 1991). Clones corresponding to low-abundance differentially expressed mRNAs will not be detected by this screening procedure.

To avoid missing low-abundance differentially expressed genes, we recommend a second approach, in which the subtracted library is hybridized with forward- and reverse-subtracted cDNA mixtures. Clones representing mRNAs that are truly differentially expressed will hybridize only with the forward probe; clones that hybridize with the reverse probe most probably represent background (Wang and Brown 1991; Lukyanov et al. 1996). It should be noted, that the removal of the adapter sequences from the ends of the cDNA molecules generated by the SSH procedure, is critical for reducing the background caused by hybridization of the adapters in the subtracted cDNA probes to those immobilized on the nylon filters.

### 4.3 Starting RNA Materials

Normally, 2–4 mg of poly(A)+ RNA for both the tracer and driver are needed for a comprehensive subtraction scheme using both forward and reverse SSH. The resulting PCR products can be used for subtracted cDNA library construction and differential screening experiments. However, in some cases, such

amount of poly(A)+ RNAs may be difficult to obtain. To circumvent this problem, an amplification step for both the driver and tracer poly(A)+ RNA can be incorporated to generate sufficient quantities of both cDNA samples (Gurskaya et al. 1996) before initiating the SSH procedure. Alternatively, total RNA can be used successfully as starting material for the preamplification (Lukyanov et al. 1997; Zhumabayeva et al. 2001a, b). However, preamplification of either poly(A)+ or total RNA samples invariably increases the background in the final PCR products and may result in the loss of some sequences. The utilization of preamplifiation should be minimized whenever it is possible.

### 4.4 Size of cDNA Fragments

For an efficient SSH procedure, the starting tracer and driver cDNAs have to be cleaved into smaller fragments. A four-base cutter, *Rsa* I, is recommended for this purpose since it generates optimal fragments (average ~600 bp) for SSH. Although this step may not be a desirable manipulation for obtaining full-length differentially expressed cDNAs, dividing each cDNA into multiple fragments has two important advantages. First, long DNA fragments may form complex networks which prevent the formation of appropriate hybrids needed to position two independent adapters, Ad1 and Ad2R, at the ends of the target molecules. Second, small cDNA fragments provide better representation of individual genes, since cDNAs derived from related but distinct members of gene families may cross-hybridize with each other, thereby eliminating them from the final subtracted cDNA products (Ko 1990). Dividing the cDNAs into smaller and different portions increases the possibility that a particular differentially expressed member of a gene family will contain a smaller fragment that is sufficiently different from other homologous members and can be enriched in the final subtracted cDNA mixture (Wang and Brown 1991; Hubank and Schatz 1994). Once a small cDNA fragment is cloned and sequenced, numerous approaches, such as 5′- or 3′-rapid amplification of cDNA ends (RACE) (see Chapter 2), can be used to quickly obtain corresponding full-length cDNAs (reviewed in Luk'ianov et al. (1999)).

Another drawback of the SSH technique is the typical small inserts in the final subtracted cDNA libraries (average ~200 bp).This problem is generated by several factors related to the SSH procedure, such as more efficient hybridization of shorter fragments, preferential amplification of these fragments by PCR, and higher efficiency of subcloning of short fragments in plasmid vectors than those of longer ones. To minimize this undesirable selection for shorter fragments, several modifications of the SSH procedure can be incorporated to increase the representation of larger inserts in the final subtracted products, such as size selection of the subtracted cDNA products before subcloning. To minimize this problem in the current of SSH protocol described here, we use adapters with identical sequences for the first 22 nucleotides at their 5′-ends (Ad 1 and Ad2R), instead of being completely different in sequence as in the original description (Ad1 and Ad2) (Siebert et al. 1995). These sequence changes introduce

short complimentary inverted terminal repeats on the end of the cDNA molecule which carry different adapters on their ends and allow the primary amplification to be carried out with a single PCR primer. This introduces weak suppression PCR effect during the primary amplification since the length of complementary part is equal of the length of the primer. In this condition the amplification of very short (less than 200 bp) DNA fragments significantly diminished (Jin et al. 1997) and the risk of nonspecific amplification also decreases (Rebrikov et al. 2004; Takarada 1994.

These equalization steps are not intended to eliminate the shorter fragments which may represent truly differentially expressed cDNAs, but instead are designed to balance representation of different fragment sizes, thereby increasing the complexity of the subtracted cDNA library.

## 5. DETAILED PROTOCOLS: MATERIALS

### 5.1 Oligonucleotides

The following oligonucleotides are used at a concentration of 10 μM. Whenever possible, oligonucleotides should be HPLC- or gel-purified.

1. cDNA synthesis primers: 5′-*TTTTGTACAAGCT(T)*$_{30}$-3′
2. Adapters:
   (Ad1) – 5′-*CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT*-3′
   3′-*GGCCCGTCCA*-5′
   (Ad2) – 5′-*TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT*-3′
   3′-*GCCTCCCGCCA*-5′
   (Ad2R) – 5′-*CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGT*-3′
   3′-*GCCGGCTCCA*-5′
3. PCR primers: (P1) – 5′-*CTAATACGACTCACTATAGGGC*-3′
   (P2) – 5′-*TGTAGCGTGAAGACGACAGAA*-3′
   Nested primer 1 (NP1) – 5′-*TCGAGCGGCCGCCCGGGCAGGT*-3′
   Nested primer 2 (NP2) – 5′-*AGGGCGTGGTGCGGAGGGCGGT*-3′
   Nested primer 2R (NP2R) – 5′-*AGCGTGGTCGCGGCCGAGGT*-3′
4. MOS PCR primer (NP2Rs) – 5′-*GGTCGCGGCCGAGGT*-3′
5. Blocking solution: a mixture of the cDNA synthesis primer, nested primers (NP1 and NP2R), and their respective complementary oligonucleotides (2 mg/ml each).

### 5.2 Buffers and Enzymes

1. AMV reverse transcriptase (20 U/μl; Life Technologies, Gaithersburg, MD).
2. 5X First strand buffer: 250 mM Tris-HCl, pH 8.5,40 mM $MgCl_2$, 150 mM KCl, and 5 mM dithiothreitol (DTT).

