CHAPTER 2

SELECTIVE SUPPRESSION OF POLYMERASE CHAIN REACTION AND ITS MOST POPULAR APPLICATIONS

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Abstract: This chapter is devoted to methods based on suppression polymerase chain reaction (PCR) effect (cDNA library construction starting from a small amount of total RNA; suppression subtractive hybridization, SSH; ordered differential display, ODD; Marathon cDNA RACE; genome walking; variants of coincidence cloning, CC; normalization of cDNA libraries; multiplex PCR, mPCR; to *in vitro* cloning). Taken together, these approaches allow one to analyze complex DNA samples, from searching sequences of interest to determining complete structures of the respective genes.

Keywords: Selective PCR suppression, PCR suppression, suppression of the PCR, PS, pan handle, suppression adapter, inverted repeats, annealing temperature, primer concentration, suppression sequence, preparation of full-size cDNA, small amount of biological material, selective amplification, differential display, ordered differential display, targeted differential display, shortcoming, disadvantage, normalized cDNA libraries, evolutionary conserved sequences, search for promoter sites, chromosome walking, *in vitro* cloning, multiplex PCR.

Abbreviations: cDNA, complementary DNA; dNTP, deoxyribonucleotidetriphosphate; ITR, inverted terminal repeat; mRNA, messenger RNA; ODD, ordered differential display; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; PS, PCR suppression effect; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SSP, selective suppression of PCR; SSH, suppression subtractive hybridization; TGDD, targeted genomic differential display.

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TABLE OF CONTENTS

1. INTRODUCTION

The most important processes in various biological systems (cell differentiation and morphogenesis during embryonic development and regeneration, apoptosis or malignization of cells, etc.) are under the control of specific regulatory genes. To understand the underlying molecular mechanisms, the genes involved in their regulation should be revealed and studied. At present, the majority of methods of molecular biology involved in unraveling such problems are based on polymerase chain reaction (PCR), which has made it possible to work with small amounts of biological material. However, the use of PCR requires information on the sequence of the DNA under study. When the sequence is partially or completely unknown, the PCR often encounters difficulties.

The selective suppression of PCR (SSP) phenomenon discovered in this laboratory (Launer et al. 1994) generated a number of highly effective, mutually complementary methods of finding and analyzing new functionally important DNA and RNA sequences when information, complete or partial, on their primary structure is absent. The use of SSP enables one to skip labor-intensive and not very effective methods of physical fractionation of DNA and makes the methods for search and analysis of genetic sequences easier, more rapid, and more reproducible.

2. SELECTIVE PCR SUPPRESSION

SSP consists in inhibiting the amplification of DNA molecules flanked by inverted terminal repeats (ITRs) in PCR with a primer corresponding to the external part of the ITR, provided that the primer is considerably shorter than the ITR (Figure 1).

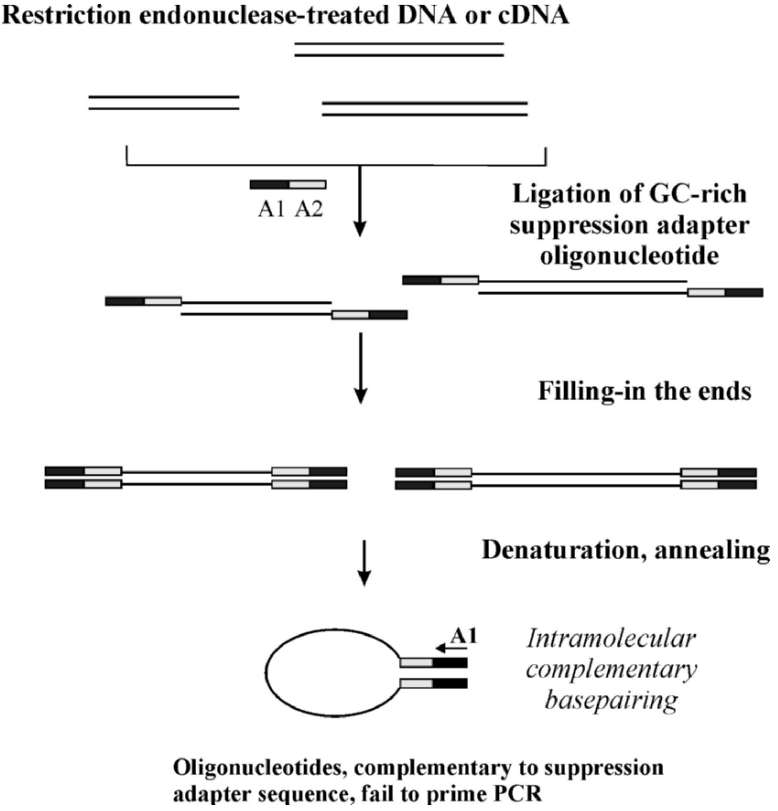

Figure 1. Schematic representation of the PCR suppression effect. DNA molecules flanked by inverted terminal repeats form intramolecular terminal duplexes, thus preventing terminal adapter-specific primer annealing and, consequently, inhibiting the PCR.

The principle of PCR suppression is that the target DNA is designed to adopt a hairpin form. In general, in all its applications, PS allows the amplification of wanted sequences and simultaneously suppresses the amplification of unwanted ones. Pan handle-like stem-loop DNA constructs for PS are created by ligation of long GC-rich adapters to DNA or cDNA restriction fragments (Figure 1) (Launer et al. 1994). As a result, each single-stranded DNA (ssDNA) fragment is flanked by terminal inverted repeats (i.e. by self-complementary ends). During PCR, on denaturing and annealing, the self-complementary ends of each single strand form duplex stems, converting each fragment into a large pan handle-like stem-loop structure. The formation of stable duplex structures at the fragment ends makes the PCR with the adapter-primer (A-primer) alone relatively inefficient, because the intramolecular annealing of the complementary termini is kinetically favored and more stable than the intermolecular annealing of shorter A-primers. This effect is therefore called PCR suppression (Launer et al. 1994).

