Identification of Differential Genes by Suppression Subtractive Hybridization: II. Subtractive Hybridization

Denis V. Rebrikov


INTRODUCTION

Suppression subtractive hybridization (SSH) is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. This technique can be used to compare two mRNA populations and obtain cDNAs representing genes that are either overexpressed or exclusively expressed in one population as compared to another. It can also be used for comparison of genomic DNA populations. This protocol describes a method for subtractive hybridization using adapter-ligated tester and RsaI-digested driver DNA samples.

RELATED INFORMATION

An overview of the SSH method is given in Identification of Differential Genes by Suppression Subtractive Hybridization: An Overview (Rebrikov 2008a). Protocols that describe subsequent steps in the SSH method are also available; see the following:

Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library (Rebrikov 2008b)
Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs (Rebrikov 2008c)
Identification of Differential Genes by Suppression Subtractive Hybridization: IV. Mirror Orientation Selection (MOS) (Rebrikov 2008d)
Identification of Differential Genes by Suppression Subtractive Hybridization: V. PCR-Based DNA Dot Blot (Rebrikov 2008e)
Identification of Differential Genes by Suppression Subtractive Hybridization: VI. Differential Hybridization with Tester and Driver DNA Probes (Rebrikov 2008f)

MATERIALS

Reagents

Driver and tester DNA from Steps 7 and 14, respectively, in Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library (Rebrikov 2008b)

This protocol uses 15 ng each of Ad1- and Ad2R-ligated tester DNA and 450 ng of driver DNA. The ratio of driver to tester can be changed if a different subtraction efficiency is desired.

Mineral oil

SSH dilution buffer
SSH hybridization buffer (4X)

**Equipment**

- Microcentrifuge
- Microcentrifuge tubes (0.5 mL)
- Thermal cyclers

*At least two thermal cyclers are needed; one must be preset to 68°C (see Step 8).*

**METHOD**

**First Hybridization**

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

<table>
<thead>
<tr>
<th>Component</th>
<th>Hybridization 1-1</th>
<th>Hybridization 1-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Driver DNA</td>
<td>1.5 µL</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>Ad1-ligated tester 1-1</td>
<td>1.5 µL</td>
<td>--</td>
</tr>
<tr>
<td>Ad2R-ligated tester 1-2</td>
<td>--</td>
<td>1.5 µL</td>
</tr>
<tr>
<td>4X SSH hybridization buffer</td>
<td>1.0 µL</td>
<td>1.0 µL</td>
</tr>
</tbody>
</table>

2. Overlay each sample with one drop of mineral oil. Centrifuge briefly.

3. Incubate the samples in a thermal cycler at 98°C for 1.5 min.

4. Incubate samples at 68°C for the time listed below (based on the sample type), and then proceed immediately to the second hybridization (Step 5).

<table>
<thead>
<tr>
<th>Sample type</th>
<th>First hybridization time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial genome subtraction</td>
<td>1-3 h</td>
</tr>
<tr>
<td>Eukaryotic genome subtraction</td>
<td>3-5 h</td>
</tr>
<tr>
<td>cDNA subtraction</td>
<td>7-12 h</td>
</tr>
</tbody>
</table>

**Second Hybridization**

*For each experimental (driver) DNA, perform the following series of steps.*

5. Mix the following reagents in a sterile 0.5-mL microcentrifuge tube:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount per reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Driver DNA</td>
<td>1 µL</td>
</tr>
<tr>
<td>4X SSH hybridization buffer</td>
<td>1 µL</td>
</tr>
<tr>
<td>H2O</td>
<td>2 µL</td>
</tr>
</tbody>
</table>

6. Place 1 µL of this mixture in a 0.5-mL microcentrifuge tube. Overlay it with one drop of mineral oil.
7. Incubate the mixture in a thermal cycler at 98°C for 1.5 min.

8. Remove the tube of freshly denatured driver DNA from the thermal cycler, and immediately place it in a second thermal cycler equilibrated to 68°C.  
*Using a second thermal cycler ensures rapid equilibration of specimens.*

9. Add the hybridization 1-1 and 1-2 samples from Step 4, in that order.  
*This ensures that the two hybridization samples are mixed only in the presence of excess of freshly denatured driver DNA.*

10. Incubate the hybridization reaction at 68°C overnight.

11. Add 100 µL of SSH dilution buffer to the tube and mix well by pipetting.

12. Incubate the mixture in a thermal cycler at 72°C for 7 min.  
The hybridization reaction is now complete. *It can be stored at -20°C or used in Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs* (Rebrikov 2008c) or *Identification of Differential Genes by Suppression Subtractive Hybridization: IV. Mirror Orientation Selection (MOS)* (Rebrikov 2008d).