3. 20X Second strand enzyme cocktail: DNA polymerase I (6 U/µl, New England Biolabs, Beverly, MA).
4. RNase H (0.25 U/µl, Epicentre Technologies, Madison,WI).
5. T4 DNA ligase (3 U/µl, New England Biolabs).
6. 5X Second strand buffer: 500 mM KCl, 50 mM ammonium sulfate, 25 mM $MgCl_2$, 0.75 mM b-NAD, 100 mM Tris-HCl, pH 7.5, 0.25 mg/ml BSA.
7. T4 DNA polymerase (3 U/µl, New England Biolabs).
8. 10X *Rsa* I restriction buffer: 100 mM Bis-Tris propane/HCl, pH 7.0, 100 mM $MgCl_2$, and 1 mM DTT.
9. *Rsa* I (10 U/ml, New England Biolabs).
10. T4 DNA ligase (400 U/µl: contains 3 mM ATP [New England Biolabs]).
11. 5X DNA ligation buffer: 250 mM Tris-HCl, pH 7.8, 50 mM $MgCl_2$,10 mM DTT, 0.25 mg/ml BSA.
12. 4X Hybridization buffer: 4 M NaCl, 200 mM HEPES, pH 8.3, 4 mM cetyltrimethyl ammonium bromide (CTAB).
13. Dilution buffer: 20 mM HEPES-HCl, pH 8.3, 50 mM NaCl, 0.2 mM EDTA.
14. Advantage cDNA PCR Mix (BD Biosciences Clontech, Palo Alto, CA). This mix contains a mixture of KlenTaq-1 and DeepVent DNA polymerases (New England Biolabs, Beverly, MA) and TaqStart antibody (BD Biosciences Clontech); 10X reaction buffer (40 mM Tricine-KOH (pH 9.2 at 22°C), 3.5 mM $Mg(OAc)_2$, 10 mM KOAc, 75 mg/ml BSA). The TaqStart antibody provides automatic hot start PCR (Kellogg et al. 1994). Alternatively, *Taq* DNA polymerase can be used alone, but five additional thermal cycles will be needed in both the primary and secondary PCR, and the additional cycles may cause higher background. If the Advantage cDNA PCR Mix is not used, manual hot start or hot start with wax beads is strongly recommended to reduce nonspecific DNA synthesis.
15. 10 mM each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ).
16. 20X EDTA/glycogen mix: 0.2 M EDTA; 1 mg/ml glycogen.
17. 4 M $NH_4OAc$, TN buffer: 10 mM Tris-HCl, 10 mM NaCl.
18. ExpressHyb hybridization solution (BD Biosciences Clontech).
19. Sterile deionized water.

Please note that the cycling parameters in this protocol have been optimized using the MJ Research PTC-200 DNA thermal cycler. For a different type of thermal cycler, the cycling parameters must be optimized for that machine. We recommend performing subtractions in both directions (tracer–driver and in parallel driver–tracer) for each DNA pair being compared. This forward- and reverse-subtracted DNA may be useful for differential screening of the resulting subtracted libraries. We also recommend performing self-subtractions (with both tracer and driver prepared from the same DNA sample) as a control experiment for fast examination of subtraction efficiency (see Note 1). For models such as RNA or DNA injections, or viral infections, it is extremely important to add appropriate DNA into driver sample (see Note 2).

## 6. DETAILED PROTOCOLS: METHODS

### 6.1 Preparation of Subtracted cDNA or Genomic DNA Library

#### *6.1.1 RNA and DNA isolation*

Two micrograms of genomic DNA or RNA is required per subtraction. Most commonly used methods for isolation of RNA and genomic DNA are appropriate for subtraction experiments (Chomczynski and Sacchi 1987). Nevertheless, the quality of DNA or RNA is very important for successful experiment. Whenever possible, samples being compared should be purified side by side utilizing the same reagents and protocol. Alternatively, commercially available kits from different vendors can be used for RNA and DNA isolation.

If genomic DNA is used as a starting material, the next step is *Rsa* I or *Alu* I digestion (Section 6.1.3). If RNA is used as a starting material, the next step is cDNA synthesis (Section 6.1.2).

*Note:* For simplicity, the term "cDNA" will be used throughout the protocol, but the protocol is suitable for genomic DNA subtraction with no changes in the amount of any reagents required to perform subtraction.

#### *6.1.2 cDNA synthesis*

There are two steps involved in cDNA synthesis: first strand cDNA synthesis and second strand cDNA synthesis. During first strand cDNA synthesis, AMV reverse transcriptase synthesizes cDNA using poly(A)+ RNA as a template. During second strand cDNA synthesis, DNA polymerase I uses first strand cDNA as a template. The following protocol is recommended for generating a subtracted library from 2 µg of poly(A)+ RNA. If enough poly(A)+ RNA is not available, the switch mechanism at the 5′-end of RNA templates (SMART) amplification technology (BD Biosciences Clontech), or FirstChoice RLM-RACE kit (Ambion) can be used to preamplify high-quality cDNA from total RNA (11) (see Note 3).

*First strand cDNA synthesis*

Perform this procedure individually with each tracer and driver poly(A)+ RNA sample.

1. For each tracer and driver sample, combine the following components in a sterile 0.5-ml microcentrifuge tube (do not use a polystyrene tube):
   – Poly(A) + RNA (2 µg) to 2–4 µl
   – cDNA synthesis primer (10 µM) to 1 µl
   – If needed, add sterile $H_2O$ to a final volume of 5 µl
2. Incubate the tubes at 70°C in a thermal cycler for 2 min
3. Cool at room temperature for 2 min and briefly centrifuge using a microcentrifuge at maximum rotation speed (we recommend 6000–7000 rpm)
4. Add the following to each reaction tube:
   – 2 µl 5X First strand buffer
   – 1 µl dNTP mixture (each 10 mM)
   – 0.5 µl Sterile $H_2O$

(Optional: to monitor the progress of cDNA synthesis, dilute 0.5 µl of [$\alpha^{32}$P] dCTP (10 mCi/ml, 3000 Ci/mM) with 9 µl of $H_2O$ and replace the $H_2O$ above with 1 µl of the diluted label.)
- 0.5 µl 0.1 M DTT
- 1 µl AMV reverse transcriptase (20 U/µl)

5. Gently vortex and briefly centrifuge the tubes
6. Incubate the tubes at 42°C for 1.5 h in an air incubator
7. Place the tubes on ice to terminate first strand cDNA synthesis and immediately proceed to second strand cDNA synthesis

*Second strand cDNA synthesis*

1. Add the following components (previously cooled on ice) to the first strand synthesis reaction tubes:
   - 48.4 µl Sterile water
   - 16.0 µl 5X Second strand buffer
   - 1.6 µl dNTP mix (10 mM)
   - 4.0 µl 20X Second strand enzyme cocktail
2. Mix the contents and briefly spin the tubes. The final volume should be 80 µl.
3. Incubate the tubes at 16°C (water bath or thermal cycler) for 2 h.
4. Add 2 µl (6 U) of T4 DNA polymerase. Mix contents well.
5. Incubate the tube at 16°C for 30 min in a water bath or a thermal cycler.
6. Add 4 µl of 0.2 M EDTA to terminate second strand synthesis.
7. Perform phenol:chloroform extraction and ethanol precipitation (see Note 4).
8. Dissolve pellet in 50 µl of TN buffer.
9. Transfer 6 µl to a fresh microcentrifuge tube. Store this sample at ?20°C until after *Rsa* I digestion. This sample will be used for agarose gel electrophoresis to estimate yield and size range of the ds cDNA-synthesized products.