In the presence of both A-primers and target-primers (T-primers, targeted at the gene-specific sequences in the single-stranded loops), however, PCR is efficient. The T-primer anneals to its target and is used by DNA polymerase to initiate DNA synthesis. The newly synthesized product has two termini, which are not complementary and, thus, cannot fold into a stem-loop structure. This fragment is, therefore, not subject to the PS effect, and is efficiently amplified. Consequently, only the fragments containing the target are exponentially amplified by PCR, while the background fragments without the target remain inert.

The inhibition of amplification depends on many parameters, of which the following are the major ones:

1. The difference in the annealing temperatures of ITR and the amplification primer. The ratio of the length and GC content of the whole ITR and the sequence corresponding to the primer affects the SSP effectiveness considerably. The use of ITR 40–50 bp long with an increased GC content in its internal (suppression) part and of an amplification primer corresponding to the external 20–25 bp segment of the ITR appears to be optimum.
2. The length of the ITR-containing DNA molecule. The longer the molecule, the less probable is the encounter and intramolecular hybridization of its termini. Thus, SSP effect does not inhibit, or inhibits sparingly, amplification of very long DNA (the threshold value depends on the conditions and is usually 6–8 kbp).
3. Primer concentration in PCR. SSP depends on competition between intramolecular hybridization and primer annealing. Therefore, SSP is more effective at low-primer concentrations.

SSP can be used to suppress amplification of an unwanted DNA fraction, which requires suitable long ITR, i.e. suppression sequences, to be introduced into the DNA.

3. PREPARATION OF ITR-CONTAINING DNA SAMPLES

Two main methods for the attachment of suppression sequences to DNA molecules were developed:

1. Ligation of double-stranded DNA (dsDNA) fragments with a pseudodouble-stranded, or so-called suppression, adapter (Siebert et al. 1995)
2. PCR with a long (suppression) primer whose 3′-terminal part is also present within the DNA fragments (Launer et al. 1994)

The first method is highly versatile, since it does not require any special sequences to be introduced into DNA. For example, a DNA sample for ligation can be obtained by the synthesis of double-stranded cDNA or by the treatment of cDNA or genomic DNA with restriction endonucleases. Apparently, blunt-end (rather than staggered-end) DNA fragments are optimum because, in this case, blunt-end suppression adapters can be universally used in ligation. The second technique is effective when an amplified DNA sample is being dealt with so that it harbors known sequences. This can be exemplified by cDNA samples

obtained through addition of a homopolymer sequence to the first cDNA strand followed by PCR with a T-primer or C- and T-primers.

4. STRATEGY OF EMPLOYMENT OF SSP

SSP can be used in the analysis of complex mixtures of DNA fragments (cDNA or fragmented genomic DNA) to suppress an undesirable DNA fraction while retaining exponential amplification of target sequences. By and large, SSP allows the selection of asymmetrically flanked DNA molecules from mixtures with symmetrically flanked ones. Before the discovery of SSP, such a problem could not be solved using PCR. Three major schemes of SSP employment can be outlined.

The first scheme (Figure 2A) is based on the addition of one suppression sequence to all DNA molecules, followed by amplification with two primers of which one corresponds to the external part of the suppression sequence while the other is complementary to the target DNA (gene-specific or oligo(dT)-containing primer). In the course of PCR, DNA molecules bearing the sequence of the second primer are selected. This scheme underlies methods such as the search for the genomic DNA or cDNA sequences belonging to a known fragment (Siebert et al. 1995; Chenchik et al. 1996), the construction of cDNA libraries on the basis of low amounts of total RNA (Lukyanov et al. 1997), and mRNA ordered differential display (ODD) (Matz et al. 1997).

The second scheme (Figure 2B) is based on an addition of two different suppression sequences to DNA followed by amplification with primers corresponding to their external parts. In the course of PCR, asymmetrically flanked DNA molecules – bearing different suppression sequences at their ends – are selected. This scheme underlies the *in vitro* cloning method (Lukyanov et al. 1996).

The third scheme (Figure 2C) is a sophisticated variant of the previous one. Two DNA samples are supplied with different 5′-terminal (but not 3′-terminal) suppression sequences. The samples are then denatured and hybridized together. After filling in the 3′-termini, PCR is carried out with primers corresponding to the external parts of the suppression sequences. As in the second scheme, asymmetrically flanked DNA molecules are selected, but in this case each of these DNA must anneal with the complementary strand from the alternative sample. Such selection of heteroduplexes enables subtractive hybridization (SH) of cDNA (Launer et al. 1994), cDNA normalization (Gurskaya et al. 1996), modified coincidence cloning (Azhikina et al. 2004; Chalaya et al. 2004; Buzdin et al. 2006), and a search for evolutionarily conserved cDNA.