**REFERENCES**


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**Recipe**

**SSH dilution buffer**

- 20 mM HEPES-HCl (pH 8.3)
- 50 mM NaCl
- 0.2 mM EDTA
### SSH hybridization buffer (4X)

- 4 M NaCl
- 200 mM HEPES (pH 8.3)
- 4 mM CTAB

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**Topic Introduction**

**Identification of Differential Genes by Suppression Subtractive Hybridization: An Overview**

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**INTRODUCTION**

Suppression subtractive hybridization (SSH) is one of the most powerful and popular methods for generating subtracted cDNA or genomic DNA libraries. This technique can be used to compare two mRNA populations and obtain cDNAs representing genes that are either overexpressed or exclusively expressed in one population as compared to another. It can also be used for comparison of genomic DNA populations. We have used SSH in studies of regeneration and development on various types of model organisms (including freshwater planaria regeneration, *Xenopus laevis* development, and mammalian brain cortex development). We also use SSH for the analysis of strain-specific genes in bacteria with different characteristics. During these studies, a large number of differentially regulated and differentially presented genes have been identified, including transcriptional regulation factors and restriction modification enzymes. This article describes the SSH method and considerations for its use.

**RELATED INFORMATION**

A series of detailed protocols for SSH is available; see the following:

- Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library (Rebrikov 2008a)
- Identification of Differential Genes by Suppression Subtractive Hybridization: II. Subtractive Hybridization (Rebrikov 2008b)
- Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs (Rebrikov 2008c)
- Identification of Differential Genes by Suppression Subtractive Hybridization: IV. Mirror Orientation Selection (MOS) (Rebrikov 2008d)
- Identification of Differential Genes by Suppression Subtractive Hybridization: V. PCR-Based DNA Dot Blot (Rebrikov 2008e)
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**AN OVERVIEW OF THE SSH PROCEDURE**
The SSH method (Fig. 1) is based on a suppression PCR effect, introduced by Sergey Lukyanov (Lukyanov et al. 1994). A key feature of the method is simultaneous normalization and subtraction steps. The normalization step equalizes the abundance of DNA fragments within the target population, and the subtraction step excludes sequences that are common to the two populations being compared (Gurskaya et al. 1996). SSH eliminates any intermediate steps demanding the physical separation of single-stranded (ss) and double-stranded (ds) DNAs, it requires only one round of subtractive hybridization, and it can achieve a >1000-fold enrichment for differentially presented DNA fragments.

**Figure 1.** Overview of the SSH and MOS procedures. The DNA in which specific sequences are to be found is called "tester" and the reference DNA is called "driver."

### Preparation of a Subtracted DNA Library

The DNA population in which specific fragments are to be found is called the tester. The reference DNA population is called the driver. The generation of tester and driver DNAs begins with either poly(A)+ mRNA, followed by conversion to cDNA, or genomic DNA. After isolation and synthesis, the tester and driver DNAs are digested with a four-base-cutting restriction enzyme that yields blunt ends (e.g., RsaI). The enzyme should be selected according to the GC-content of the target organism, and should yield blunt-ended fragments averaging around 0.5 to 1 kb.

The tester DNA is then subdivided into two portions, and each portion is ligated to a different pseudo-double-stranded (ds) adapter (adapter 1 [Ad1] and adapter 2R [Ad2R]). These adapters will serve as primer-binding sites for PCR amplification in later steps. Tester and driver DNA are then incubated and treated so that only appropriate adapter-labeled molecules will be amplified during the PCR steps (Fig. 2). These methods are described step by step in the following protocol: Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library (Rebrikov 2008a).
Subtractive Hybridization

It is strongly recommended that subtractions be performed in both directions for each tester/driver DNA pair. Forward subtraction is designed to enrich for differentially presented molecules present in the tester but not in the driver; reverse subtraction is designed to enrich for differentially presented sequences present in the driver but not in the tester. The availability of such forward- and reverse-subtracted DNAs is useful for differential screening of the resulting subtracted tester DNA library. For a protocol describing subtractive hybridization, see Identification of Differential Genes by Suppression Subtractive Hybridization: II. Subtractive Hybridization (Rebrikov 2008b).

We also recommend performing self-subtractions, with both tester and driver prepared from the same DNA sample, as a control to determine subtraction efficiency. These controls should yield little, if any, PCR product after amplification. For a protocol describing PCR amplification of subtracted DNA samples, see Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs (Rebrikov 2008c).

Cloning of Subtracted DNAs

Once a subtracted sample has been confirmed to be enriched in DNAs derived from differentially presented genes, the PCR products can be subcloned using several conventional cloning techniques. For site-specific cloning using the adapter sequences used in this series of protocols, cleave at the EagI (NotI) and XmaI (SmaI, SrfI) sites and then ligate the products into an appropriate plasmid vector. Keep in mind that some or all of these sites might also be present in the DNA fragments.