### *6.1.3 Rsa I digestion*

Perform the following procedure with each experimental double-stranded tracer and driver cDNA. This step generates shorter, blunt-ended double-stranded cDNA fragments optimal for subtractive hybridization.

1. Add the following reagents into the tube from Section 1.2.2, step 8:
   - 43.5 µl double-stranded cDNA
   - 5.0 µl 10X *Rsa* I restriction buffer
   - 1.5 µl *Rsa* I (10 U/µl)
2. Mix and incubate at 37°C for 2–4 h.
3. Use 5 µl of the digest mixture and analyze on a 2% agarose gel along with undigested cDNA (Section 6.1.2, step 9 or Section 6.1.1 for genomic DNA) to analyze the efficiency of *Rsa* I digestion.

*Note:* Continue the digestion during electrophoresis and terminate the reaction only after you are satisfied with the results of the analysis.

4. Add 2.5 µl of 0.2 M EDTA to terminate the reaction.
5. Perform phenol:chloroform extraction and ethanol precipitation (see Note 4–6).
6. Dissolve each pellet in 6 µl of TN buffer (see Note 7) and store at –20°C.

Driver cDNA preparation is now complete.

### *6.1.4 Adapter ligation*

It is strongly recommended that you perform subtractions in both directions for each tracer or driver cDNA pair. Forward subtraction is designed to enrich differentially expressed transcripts present in tracer but not in driver; reverse subtraction is designed to enrich differentially expressed sequences present in driver but not in tracer. The availability of such forward- and reverse-subtracted cDNAs will be useful for differential screening of the resulting subtracted tracer cDNA library (see Section 6.4).

The tracer cDNAs are ligated separately to Ad1 (tracer 1-1 and 2-1) and Ad2R (tracer 1-2 and 2-2). It is highly recommended that a third ligation of both adapters 1 and 2R to the tracer cDNAs (unsubtracted tracer control 1-c and 2-c) be performed and used as a negative control for subtraction. Please note that the adapters are not ligated to the driver cDNA.

1. Dilute 1 µl of each *Rsa* I-digested tracer cDNA from Section 6.1.3 with 5 µl of TN buffer.
2. Prepare a master ligation mix of the following components for each reaction:
   – 3 µl Sterile water
   – 2 µl 5X Ligation buffer
   – 1 µl T4 DNA ligase (400 U/µl)

Please note that ATP required for ligation is in the T4 DNA ligase (3 mM initial, 300 µM final).

3. For each tracer cDNA mixture, combine the following reagents in a 0.5-ml microcentrifuge tube in the order shown. Pipet the solution up and down to mix thoroughly.

| Tube no. | 1 | 2 |
|---|---|---|
| Component | Tracer 1-1 (µl) | Tracer 1-2 (µl) |
| Diluted tracer cDNA | 2 | 2 |
| Adapter Ad1 (10 µM) | 2 | – |
| Adapter Ad2R (10 µM) | – | 2 |
| Master ligation mix | 6 | 6 |
| Final volume | 10 | 10 |

4. In a fresh microcentrifuge tube, mix 2 µl of tracer 1-1 and 2 µl of tracer 1-2. This is your unsubtracted tracer control 1-c. Do the same for each tracer cDNA sample. After ligation, approximately one third of the cDNA molecules in each unsubtracted tracer control tube will have two different adapters on their ends, suitable for exponential PCR amplification with adapter-derived primers.
5. Centrifuge the tubes briefly and incubate at 16°C overnight.
6. Stop the ligation reaction by adding 1 µl of 0.2 M EDTA.
7. Heat samples at 72°C for 5 min to inactivate the ligase.
8. Briefly centrifuge the tubes. Remove 1 µl from each unsubtracted tracer control (1-c, 2-c, . . .) and dilute into 1 ml of $H_2O$. These samples will be used for PCR amplification.

Preparation of your experimental adapter-ligated tracer cDNAs 1-1 and 1–2 is now complete.

Perform ligation efficiency test before proceeding to the next section (see Note 8).

### *6.1.5 Subtractive hybridization*

*First hybridization*

1. For each tracer sample, combine the reagents in the following order:

| Component | Hybridization 1.1 (µl) | Hybridization 1.2 (µl) |
|---|---|---|
| Rsa I-digested driver cDNA (Section 6.1.3, step 7) | 1.5 | 1.5 |
| Ad1-ligated tracer 1-1 (Section 6.1.4, step 8) | 1.5 | – |
| Ad2R-ligated tracer 1-2 (Section 6.1.4, step 5) | – | 1.5 |
| 4X Hybridization buffer | 1.0 | 1.0 |
| Final volume | 4.0 | 4.0 |

2. Overlay samples with one drop of mineral oil and centrifuge briefly.
3. Incubate samples in a thermal cycler at 98°C for 1.5 min. Incubate samples at 68°C for 8 h (see Note 9) and then proceed immediately to the second hybridization (see Note 21).

*Second hybridization*

Repeat the following steps for each experimental driver cDNA.

1. Add the following reagents into a sterile 0.5-µl microcentrifuge tube:
   – 1 µl Driver cDNA (Section 6.1.3, step 6)
   – 1 µl 4X Hybridization buffer
   – 2 µl Sterile water
2. Place 1 µl of this mixture in a 0.5-ml microcentrifuge tube and overlay it with one drop of mineral oil.
3. Incubate in a thermal cycler at 98°C for 1.5 min (see Note 10).
4. Remove the tube of freshly denatured driver from the thermal cycler (see Note 11).
5. To the tube of freshly denatured driver cDNA, add hybridized sample 1.1 and hybridized sample 1.2 (from first hybridization) in that order. This ensures that the two hybridization samples are mixed only in the presence of excess driver cDNA.
6. Incubate the hybridization reaction at 68°C overnight.
7. Add 100 µl of dilution buffer to the tube and mix well by pipetting.
8. Incubate in a thermal cycler at 72°C for 7 min.
9. Store hybridization solution at –20°C (see Note 12).