5. SSP-BASED PROCEDURES

Currently, studies on the effects of gene expression on various biological processes often employ the following strategy: (1) construction of cDNA libraries from biological samples under study; (2) screening of these libraries for

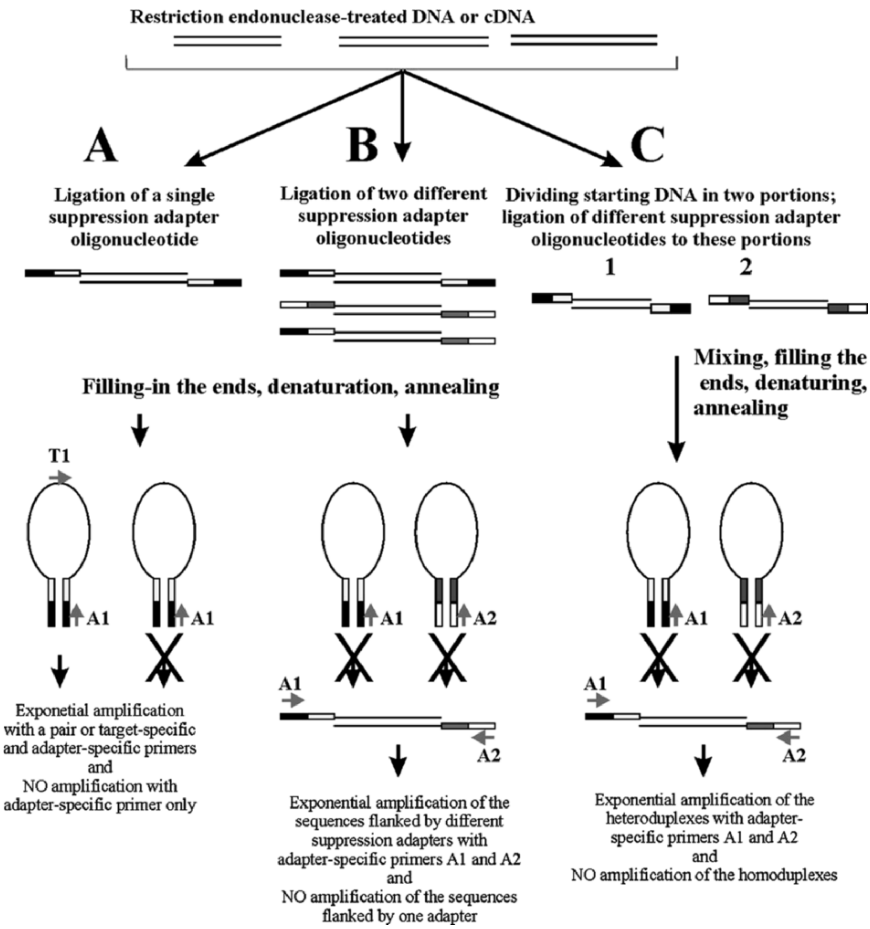

Figure 2. Outline of the major strategies using PCR suppression effect. (A) Single suppression adapter is ligated to the sample; (B) two different suppression adapters are ligated; (C) the sample is subdivided in two portions, which are further ligated to two different suppression adapters.

genes (more exactly, their fragments) differentially expressed or interesting in other aspects; and (3) preparation of full-size cDNA and genomic copies of the genes of interest. All these stages can be carried out using SSP-based methods that are considered in detail in section 5.1.

PS effect has already found applications in a variety of methods (reviewed in Luk'ianov et al. (1999)). In this book, suppression SH, multiplex PCR (mPCR), and targeted genomic differential display (TGDD) will be discussed:.

5.1 Construction of cDNA Libraries from a Small Amount of Biological Material

cDNA libraries are needed for a wide range of problems related to the functional and structural aspects of gene expression. The methods for their construction from relatively large amounts of biological material were developed long ago and are now routine genetic engineering procedures. However, when biological material is scarce and therefore poly(A) + RNA is insufficiently available (this is the standard situation nowadays), the conventional methods are of no avail.

The advent of PCR gave impetus to new methods of constructing cDNA libraries from small amounts of total RNA. The use of PCR, however, requires information on at least a partial sequence of the DNA to be amplified. Since mRNAs of the overwhelming majority of genes contain a poly(A) sequence, PCR can be carried out with an oligo(dT)-containing primer (T-primer). Exponential amplification needs an artificial sequence to be attached to all cDNA molecules for annealing of a second primer.

Several methods for the introduction of such a sequence have been described: (1) addition of a homopolymer sequence to the 3′-terminus of the first strand of cDNA using terminal deoxynucleotidyl transferase (tailing) (Frohman et al. 1988); (2) ligation of a synthetic single-stranded oligonucleotide to the first strand of cDNA (Edwards et al. 1991) or to mRNA (Liu and Gorovsky 1993) using T4 RNA ligase; and (3) ligation of a double-stranded oligonucleotide adapter to double-stranded cDNA using T4 DNA ligase (Akowitz and Manuelidis 1989).

Ligation of double-stranded molecules using T4 DNA ligase is more effective and reproducible than tailing or ligation of single-stranded substrates using T4 RNA ligase (Chenchik et al. 1996). However, the method (Akowitz and Manuelidis 1989) using T4 DNA ligase has the substantial drawback that it can be applied only to poly(A) + RNA. If cDNA is synthesized on the basis of total RNA, even the use of a T-primer does not prevent the synthesis of a large excess of background cDNA on ribosomal RNA as a template. On the basis of SSP, a method was developed that involves T4 DNA ligase-mediated ligation of a suppression adapter to double-stranded cDNA, followed by PCR with a T-primer and a primer corresponding to the external part of the adapter (Figure 2A) (Lukyanov et al. 1997).