The number of independent colonies obtained for each library depends on the number of differentially expressed genes, as well as the subtraction and subcloning efficiencies. Additional colonies can be easily obtained by performing further subclonings of the secondary PCR products. Typically, it is necessary to analyze 500-1000 clones from a subtracted library to ensure that genes representing low-abundance transcripts are not lost. Sequence data from various studies show that the majority of the clones will be picked repeatedly, two to six times, indicating a degree of redundancy. This finding confirms the high level of normalization of SSH libraries, suggesting that the libraries contain both high- and low-abundance differentially presented DNAs.

Differential Screening of the Subtracted DNA Library

Two approaches can be utilized for differential screening of the arrayed subtracted DNA clones: colony dot blots and PCR-based DNA 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 DNA dot blots. The DNA dot blot approach is highly recommended and
MIRROR ORIENTATION SELECTION (MOS)

High background in the SSH-generated subtracted library can be reduced using MOS. The MOS technique is based on the rationale that after PCR amplification, during SSH, background molecules will be present in one orientation only, relative to the adapter sequences. Genuine SSH clones will be present in both sequence orientations (Rebrikov et al. 2000), as detailed in Figure 3.

The MOS procedure is described in Identification of Differential Genes by Suppression Subtractive Hybridization: IV. Mirror Orientation Selection (MOS) (Rebrikov 2008d). We recommend the use of MOS in the following cases:

- Use MOS if the percentage of differentially expressed clones found during differential screening is very low (e.g., 1%-5%). MOS can increase the number of differential clones up to 10-fold.

- Use MOS if most of the differentially expressed clones found are false positives. The MOS procedure can decrease the portion of false-positive clones several-fold.

- Use MOS if the primary PCR in SSH requires more than 30 cycles (but no more than 36 cycles) to generate visible PCR product. If this is the case, the problems described in the previous two items will usually appear.

- If the complexity of tester and driver samples is very great, or if the difference in gene expression between tester and driver is very small, use MOS. Plan to perform MOS from the beginning of the experiment.

- If the SSH subtracted library has already been made and found, upon differential screening, to contain high background, the option to perform MOS on the SSH-generated library should be considered. The hybridization mix generated in Step 12 of Identification of Differential Genes by Suppression Subtractive Hybridization: II. Subtractive Hybridization (Rebrikov 2008b) can be used for PCR amplification using MOS.
FINAL CONSIDERATIONS

The most critical step in SSH is the choice of cells or tissue for comparison. The two samples should be related, with stable expression of the phenotypic difference of interest. If the two samples are too distantly related, a large number of irrelevant, differentially expressed genes will be identified.

For systems where there is experimentally induced overexpression of a known set of genes, such as viral infections, it is extremely important to add these known sequences to the driver DNA sample. For example, when searching for p53-up-regulated genes in a p53-overexpressing cell line, add RsAl-digested p53 cDNA to the RsAl-digested driver sample to a level of 10% of the total driver DNA concentration. This must be done after the preparation of the adapter-ligated tester sample. Addition of this exogenous DNA before RsAl digestion will cause disproportional representation of this material in the samples.

The level of enrichment of a particular DNA depends greatly on its original abundance, the ratio of its concentration in the samples being subtracted, and the number of other differentially presented genes (Jin et al. 1997; Akopyants et al. 1998). 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 the success of SSH for any given application. For instance, the high complexity of eukaryotic genomic DNA makes SSH very difficult. Some cDNA subtractions can also be very challenging due to the nature of the starting samples. Subtracted libraries generated from complex samples may contain very high background. An especially challenging problem is the generation of so-called "false-positive" clones that show a differential signal in a primary screening procedure but are not confirmed by subsequent detailed analysis. To overcome this problem, MOS can be used to decrease substantially the number of background clones (Rebrikov et al. 2000).

To obtain the maximum data from a cDNA or genomic DNA subtraction experiment, it is important to achieve the highest efficiency of subtraction. The power of SSH subtraction makes it possible to achieve a level of 90%-95% differentially expressed clones in the cDNA-subtracted library (Diatchenko et al. 1996; Zuber et al. 2000). In cases where differentially expressed clones represent the majority of the clones in the subtracted library, the time-consuming process of differential screening can be omitted. Whenever possible, the researcher should consider designing the experiment to yield the highest level of difference between the tester and driver RNA populations, possibly by choosing the time point with the highest fold induction of control gene, for example. In the case of a sample comprising a mixed cell population, homogeneity can often be achieved by fine dissection of fixed or frozen tissues and/or by cell sorting. However, when this is not possible, the MOS procedure should be applied.

REFERENCES


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This article has been cited by other articles:

**Cold Spring Harbor Protocols**

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**Identification of Differential Genes by Suppression Subtractive Hybridization: An Overview**


[Abstract] [Full Text]

**Cold Spring Harbor Protocols**

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**Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library**


[Abstract] [Full Text]
| Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs |
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| CSH Protocols, July 1, 2008; 2008(8): pdb.prot4857 - pdb.prot4857. |
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