*PCR amplification*

Differentially presented DNAs are selectively amplified during the reactions described in this section. Each experiment should have at least four reactions: subtracted tracer cDNAs, unsubtracted tracer control (1-c), reverse-subtracted tracer cDNAs, and unsubtracted driver control for the reverse subtraction (2-c).

PRIMARY PCR

1. Place a 1 µl aliquot of each diluted cDNA sample (i.e. each subtracted sample from Section 6.1.6, step 8, and the corresponding diluted unsubtracted tracer control from Section 6.1.4, step 8) into an appropriately labeled tube (see Note 12).
2. Prepare a master mix for all of the primary PCR tubes plus one additional tube. For each reaction combine the reagents in the order shown:

| Reagent | Amount per reaction (µl) |
|---|---|
| Sterile water | 19.5 |
| 10X PCR reaction buffer | 2.5 |
| dNTP mix (10 mM) | 0.5 |
| PCR primer P1 (10 µM) | 1.0 |
| 50X Advantage cDNA PCR Mix | 0.5 |
| Total volume | 24.0 |

3. Place 24 µl aliquot of master mix into each reaction tube prepared in step 1.
4. Overlay with 50 µl of mineral oil. Skip this step if an oil-free thermal cycler is used.
5. Incubate the reaction mixture in a thermal cycler at 75°C for 5 min to extend the adapters (see Note 13). Do not remove the samples from the thermal cycler.
6. Immediately commence 26 cycles of:
   95°C 10 s
   66°C 10 s
   72°C 1.5 min
7. Analyze 4 µl from each tube on a 2% agarose/EtBr gel run in 1X TAE buffer (see Notes 14 and 15).

SECONDARY PCR

1. Dilute 2 µl of each primary PCR mixture in 38 µl of water.
2. Place 1 µl aliquot of each diluted primary PCR product mixture from step 1 into an appropriately labeled tube.
3. Prepare a master mix for the secondary PCR samples plus one additional reaction by combining the reagents in the following order:

| Reagent | Amount per reaction (µl) |
|---|---|
| Sterile water | 18.5 |
| 10X PCR reaction buffer | 2.5 |
| Nested PCR primer NP1 (10 µM) | 1.0 |
| Nested PCR primer NP2R (10 µM) | 1.0 |
| dNTP mix (10 mM) | 0.5 |
| 50X Advantage cDNA PCR Mix | 0.5 |
| Total volume | 24.0 |

4. Place 24 µl aliquot of master mix into each reaction tube from step 2.
5. Overlay with one drop of mineral oil. Skip this step if an oil-free thermal cycler is used.
6. Immediately commence 10–12 cycles of:
   95°C 10 s
   68°C 10 s
   72°C 1.5 min
7. Analyze 4 µl from each reaction on a 2% agarose/EtBr gel.
8. Store PCR products at −20°C. This PCR product is now enriched for differentially presented DNAs. At this point if you are not going to perform MOS, please go to Section 6.3 (cloning of subtracted library) in this method section.

### 6.2 Mirror Orientation Selection

The major drawback of SSH is the presence of background clones that represent nondifferentially expressed DNA species in the subtracted libraries. In some difficult cases, the number of background clones may considerably exceed the number of target clones. To overcome this problem, we recommend MOS – a simple procedure that substantially decreases the number of background clones in the libraries generated by SSH (see Note 16).

We recommend the use of MOS in the following cases:

- If the percentage of differentially expressed clones found during differential screening is very low (e.g. 1–5%). The MOS procedure can increase the number of differential clones up to tenfold.
- If most of the differentially expressed clones found are false positive clones (i.e. clones that appear to be differentially expressed in the differential screening procedure, but turn out not to be differentially expressed in the Northern blot or reverse transcription–PCR analysis). The MOS procedure decreases the portion of false positive clones by several folds.
- If the primary PCR in SSH requires more than 30 cycles (but not more than 36 cycles, see Note 15) to generate agarose gel detectable PCR product. If the primary PCR requires more than 30 cycles, the problems described in the previous two items will usually appear. If you want to perform MOS, please follow the following procedure for PCR amplification using the second hybridization solution (Section 6.1.6, step 9).

#### *6.2.1 PCR amplification for MOS*

If the complexity of tracer and driver samples is very high or if the difference in gene expression between tracer and driver is very small, one can plan to perform MOS from the beginning of the experiment. In that case, after subtractive hybridization (Section 6.1.5), perform PCR amplification using the following protocol instead of using protocol in Section 6.1.6. If you have already made the

SSH subtracted library and found high background upon differential screening, you may perform MOS on the SSH-generated library. You can use the hybridization mix generated in Section 6.1.6, step 9) for PCR amplification using the following protocol:

PRIMARY PCR

1. Transfer 10 µl of each diluted second hybridization (from Section 6.1.5) into appropriately labeled tubes (see Note 12)
2. Prepare a master mix for the primary PCR-1. For each reaction, combine the reagents as follows:

| Component | Amount per reaction (µl) |
|---|---|
| Sterile water | 92.5 |
| 10X PCR buffer | 12.5 |
| dNTP mixture (10 mM each) | 2.5 |
| PCR primer | 15.0 |
| 50X polymerase mixture | 2.5 |
| Total volume | 115 |

3. Mix well and briefly centrifuge the tube
4. Place 115 µl aliquot of master mix into each reaction tube from step 1
5. Place 125 µl aliquot of final mix into five 0.5 µl PCR tubes (25 µl per tube)
6. Overlay with one drop of mineral oil
7. Incubate the reaction mixture in a thermal cycler at 72°C for 5 min to extend the adapters (see Note 13)
8. Immediately commence thermal cycling (see Note 17 to calculate the number of PCR cycles you need):
   95°C 10 s
   66°C 10 s
   72°C 1.5 min
9. Combine 2 µl of each (of 5) primary PCR-1 product in one tube and add 390 µl of water
10. Place 1 µl aliquot of each diluted primary PCR-1 product mixture from step 9 into an appropriately labeled PCR tube
11. Prepare master mix for primary PCR-2 as follows:

| Component | Amount per reaction (µl) |
|---|---|
| Sterile water | 19.5 |
| 10X PCR buffer | 2.5 |
| dNTP mixture (10 mM each) | 0.5 |
| PCR primer | 11.0 |
| 50X polymerase mixture | 0.5 |
| Total volume | 24 |