Such PCR is accompanied by selective amplification of the cDNA fraction comprising the T-primer structure. At the same time, SSP inhibits amplification of the rest of cDNA, which is adapter-flanked from both sides. Thus, a cDNA library can be constructed based on a small amount of total RNA without isolating poly(A) + RNA fraction. We have shown the potential of this procedure on a model system (Lukyanov et al. 1997). The libraries so constructed were subsequently used to reveal and analyze functionally important expressing sequences in various biological systems (Bogdanova et al. 1997, 1998; Kazanskaya et al. 1997). This method enables representative, essentially full-size,

cDNA libraries to be constructed from small (10–100 ng) amounts of total RNA that are of as high a quality as libraries produced by conventional methods from 5 to 10 mg of poly(A) + RNA.

5.2 Detection of Differentially Expressed Genes

Understanding the molecular mechanisms of biological processes requires the search for, and study of, genes expressed differentially in these processes. The identification methods for such genes are based on the detection of mRNA molecules that are diversely represented in various tissues and at various stages of a biological process. Changes in composition and content of cellular mRNA can be analyzed in several modes. Apart from microarray hybridizations, two strategies are most widely used: differential display and SH.

5.2.1 Differential display

The differential display technique was proposed in 1992 (Liang and Pardee 1992) and is currently in great demand in searches for differentially expressed genes. This technique is based on the employment of a short oligonucleotide primer that has a low annealing temperature and can direct PCR amplification of a limited pool of cDNA fragments. Comparative polyacrylamide gel electrophoresis (PAGE) analysis of such cDNA samples allows the identification of differentially represented cDNA fragments. Use of an arbitrary primer, however, precludes systematic comparison of the samples by all mRNA types: characteristic sets of DNA fragments are random, and the overwhelming majority of these fragments correspond to the types of transcripts most abundant in the original samples.

In addition, use of oligonucleotide primers with a low annealing temperature is accompanied by nonspecific amplification and leads to numerous artifacts. A totally different approach, involving a systematic comparison of samples by all mRNA types and based on the separation of 3′-terminal restriction fragments of cDNA, was proposed by Ivanova and Belyavsky (1995). Its drawbacks are its limited sensitivity and labor-intensive character. We have developed a method of mRNA ODD that is also based on an analysis of 3′-terminal restriction fragments of cDNA (Matz et al. 1997). However, to select these fragments, our approach uses PCR rather than physical separation (Figure 3). This results in selective amplification of 3′-terminal fragments of cDNA (from T-primer to the nearest restriction site), which substantially simplifies the method and enhances its sensitivity. The effectiveness of the ODD method was exemplified by revealing sequences differentially expressed along the anterior–exterior axis of planarian (Matz et al. 1997).

In TGDD, the PS effect is used to amplify a set of genomic or cDNA fragments with a single primer targeting repetitive sequences. Because of PS, the vast amount of other genomic fragments that do not have the targeted repeat remains unamplified. The PCR product consists of many fragments, each containing part of the

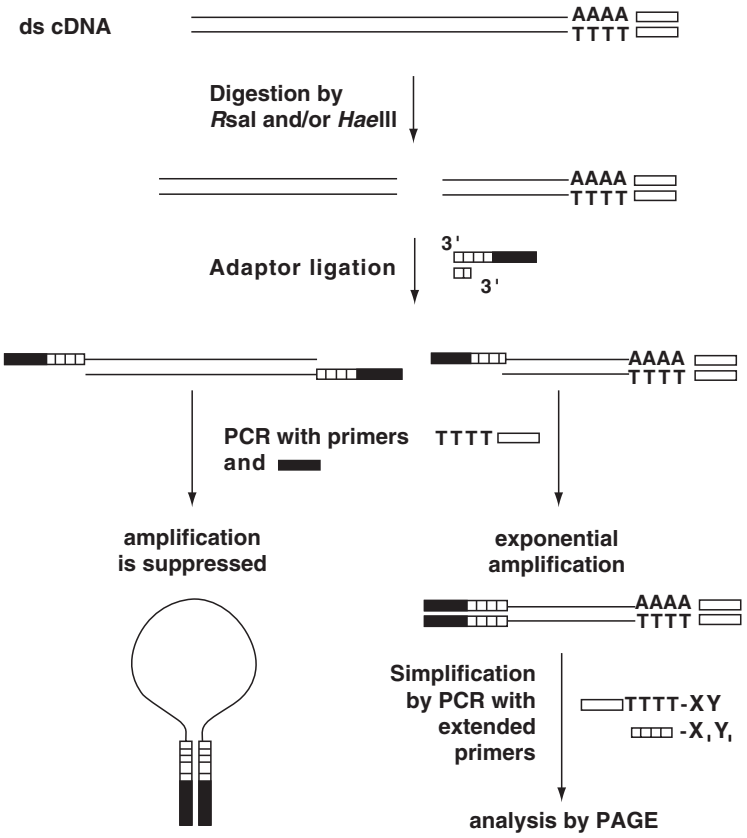

Figure 3. Schematic view of the ordered differential display (ODD) approach, which takes advantage of the PCR suppression effect for the specific amplification of cDNA 3′ regions.

repeat and one of the flanking regions. After resolution of the PCR products on a sequencing gel, a stable pattern of fragments specific for each genome or cDNA is obtained, with any differences revealed by their different mobility (Figure 4) (Broude et al. 1997, 1999; Lavrentieva et al. 1999; Vinogradova et al. 2002). Single-nucleotide polymorphisms, insertions, and deletions were detected when differential fragments were isolated and subjected to sequence analysis (Broude et al. 1999). In TGDD, the complexity of the displayed fragments is controlled by the 3′-anchoring of the primers, essentially in the same way as in the amplified fragment length polymorphism approach (Vos et al. 1995) and in the ordered cDNA differential display (Matz et al. 1997). This allows nonoverlapping sets of genomic fragments to be surveyed with different 3′-anchors in different reactions.

