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 screening a subtracted cDNA library by differential hybridization using (1) a tester-specific subtracted probe (forward-subtracted probe), (2) a driver-specific subtracted probe (reverse-subtracted probe), (3) a cDNA probe synthesized directly from tester mRNA (or tester genomic DNA), and (4) a cDNA probe synthesized directly from driver mRNA (or driver genomic DNA).

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: II. Subtractive Hybridization (Rebrikov 2008c)

Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs (Rebrikov 2008d)

Identification of Differential Genes by Suppression Subtractive Hybridization: IV. Mirror Orientation Selection (MOS) (Rebrikov 2008e)

Identification of Differential Genes by Suppression Subtractive Hybridization: V. PCR-Based DNA Dot Blot (Rebrikov 2008f)

MATERIALS

Reagents

Blocking solution

The blocking solution should contain 2 mg/mL each of unpurified NP1 (TCGAGCGGCCGCCGGCAGGT), NP2R (AGCGTGGTCGCGGCCGAGGT), cDNA synthesis primers (in case of cDNA; TTTTGTACAAGCTT), and their complementary oligonucleotides.
ExpressHyb Hybridization kit (Clontech)

- High-stringency wash buffer (0.2X SSC and 0.5% [w/v] SDS), prewarmed to 68°C
- Low-stringency wash buffer (2X SSC and 0.5% [w/v] SDS), prewarmed to 68°C

Membranes, as prepared in Identification of Differential Genes by Suppression Subtractive Hybridization: V. PCR-Based DNA Dot Blot (Rebrikov 2008f)

Probes, tester and driver

The tester- and driver-specific subtracted probes used in this protocol are the secondary PCR products of the subtracted DNA pool (from Step 13 in Identification of Differential Genes by Suppression Subtractive Hybridization: III. PCR Amplification of Differentially Presented DNAs [Rebrikov 2008d]. The cDNA probes can be synthesized from the tester and driver poly(A)+ RNA, and can be used as either single-stranded or double-stranded cDNA probes (see Steps 1 and 2, respectively, in Identification of Differential Genes by Suppression Subtractive Hybridization: I. Preparation of Subtracted cDNA or Genomic DNA Library [Rebrikov 2008b]). Alternatively, unsubtracted tester and driver DNA or preamplified cDNA from total RNA (Chenchik et al. 1998) can be used if enough poly(A)+ RNA is not available. Do not use MOS PCR products for probe preparation.

- Random primer radiolabeling kit
- Salmon sperm DNA, sheared (10 mg/mL)
- SDS (0.5%, w/v) (optional; see Step 9)
- SSC (20X)

Equipment

- Hybridization oven, preset to 72°C (then 68°C; see Steps 3-7)
- Ice
- Plastic wrap (optional; see Step 9)
- Water bath, boiling
- X-ray film and developer (or phosphorimager)

METHOD

1. Label and purify the tester and driver DNA probes. Use a commercially available random primer radiolabeling kit.

   Radiolabeled probes should register at least $10^7$ cpm per 100 ng of subtracted DNA. The specific activities of each probe should be approximately equal.

2. Prepare a sufficient volume of prehybridization solution for each membrane:

   i. Combine 50 µL of 20X SSC, 50 µL of sheared salmon sperm DNA (10 mg/mL), and 10 µL of blocking solution.

   ii. Boil the solution for 5 min, then chill on ice.

   iii. Combine the solution with 5 mL of ExpressHyb Hybridization Solution (Clontech).

   This is the prehybridization solution. It is important to include the blocking solution in the prehybridization solution: Subtracted probes contain the same adapter sequences as arrayed clones, so these probes hybridize to all arrayed clones regardless of their sequences.

3. Place each membrane in the prehybridization solution from Step 2.iii. Prehybridize for 40-60 min with
continuous agitation at 72°C.

The hybridization conditions given here have been optimized for Clontech’s ExpressHyb solution; the optimal hybridization conditions for other systems should be determined empirically.

4. Prepare hybridization probes:
   i. Mix 50 µL of 20X SSC, 50 µL of sheared salmon sperm DNA (10 mg/mL), 10 µL of blocking solution, and purified probe from Step 1.
   
   ii. Boil the probe solution for 5 min, then chill on ice.
   
   iii. Add the probe solution to the prehybridization solution.

5. Hybridize the membranes overnight with continuous agitation at 72°C.

6. Wash the membranes with prewarmed low-stringency buffer for 20 min at 68°C. Repeat this wash three times.

7. Wash the membranes with prewarmed high-stringency buffer for 20 min at 68°C. Repeat this wash once.

8. Perform autoradiography.

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

To minimize hybridization background, store the membranes at −20°C in plastic wrap when they are not in use.

REFERENCES


Caution

Radioactive substances

Radioactive substances: When planning an experiment that involves the use of radioactivity, consider the physico-chemical properties of the isotope (half-life, emission type, and energy), the chemical form of the radioactivity, its radioactive concentration (specific activity), total amount, and its chemical concentration. Order and use only as much as needed. Always wear appropriate gloves, lab coat, and safety goggles when handling radioactive material.
rays and gamma rays are electromagnetic waves of very short wavelengths either generated by technical devices or emitted by radioactive materials. They might be emitted isotropically from the source or may be focused into a beam. Their potential dangers depend on the time period of exposure, the intensity experienced, and the wavelengths used. Be aware that appropriate shielding is usually made of lead or other similar material. The thickness of the shielding is determined by the energy(s) of the X rays or gamma rays. Consult the local safety office for further guidance in the appropriate use and disposal of radioactive materials. Always monitor thoroughly after using radioisotopes. A convenient calculator to perform routine radioactivity calculations can be found at: http://www.graphpad.com/calculators/radcalc.cfm.

Caution

SDS (Sodium dodecyl sulfate)

SDS (sodium dodecyl sulfate) is toxic, an irritant, and poses a risk of severe damage to the eyes. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety goggles. Do not breathe the dust.

Recipe

SSC

For a 20X solution: Dissolve 175.3 g of NaCl and 88.2 g of sodium citrate in 800 mL of H2O. Adjust the pH to 7.0 with a few drops of a 14 N solution of HCl. Adjust the volume to 1 L with H2O. Dispense into aliquots. Sterilize by autoclaving. The final concentrations of the ingredients are 3.0 M NaCl and 0.3 M sodium citrate.

Topic Introduction

Identification of Differential Genes by Suppression Subtractive Hybridization: An Overview
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. 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)

Identification of Differential Genes by Suppression Subtractive Hybridization: VI. Differential Hybridization with Tester and Driver DNA Probes (Rebrikov 2008f)

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.

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 is described in **Identification of Differential Genes by Suppression Subtractive Hybridization: V. PCR-Based DNA Dot Blot** (Rebrikov 2008e) and **Identification of Differential Genes by Suppression Subtractive Hybridization: VI. Differential Hybridization with Tester and Driver DNA Probes** (Rebrikov 2008f).

**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 RsaI-digested p53 CDNA to the RsaI-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 RsaI 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