12. Mix well and briefly centrifuge the tube
13. Place 24 µl aliquot of master mix into each reaction tube from step 10
14. Overlay with one drop of mineral oil
15. Immediately commence thermal cycling:
    – 10 Cycles:
      94°C 30 s
      66°C 30 s
      72°C 1.5 min
16. Analyze 4 µl from each reaction on a 2% agarose/EtBr gel

SECONDARY PCR

1. Dilute 2 µl of each primary PCR-2 product generated in Section 6.2.1, step 16 in 38 µl of water
2. Place 2 µl aliquot of each diluted primary PCR-2 product into an appropriately labeled tube
3. Prepare a master mix for secondary PCR. For each reaction, combine the reagents as follows:

| Component | Amount per reaction (µl) |
|---|---|
| Sterile water | 37.0 |
| 10X PCR buffer | 5.0 |
| dNTP mixture (10 mM each) | 1.0 |
| PCR primer NP1 | 2.0 |
| PCR primer NP2R | 2.0 |
| 50X polymerase mixture | 1.0 |
| Total volume | 48.0 |

4. Mix well and briefly centrifuge the tube
5. Place 48 µl aliquot of master mix into each reaction tube from step 2
6. Overlay with one drop of mineral oil
7. Immediately commence thermal cycling:
    – 10 Cycles:
      95°C 10 s
      68°C 10 s
      72°C 1.5 min
8. Analyze 4 µl from each tube on a 2% agarose/EtBr gel
9. The PCR product of secondary PCR is purified by phenol or chloroform extraction and ethanol precipitation (see Note 4)
10. Dissolve the pellet in 20–40 µl of NT buffer up to concentration 20 ng/µl of DNA
11. Analyze 2 µl of purified PCR product from step 9 on a 2% agarose/EtBr gel
12. Dilute 1 µl of purified PCR product from step 9 in 1.6 ml of water (this will be your undigested control)
13. Store at –20°C

### *6.2.2 Xma I digestion*

1. Add the following reagents into the tube:
   – 12 µl $H_2O$
   – 2 µl 10X *Xma* I restriction buffer
   – 5 µl DNA (Section 6.2.1, secondary PCR, step 10)
   – 1 µl *Xma* I (10 U/µl)
2. Mix and incubate at 37°C for 2 h
3. Add 2 µl of 0.2 M EDTA to terminate the reaction
4. Incubate at 70°C for 5 min to inactivate enzyme
5. Store at –20°C.

### *6.2.3 MOS hybridization*

Combine the following reagents in a fresh 1.5-ml tube:
– 2 µl $H_2O$
– 1 µl *Xma* I-digested DNA
– 1 µl 4X Hybridization buffer
Place 2 µl of this mixture in a 0.5-ml microcentrifuge tube and overlay with one drop of mineral oil.
– Incubate in a thermal cycler at 98°C for 1.5 min
– Incubate in a thermal cycler at 68°C for 3 h
– Add 200 µl of dilution buffer to the tube and mix by pipetting
– Heat in a thermal cycler at 70°C for 7 min.
– Store at –20°C.

### *6.2.4 MOS PCR amplification*

1. Prepare a master mix for all MOS PCR reactions as follows:

| Component | Amount per reaction (µl) |
|---|---|
| Sterile water | 19.5 |
| 10X PCR buffer | 2.5 |
| dNTP mixture (10 mM each) | 0.5 |
| MOS PCR primer (NP2Rs) | 1.0 |
| 50X polymerase mixture | 0.5 |
| Total volume | 24.0 |

2. Add 1 µl of each diluted cDNA sample (after hybridization and the corresponding undigested control) to an appropriately labeled tube containing 24 µl of master mix.
3. Overlay with one drop of mineral oil.
4. Incubate the reaction mix in a thermal cycler at 72°C for 2 min to extend the adapters. (Do not remove the samples from the thermal cycler.)
5. Immediately commence thermal cycling:
   – 19 Cycles:
   94°C 30 s

62°C 30 s
72°C 1.5 min
6. Analyze 4 µl from each tube on 2% agarose/EtBr gel.

### 6.3 Cloning of Subtracted cDNAs

Once a subtracted sample is confirmed to be enriched in cDNAs derived from differentially expressed genes, the PCR products (from Section 6.1.5, secondary PCR or from Section 6.2.4, MOS PCR amplification) can be subcloned using several conventional cloning techniques. The following describes two such methods that are currently used.

#### *6.3.1 T/A cloning*

Use 3 µl of the secondary PCR product (Section 6.1.5, step 8) or MOS PCR product (Section 6.2.4, step 6) for cloning with a T/A-based system, such as the Advantage PCR Cloning Kit (Invitrogen), according to the manufacturer's protocol. The library may be transformed into bacteria (electrocompetent cells) by electroporation (1.8 kV) using a pulser (BioRad) and plated onto agar plates containing ampicillin, X-Gal, and IPTG. Recombinant (white colonies) clones are picked and used to inoculate LB medium in 96-well microtiter plates. Bacteria should be allowed to grow at 37°C for 4 h before insert amplification (Section 6.4.1). Typically, $10^4$ independent clones from 1 µl of secondary PCR product can be obtained using the above cloning system and electroporation. It is important to optimize the cloning efficiency because a low cloning efficiency will result in a high background.

#### *6.3.2 Site-specific or blunt-end cloning*

For site-specific cloning, cleave at the *Eag* I, *Not* I, and *Xma* I (*Sma* I, *Srf* I) sites embedded in the adapter sequences and then ligate the products into an appropriate plasmid vector. Keep in mind that all of these sites might be present in the cDNA fragments. The *Rsa* I site in the adapter sequences can also be used for blunt-ended cloning. Commercially available cloning kits are suitable for these purposes. The number of independent colonies obtained for each library depends on the estimated number of differentially expressed genes, as well as the subtraction and subcloning efficiencies. In general, 500 colonies can be initially arrayed and studied. The complexity of the library can be increased by additional subcloning of secondary PCR products (from Section 6.1.5) or MOS PCR products (from Section 6.2.4).

### 6.4 Differential Screening of the Subtracted cDNA Library

Two approaches can be utilized for differential screening of the arrayed subtracted cDNA clones; cDNA dot blots and colony dot blots. For colony dot blots, bacterial colonies are spotted on nylon filters, grown on antibiotic

plates, and processed for colony hybridization. This method is cheaper and more convenient, but it is less sensitive and gives a higher background than PCR-based cDNA dot blots. The cDNA array approach is highly recommended (Section 6.4.2).

### *6.4.1 Amplification of cDNA inserts by PCR*

For high-throughput screening, a 96-well format PCR from one of several thermal cycler manufacturers is recommended. Alternatively, single tubes can be used.