In contrast to other PCR-based display methods in which repeated sequences have been targeted (Weising et al. 1995; Donohue et al. 1997), TGDD shows high

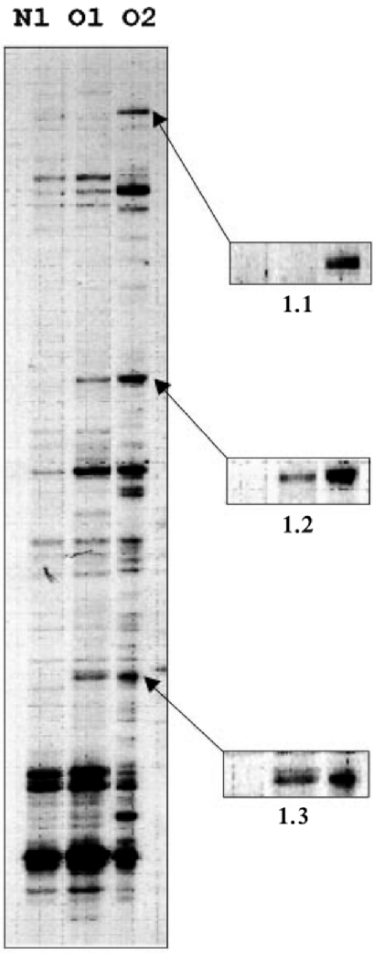

Figure 4. Representative electrophoregram showing results of the PS-assisted differential display of the endogenous retrovirus – driven transcripts in a normal (N1) and two cancerous tissues (O1 and O2). The fragments 1.1, 1.2, and 1.3 are abundant in O1 and O2 samples, but are hardly detectable in N1. The differential fragments are then removed from gels and sequenced, with their differential status to be confirmed by any independent approach like RT-PCR or Northern blot.

specificity: ~90% of the clones obtained the targets (Broude et al. 1999; Lavrentieva et al. 1999). TGDD was applied for the genome-wide recovery of polymorphic human genomic loci containing endogenous retroviral inserts (Lavrentieva et al. 1999) and demonstrated excellent performance, with high specificity, robustness, and high discrimination power. It can be adapted for the display of any type of repeats and its application is especially promising in cases where repeat instability is anticipated. Indeed, TGDD modifications were recently successfully employed for the identification of differentially expressed human endogenous retroviruses (Vinogradova et al. 2002) and for the recovery of polymorphic insertions of other mammalian repetitive elements – human L1 retrotransposons (Badge et al. 2003).

Shortcomings of the method should also be mentioned. These are common to all PCR-based methods with simultaneous amplification of a multitude of

genomic fragments: (1) there is biased amplification of certain fragments, which leads to overrepresentation of some of the fragments and the loss of others during amplification; and (2) the complete digestion of all the DNA samples is paramount for reliable DNA comparisons.

5.2.2 Subtractive hybridization of cDNA

SH of cDNA is a process of exhaustive hybridization of two cDNA samples named *driver* and *tracer* that is meant to reveal sequences (targets) that are present in tracer (also frequently called tester) but are absent from, or at a lower level in, driver. SH involves hybridization of tracer with excess driver, followed by the separation of hybrid molecules from target molecules.

The use of SH of cDNA resulted in the detection of a considerable number of functionally important genes involved in embryonic development, cell differentiation, tumor transformation, and metastasizing. However, the low representativeness of the resulting enriched libraries, moderate enrichment degrees, and labor-intensity of procedures for the purification of the enriched fraction considerably hinders the use of this approach when the amount of biological material is limited and in the identification of genes whose transcripts are sparingly presented in the cell (1–10 copies per cell).

Our cDNA SH procedure (Diatchenko et al. 1996, 1999) allows one to overcome the problem of searches for rare transcripts by introducing a step of normalization of concentrations of various transcripts in the cDNA sample under study. This is achieved by using SSP (Figure 2C), which led to this method being named suppression subtractive hybridization (SSH). The effectiveness of this procedure was confirmed in model experiments with the use, as a target, of an exogenous viral DNA added to tracer in predetermined concentrations (Launer et al. 1994). This method, which will be described in detail in Chapter 3, was employed in revealing sequences differentially expressed in various biological processes, such as activation of immune response in a culture of immunocompetent cells (Gurskaya et al. 1996), alteration of the metastatic potential of tumor cells, or regeneration of planarian (Bogdanova et al. 1998), and for constructing human tissue-specific cDNA libraries (Diatchenko et al. 1996). The important advantages of SSH are: (1) it incorporates two hybridization steps, leading to efficient normalization of cDNA concentrations; (2) it requires only one subtraction round; and (3) it does not require physical separation of single-stranded and double-stranded fractions. The frequency of false positives is low and ~90% of clones are different (Diatchenko et al. 1996). As a result, SSH has become one of the most popular and efficient methods for subtractive expression studies (von Stein et al. 1997; Zhang and DuBois 2001).

Currently, the SSH technique is widely used (e.g. Chu et al. 1997; Hudson et al. 1997; Mueller et al. 1997; Yokomizo et al. 1997). Elements of the proposed technique of SH were successfully employed in the solution of some other problems, for example, constructing normalized cDNA libraries (Lukyanov et al. 1996; Luk'ianov et al. 1999) and revealing evolutionarily conserved sequences (Chalaya et al. 2004).

5.3 Search for 5′- and 3′-Terminal Fragments of cDNA

One of the most important and technically difficult objectives associated with the characterization of genes is the preparation of full-size cDNA. Conventional methods for detection of genetic sequences (screening of cDNA libraries, cloning of conserved genes using PCR with degenerated oligonucleotide primers, identification of differentially expressed genes using mRNA differential display, or cDNA SH) usually allow identification of only a fragment of cDNA. To clone full-size cDNA more rapidly and effectively, a number of methods jointly referred to as rapid amplification of cDNA ends (RACE) were recently proposed for *in vitro* amplification of the ends of cDNA (for review, see Schaefer 1995).

The majority of RACE methods now known are based on the introduction into the 3′-end of the first cDNA strand of an additional nucleotide sequence, which subsequently serves as the annealing site for a PCR primer. However, the problem of amplification suppression of nonspecific sequences upon use of the RACE strategy for rare genes is even more complicated than in constructing cDNA libraries. SSP makes it possible to overcome this difficulty. The cDNA sample is ligated with a suppression adapter and then amplified with a primer corresponding to the external part of the suppression adapter and a gene-specific primer (Figure 5; see also a more generalized scheme in Figure 2A). Only molecules containing the annealing site for the specific primer, i.e. 5′- or 3′-terminal sequences (depending on the direction of the specific primer), are thereby amplified. Amplification of the remainder of the molecules is prevented by SSP. The effectiveness of this method was tested in model experiments on known genetic sequences (Chenchik et al. 1996; Ackerman et al. 1997; Fleury et al. 1997; Loftus et al. 1997; Meyerson et al. 1997; Yang et al. 1997) and is currently widely used in many laboratories.

5.4 Search for Promoter Sites (Chromosome Walking)

Analysis of the genomic organization of the sequences isolated and cloning of regulatory regions of genes is important in the structure-functional analysis of genes. However, existing PCR-based techniques of chromosome walking are rather labor-intensive and ineffective. We proposed a novel technique of PCR walking on genomic DNA based on SSP (Siebert et al. 1995). To produce an ITR-containing DNA sample, genomic DNA is treated with a restriction endonuclease and ligated with a suppression adapter. Isolation of regulatory regions of the gene under study is carried out in subsequent PCR on the resulting DNA sample with gene-specific and adapter primers according to the scheme for isolation of full-size cDNA. This technique has also been widely used (e.g. Johansson and Karlsson 1996; Rosenberg and Dyer 1996; Takenoshita et al. 1996; Chong et al. 1997; Morii et al. 1997; Wade et al. 1997).

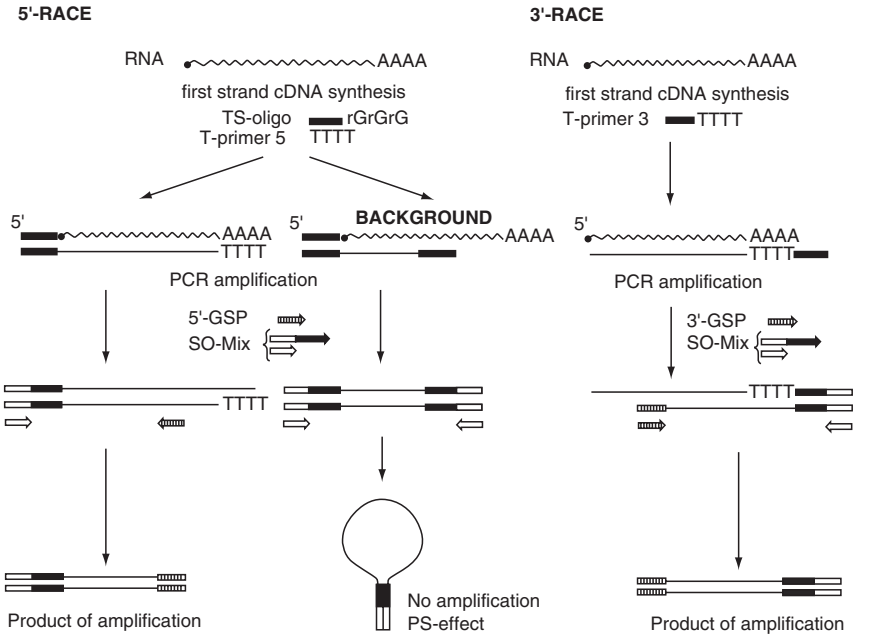

Figure 5. Rapid amplification of the cDNA ends (RACE) technique based on the PS effect. This fast and cost-effective procedure permits reliable cloning and identification of both 3′ and 5′ cDNA terminal regions.

5.5 *In Vitro* Cloning

Although the majority of classical genetic engineering methods were recently improved or displaced by more effective PCR-based techniques, traditional methods retained their leading role in the molecular cloning of DNA (cloning in bacterial, phage, and other *in vivo* systems). Based on SSP, a new method, named *in vitro* cloning, was proposed (Lukyanov et al. 1996), which allows PCR amplification and then sequencing of individual DNA molecules of unknown sequence without *in vivo* cloning.

The method of *in vitro* cloning includes the following stages (Figure 2B):

1. Simultaneous ligation of dsDNA fragments with two suppression adapters
2. Multiple dilution of the resulting sample to adjust the DNA content in the volume to be used in amplification to single molecules
3. PCR amplification of single DNA molecules using primers complementary to the external parts of the adapters.