1. Randomly pick 96 white bacterial colonies
2. Grow each colony in 100 µl of LB-amp medium in a 96-well plate at 37°C for at least 2 h (up to overnight) with gentle shaking
3. Prepare a master mix for 100 PCR reactions (see Note 18):

| Reagent | Amount per reaction (µl) |
|---|---|
| 10X PCR buffer | 2.0 |
| Nested primer NP1* | 0.6 |
| Nested primer NP2R* | 0.6 |
| dNTP mix (10 mM) | 0.4 |
| $H_2O$ | 15.0 |
| 50X Advantage cDNA PCR Mix | 0.4 |
| Total volume | 19.0 |

Alternatively, primers flanking the insertion site of the vector can be used in PCR amplification of the inserts.

4. Place 19 µl aliquot of the master mix into each tube or well of the reaction plate
5. Transfer 1 µl of each bacterial culture (from step 2) to each tube or well containing master mix (see Note 19)
6. Perform PCR in an oil-free thermal cycler with the following conditions:
   – 1 cycle:
     94°C 2 min
   – Then 22 cycles:
     94°C 30 s
     68°C 3 min
7. Analyze 5 µl from each reaction on a 2% agarose/EtBr gel (see Note 20).

### *6.4.2 Preparation of cDNA dot blots of the PCR products*

1. For each PCR reaction, combine 5 µl of the PCR product and 5 µl of 0.6 M NaOH (freshly made or at least freshly diluted from concentrated stock).
2. Transfer 1–2 µl of each mixture to a nylon membrane. This can be accomplished by dipping a 96-well replicator in the corresponding wells of a microtiter dish used in the PCR amplification and spotting it onto a dry nylon

filter. Make at least two identical blots for hybridization with subtracted and reverse-subtracted probes (Section 6.1.4; see Note 22).
3. Neutralize the blots for 2–4 min in 0.5 M Tris-HCl (pH 7.5) and wash in 2X SSC.
4. Immobilize cDNA on the membrane using a UV cross-linking device (such as Stratagene's UV Stratalinker), or bake the blots for 4 h at 68°C.

### *6.4.3 Differential hybridization with tracer and driver cDNA probes*

Label tracer and driver cDNA probes by random-primer labeling using a commercially available kit. The hybridization conditions given here are optimized for BD Biosciences Clontech's ExpressHyb solution; the optimal hybridization conditions for other systems should be determined empirically.

The following four different probes will be used for differential screening hybridization:
1. Tracer-specific subtracted probe (forward-subtracted probe)
2. Driver-specific subtracted probe (reverse-subtracted probe)
3. cDNA probe synthesized directly from tracer mRNA
4. cDNA probe synthesized directly from driver mRNA

See Note 23.
1. Prepare a prehybridization solution for each membrane:
   (a) Combine 50 µl of 20X SSC, 50 µl of sheared salmon sperm DNA (10 mg/ml), and 10 µl of blocking solution (containing 2 mg/ml of unpurified NP1, NP2R, cDNA synthesis primers, and their complementary oligonucleotides).
   (b) Boil the blocking solution for 5 min, then chill on ice.
   (c) Combine the blocking solution with 5 ml of ExpressHyb hybridization solution (BD Biosciences Clontech).
2. Place each membrane in the prehybridization solution prepared in step 1. Prehybridize for 40–60 min with continuous agitation at 72°C.

*Note:* It is important to add blocking solution in prehybridization solution. Because subtracted probes contain the same adapter sequences as arrayed clones, these probes hybridize to all arrayed clones regardless of the sequences.
3. Prepare hybridization probes:
   (a) Mix 50 µl of 20X SSC, 50 µl of sheared salmon sperm DNA (10 mg/ml) and 10 µl blocking solution, and purified probe (at least $10^7$ cpm per 100 ng of subtracted cDNA). Make sure the specific activity of each probe is approximately equal.
   (b) Boil the probe for 5 min, then chill on ice.
   (c) Add the probe to the prehybridization solution.
4. Hybridize overnight with continuous agitation at 72°C.
5. Prepare low-stringency (2X SSC/0.5% SDS) and high-stringency (0.2X SSC/0.5% SDS) washing buffers and warm them up to 68°C.
6. Wash membranes with low-stringency buffer (4X 20 min at 68°C), then wash with high stringency buffer (2X 20 min at 68°C).

7. Perform autoradiography.
8. If desired, remove probes from the membranes by boiling for 7 min in 0.5% SDS. Blots can typically be reused at least five times.

*Note:* To minimize hybridization background, store the membranes at –20°C when they are not in use.

## 7. NOTES

Suppression subtractive hybridization and MOS are available as the custom service from Evrogen (http://www.evrogen.com/).

1. Self-subtraction (with both tracer and driver prepared from one DNA sample) is recommended as the best comprehensive control. In the self-subtracted control, subtracted secondary PCR requires more cycles than unsubtracted secondary PCR. A number of other control experiments may be performed for fast analysis of SSH and MOS experiments (see below).
2. For experimental systems such as transfection, overexpression, mRNA injection, or viral infection using mammalian or viral expression systems, we strongly recommend that you use affecting RNA or DNA sequence for compensation of overexpressed sequence concentration. For example, if you are searching for p53-up-regulated genes in a p53 overexpressed cell line, add *Rsa* I-digested p53 cDNA into *Rsa* I-digested driver sample (about one tenths of driver cDNA concentration) after you prepare adapter-ligated tracer. Adding exogenous DNA/RNA earlier (in RNA sample) or before *Rsa* I digestion may cause disproportion of this material in initial DNAs.
3. We recommend the use of poly(A)+ RNA as starting material. Amplified cDNA should be used as a starting material only when enough RNA is not available. The amplification of two cDNA samples to be subtracted is a crucial procedure and any disproportion during cDNA amplification may cause artifacts in the subtraction results. Some RNA types cannot be amplified because the messages are too long and are not available for subtraction and analysis.
4. Phenol–chloroform extraction and ethanol precipitation:
   (a) Add equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1) and vortex thoroughly.
   (b) Centrifuge the tubes at 14,000 rpm for 10 min.
   (c) Remove the top aqueous layer and transfer to a fresh microcentrifuge tube.
   (d) Add equal volumes of chloroform:isoamyl alcohol (24:1) and vortex thoroughly.
   (e) Centrifuge the tubes at 14,000 rpm for 10 min.
   (f) Remove the top aqueous layer and transfer to a fresh microcentrifuge tube.
   (g) Add 0.5 volume of 4 M $NH_4OAc$, mix, then add 2.5 volumes of 95% ethanol and vortex thoroughly.
   (h) Centrifuge the tubes at 14,000 rpm for 20 min.
   (i) Remove the supernatant carefully.