The resulting PCR products, named *in vitro* clones, correspond to single DNA molecules. Owing to SSP, these clones are necessarily flanked with sequences of different adapters, which allows one to sequence the cloned DNA fragment by

any method suitable for PCR products. *In vitro* cloning can be used in solving a wide range of problems of molecular biology in the place of conventional *in vivo* cloning. This method is especially convenient when no more than several dozen clones are needed. We used this approach in the differential screening of cDNA libraries constructed by SH (Luk'ianov and Luk'ianov 1997). In addition, we developed a protocol for rapid preparation of a panel of overlapping subclones to sequence long-stretched (5 kb) DNA fragments (Fradkov et al. 1998).

5.6 Multiplex PCR

PCR suppression was also used to develop a new strategy for mPCR amplification. In mPCR, multiple DNA targets are amplified simultaneously in one tube (Edwards and Gibbs 1994). Amplification of each target in conventional PCR requires two gene-specific primers. At a high level of multiplexing, it is often difficult to avoid primer interactions and achieve efficient and uniform target synthesis. In spite of numerous studies aimed at developing an effective strategy for mPCR (Shuber et al. 1995; Henegariu et al. 1997) and minimizing primer–primer interactions (Brownie et al. 1997), mPCR still presents a challenge (Broude 2002).

Although high multiplexing levels were achieved in some studies (e.g. Fan et al. 2000), they remain exceptions. Typically, a routine mPCR does not exceed 5- to 10-plex. Suppression PCR requires one gene-specific primer per amplicon and one primer, which is common for all targets. Therefore, an *n*-plex PCR would require only *n* primers instead of *2n* in conventional PCR (Figure 6). As expected, PS-based mPCR allowed efficient amplification of several targeted sequences (Broude et al. 2001; Broude 2002), and only simple adjustment of conditions was necessary to amplify simultaneously 30 DNA targets of different length from different human chromosomes (Broude 2002).

Additionally, PS-based mPCR exhibited excellent specificity and provided allele specificity in a multiplex format (Broude et al. 2001; Broude 2002). Although the PS approach includes two additional steps compared with conventional PCR (digestion of genomic DNA with a restriction enzyme and ligation with the adapters), this does not create many problems. It was demonstrated that it is possible to use one restriction enzyme for multiple target amplifications, so that a single DNA sample can be used in many experiments. Thus, application of the suppression approach for mPCR seems to offer several advantages over traditional methods, such as higher levels of multiplexing, higher specificity, simpler primer design, and primer cost savings (Broude 2002).

Thus, the discovery of SSP has resulted in a number of mutually complementary procedures of analysis of complex DNA samples: these include construction of cDNA libraries from small amounts of biological material, SH and differential display of mRNA for revealing differentially expressed sequences, fast cloning of full-size cDNA, chromosome walking for the cloning of promoter and other genomic regions, mPCR, *in vitro* cloning, and other applications.

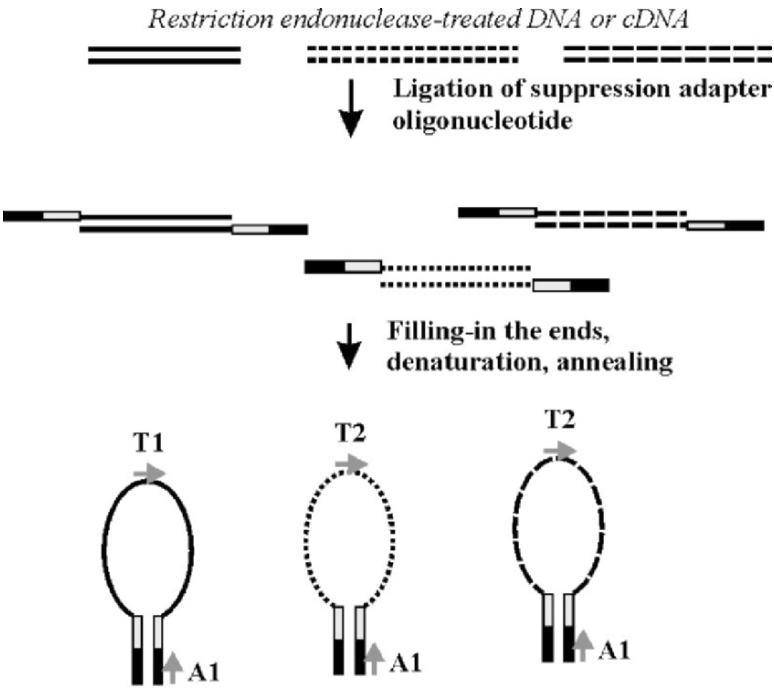

Figure 6. Scheme for the multiplex PCR amplification with only one specific primer used per one locus amplified (instead of two primers in conventional PCR).

6. DETAILED PROTOCOLS

6.1 Materials

6.1.1 Oligonucleotides

Many adapter oligonucleotides were shown to be efficient for the selective PCR suppression. We will provide here the protocols for direct suppression adapter ligation, as the direct adapter ligation is more uniform and widely used technique than the introduction of adapter sequences by PCR. The full-size adapter is usually a 40–45 nt long sequence with an inner (3′-terminal) part highly enriched in GC content. We recommend the following adapter oligonucleotides to be used for any of the above PS applications (whenever possible, oligonucleotides should be HPLC- or gel-purified):
Adapters and related oligonucleotides:

Set 1
A1A2 (adapter oligonucleotide, 44 nt long)

5′-*CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT*-3′
A3 (oligonucleotide complementary to the adapter 3′-terminal part, 10 nt long)
3′-*GGCCCGTCCA*-5′
A1 (“outer” PCR primer, 22 nt long)
5′-*CTAATACGACTCACTATAGGGC*-3′
A2 (“inner” primer for nested PCR, 22 nt long)
5′-*TCGAGCGGCCGCCCGGGCAGGT*-3′