(j) Add 200 µl of 80% ethanol.
(k) Centrifuge the tubes at 14,000 rpm for 10 min.
(l) Remove the supernatant carefully.
(m) Air-dry the pellets for 5–10 min.
(n) Dissolve the pellets in appropriate volume of TN buffer.

5. Using glycogen or any type of coprecipitants during DNA precipitation may increase viscosity of DNA solution and prevent DNA hybridization in some cases. We recommend avoiding use of these reagents if possible.
6. We do not recommend using silica matrix-based PCR purification systems at this stage.
7. Water may denature short DNA fragments and may make the adapter ligation difficult. We advise using TN buffer.
8. Ligation efficiency test:
   (a) Place 1 µl aliquot of each undiluted unsubtracted control sample (Section 6.1.4, step 8) into an appropriately labeled 0.5-ml PCR tube.
   (b) Prepare a master mix for all of the reaction tubes. Combine the reagents as follows:

| Component | Amount per reaction (µl) |
|---|---|
| $H_2O$ | 19.5 |
| 10X PCR buffer | 2.5 |
| dNTP mixture (10 mM each) | 0.5 |
| PCR primer P1 | 1.0 |
| 50X polymerase mixture | 0.5 |
| Total volume | 24.0 |

   (c) Mix and briefly centrifuge the tubes.
   (d) Place 24 µl aliquot of master mix into each of the reaction tubes prepared in step 1.
   (e) Overlay with one drop of mineral oil.
   (f) Incubate the reaction mixture in a thermal cycler at 72°C for 5 min to extend the adapters.
   (g) Immediately commence thermal cycling:
      – 15 Cycles:
        95°C 10 s
        66°C 10 s
        72°C 1.5 min
   (h) Analyze 4 µl from each tube on a 2% agarose/EtBr gel. This PCR product should have a similar pattern to that of *Rsa* I-digested DNA. If PCR products are not visible after 15 cycles, perform three more cycles and again analyze the PCR product. If PCR products are not visible after 21 cycles, the activity of the polymerase mixture needs to be examined. If there is no problem with the polymerase mixture, the ligation reaction should be repeated with fresh samples before proceeding to the next step.

9. Recommended first hybridization times for different DNA samples:

| Sample type | First hybridization time (h) |
|---|---|
| Bacterial genome subtraction | 1–3 |
| Eukaryotic genome subtraction | 3–5 |
| cDNA subtraction | 7–12 |

10. We recommend that you use two blocks thermal cycler (or two thermal cyclers nearby) for proper and fast operations.
11. We recommend transferring this tube immediately after denaturing (98°C for 1.5 min) into thermal cycler with first hybridization process (68°C) and waiting for 1 min before proceeding to the next step.
12. If hybridization mix was frozen, we recommend the following before proceeding with PCR reactions: mix hybridization samples well by pipetting, heat in a thermal cycler at 72°C for 7 min, then mix again by pipetting and use for PCR.
13. This step "fills in" the missing strand of the adapters and thus creates binding sites for the PCR primers.
14. For some complicated subtractions (with complex tissues or eukaryotic genomes), we recommend performing primary PCR two times one by one. This procedure may significantly reduce background, generated by partial disruption of PCR suppression effect. First, perform primary PCR as described in Section 6.1.5. Then perform another primary PCR as follows:
    (a) Dilute 2 µl of each primary PCR product (from step 7) in 78 µl of water.
    (b) Place 1 µl of each diluted primary PCR product from step 1 into appropriately labeled tube.
    (c) Combine the following reagents to prepare a master mix for each reaction.

| Component | Amount per reaction (µl) |
|---|---|
| 10X PCR buffer | 2.5 |
| PCR primer P1 | 1.0 |
| dNTP mix (10 Mm) | 0.5 |
| H2O | 19.5 |
| 50X Advantage cDNA PCR Mix | 0.5 |
| Total volume | 24.0 |

    (d) Mix well and briefly centrifuge the tube.
    (e) Place 24 µl aliquot of master mix into each reaction tube from step 2.
    (f) Overlay with one drop of mineral oil.
    (g) Immediately commence thermal cycling:
        – 10 Cycles:
          95°C 10 s
          66°C 10 s
          72°C 1.5 min
    (h) Analyze 4 µl from each tube on a 2% agarose/EtBr gel, then proceed to secondary PCR (Section 6.1.5).

15. If the SSH primary PCR requires more then 36 cycles, "*in vitro* cloning" will occur. As a result, only false-positive clones may be found during differential screening procedure.
16. To illustrate the utility of combining SSH and MOS for eukaryotic genome comparison, we will describe our efforts to isolate genes that are present in one freshwater planaria warm strain but are absent in another. In this study, we used two closely related strains of freshwater planaria *Girardia tigrina* that reproduce in different ways. Whereas one strain has exclusively asexual reproduction, the other reproduces both sexually and asexually. The genomes of both strains of *G. tigrina* we compared to search for genetic determinants of asexuality. Total DNA from these strains was purified using the procedure described in Section 6.1.1. The SSH and MOS combination was used to isolate genes that are differentially present in each planaria strain. Forward subtraction (AB) was performed using asexual DNA (sample A) as tracer and sexual DNA (sample B) as driver, and the forward-subtracted DNA was enriched for DNA fragments specific to the asexual strain of freshwater planaria. Reverse-subtracted DNA (BA) was enriched in DNA fragments specific to the sexual planaria strain. Self-subtractions were performed for both DNA samples to get a quick idea of subtraction efficiency. Subsequent MOS PCR analysis confirmed that the self-subtractions (as well as undigested controls) require more PCR cycles to generate visible PCR product, indicating that the subtraction was successful (Figure 4).

We anticipated that the differences between tracer and driver DNA would be small, so we proceeded with a differential screening procedure. From each forward- and reverse-subtracted library, 86 randomly selected clones were arrayed (DNA dot blot) onto nylon membranes. DNA dot blots were hybridized to probes prepared from the subtracted and reverse-subtracted libraries. Figure 5 shows typical results of differential screening of a subtracted cDNA libraries obtained using the SSH and MOS combination. Normally, MOS results for DNA or cDNA reveal the following types of clones:

(a) Clones hybzridizing to the one probe only. These correspond to the differentially presented DNA, but must be verified by Southern blot analysis. The signal intensity depends on the copy number in genomic or extrachromosomal DNA.
(b) Clones hybridizing to both subtracted probes with the same efficiency. These clones most probably do not correspond to the differentially presented DNA and thus represent a background.
(c) Clones hybridizing to both subtracted probes with different hybridization efficiencies. In the case of genomic DNA subtraction, these clones may represent genes (DNA fragments) with different number of copies per genome. In the case of cDNA subtraction, these clones do represent differentially expressed clones. In some cDNA subtractions, this difference can be a result of random fluctuation and does not represent differentially

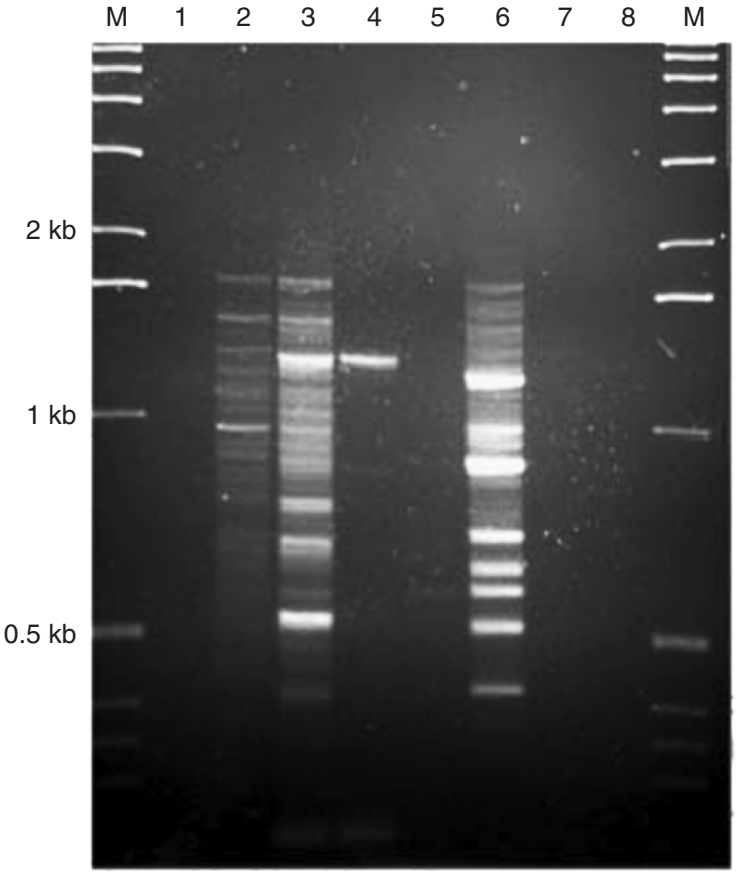


*Figure 4.* MOS stage significantly improves differential library quality in many cases. Lane 1: MOS PCR product of undigested control of BB self-subtraction. Lane 2: MOS PCR product of BB self-subtraction. Lane 3: MOS PCR product of BA experimental subtraction. Lane 4: MOS PCR product of undigested control of BA experimental subtraction. Lane 5: MOS PCR product of undigested control of AB experimental subtraction. Lane 6: MOS PCR product of AB experimental subtraction. Lane 7: MOS PCR product of AA self-subtraction. Lane 8: MOS PCR product of undigested control of AA self-subtraction.

expressed cDNA. For this reason, it is always recommended to confirm true differential expression of these clones by Northern blot analysis or reverse transcription polymerase chain reaction (RT-PCR).

(d) Clones that do not hybridize noticeably to either hybridization probe. These clones may not contain DNA insertion or may be present at very low concentration in the subtracted probe. (In most cases, they do not represent differentially presented clones.)

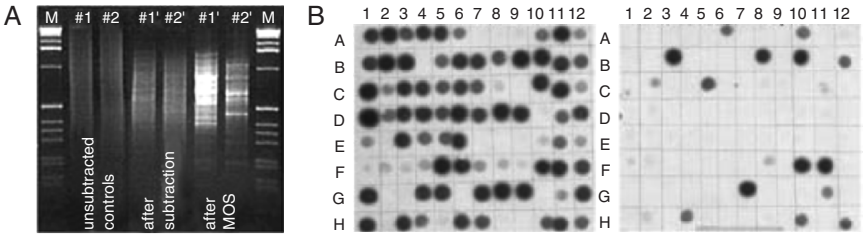


*Figure 5.* Typical results of suppression subtractive hybridization, MOS, and differential screening of the resulting libraries. (A) MOS reveals real differential bands and eliminates most of false positives. M – 1 kb DNA size marker ladder, #1 and #2 – cDNA samples 1 and 2; #1′ – sample 1 versus sample 2 subtraction; #2′ – sample 2 versus sample 1 subtraction. (B) Typical differential screening results. Randomly picked clones from #1′ subtracted cDNA library (after MOS) were hybridized with radioactively labeled #1′ (filter from the left) and #2′ (filter from the right) subtracted cDNA probes.

Differential screening revealed about 60% and 30% of the strain-specific clones in AB and BA libraries, respectively. Most of the nondifferential DNA sequences were identified as the *mariner* repetitive element, presented by ~7000 copies in each compared genome (Garcia-Fernandez et al. 1993).

17. The recommended number of primary PCR-1 cycles for MOS is the number of SSH primary PCR minus 2. For example, if the SSH primary PCR was visible on agarose/EtBr gel after 31 cycles, you will need 31 – 2 = 29 cycles of primary PCR-1 for MOS.
18. Short PCR primers NP1s and NP2s may be used for insert amplification to reduce hybridization background. However, this is not always necessary.
19. Freshly grown 96-well plates should be used for PCR before bacterial cells precipitate, otherwise 1 μl aliquots will not be equal.
20. It is possible that (5–10% of clones will not yield PCR product as a result of imperfect cloning.
21. The protocol uses 15 ng of ligated tracer cDNA and 450 ng of driver cDNA. The ratio of driver to tracer can be changed if different subtraction efficiency is desired.
22. We highly recommend that you make four identical blots. Two of the blots will be hybridized to forward- and reverse-subtracted cDNAs and the other two can be hybridized to cDNA probes synthesized from tracer and driver mRNAs.
23. The first two probes are the secondary PCR products (Section 6.1.5, step 8 or Section 6.2.1, secondary PCR, step 10) of the subtracted cDNA pool. The last two cDNA probes can be synthesized from the tracer and driver poly(A)+ RNA. They can be used as either single- or double-stranded cDNA probes. Alternatively, unsubtracted tracer and driver cDNA or preamplified cDNA from total RNA can be used if enough poly(A)+ RNA is not available. If you have made the MOS-subtracted library, you can still screen it using the same probes.

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