Set 2
B1B2 (adapter oligonucleotide, 43 nt long)
5′-*TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT*-3′
B3 (oligonucleotide complementary to the adapter 3′-terminal part, 11 nt long)
3′-*GCCTCCCGCCA*-5′
B1 (“outer” PCR primer, 21 nt long)
5′-*TGTAGCGTGAAGACGACAGAA*-3′
B2 (“inner” primer for nested PCR, 22 nt long)
5′-*AGGGCGTGGTGCGGAGGGCGGT*-3′

Set 3
C1C2 (adapter oligonucleotide, 44 nt long)
5′-*AGCAGCGAACTCAGTACAACAAGTCGACGCGTGCCCGGGCTGGT*-3′
C3 (oligonucleotide complementary to the adapter 3′-terminal part, 11 nt long)
3′-*GGGCCCGACCA*-5′
C1 (“outer” PCR primer, 21 nt long)
5′-*AGCAGCGAACTCAGTACAACA*-3′
C2 (“inner” primer for nested PCR, 23 nt long)
5′-*AGTCGACGCGTGCCCGGGCTGGT*-3′

Note: In principle, all these adapter oligonucleotide sets can be used for any of the selective PCR suppression-based techniques; however, in some particular cases adapter and related nested PCR primer sequences may interfere with genomic or cDNA-specific oligonucleotides designed by the user. To avoid this problem, one should make a preliminary *in silico* analysis of oligonucleotide compatibility in terms of formation of extended cross-hybridization patterns and primer dimers. Such an analysis will make it possible to choose the best appropriate adapter oligonucleotide set among those presented here or among any other suppression adapters. For some specific applications, custom adapter design is needed, e.g. introduction of restriction endonuclease recognition sites. Another important issue is that one should pay attention to new restriction sites that may appear upon adapter ligation, if any further manipulations with restriction enzymes are needed.

The recommended annealing temperature for any outer or inner nested PCR primer varies between 60°C and 67°C. We advise 65°C as the default T_m, or as the starting T_m if any further fine tuning of the PCR conditions is required. 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.

6.1.2 Buffers and enzymes

We exemplify here the PS adapter ligation to genomic DNA or cDNA sample. In reality, it is up to the user to choose the restriction endonuclease (*Rsa* I in this example) and the ligation enzyme (here T4 DNA ligase). We recommend blunt-end adapter ligation as a more universal approach. If sticky-end making restriction endonuclease is used to process genomic DNA or cDNA, Klenow fragment or other DNA polymerase enzyme may be employed to produce blunt-ends.

1. Rsa I restriction endonuclease (10 U/ml, New England Biolabs).
2. 10X Rsa I restriction buffer: 100 mM Bis-Tris propane/HCl, pH 7.0, 100 mM $MgCl_2$, and 1 mM dithiothreitol (DTT).
3. T4 DNA ligase (3 U/µl, New England Biolabs).
4. T4 10X DNA ligation buffer.
5. T4 DNA polymerase (3 U/µl, New England Biolabs).
6. 10X buffer for T4 DNA polymerase.
7. 10 mM each dNTP (Amersham Pharmacia Biotech, Piscataway, NJ).
8. TN buffer: 10 mM Tris-HCl, 10 mM NaCl.
9. 10X PCR buffer.

6.1.3 Starting material

Genomic DNA or double-stranded cDNA can be used for the ligation of suppression adapters. For example, in the case of suppression SH, 2 µg of genomic DNA or RNA is required per experiment. Most commonly used methods for isolation of RNA and genomic DNA are appropriate. 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. If genomic DNA is used as a starting material, the next step is restriction endonuclease digestion. If RNA is used as a starting material, the next step will be the cDNA synthesis, including two major steps: first strand cDNA synthesis and second strand cDNA synthesis, using commercially available kits.

6.2 Methods

6.2.1 Rsa I digestion

This step generates shorter, blunt-ended genomic DNA or double-stranded cDNA fragments optimal for several PS applications.

1. Add the following reagents into the sterile 1.5 ml tube:
   – 1.5 µg genomic DNA or double-stranded cDNA
   – 5.0 µl 10X *Rsa* I restriction buffer
   – 1.5 µl *Rsa* I (10 U/µl)
   – to 50 µl deionized sterile water
2. Mix and incubate at 37°C for 2–4 h

3. Analyze 5 µl of the digest mixture on a 1.5% agarose gel along with undigested 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
6. Dissolve each pellet in 5–10 µl of water and store at –20°C.

6.2.2 Adapter annealing

This step is needed to properly anneal 40–45 nt long adapter on a 8–12 nt long oligonucleotide complementary to the adapter 3′-terminal part (e.g. adapter A1A2 on oligonucleotide A3). This will create a "pseudo double-stranded" adapter which may be ligated by DNA ligase to digested genomic DNA or cDNA.

1. In PCR tube, prepare an adapter annealing mix of the following components:
   – 22.5 µl of 100 µM adapter oligonucleotide (e.g. A1A2, B1B2, C1C2)
   – 22.5 µl of 100 µM short complementary oligonucleotide (e.g. A3, B3, C3)
   – 5 µl 10X PCR buffer
2. Incubate the annealing mixture in a thermal cycler at 65°C for 10 min. Do not remove the samples from the thermal cycler.
3. Immediately commence the following program:
   60°C – 5 min
   55°C – 5 min
   50°C – 5 min
   45°C – 5 min
   40°C – 5 min
   35°C – 5 min
   30°C – 5 min
   25°C – 5 min
4. Store annealed adapter solution at –20°C.

6.2.3 Adapter ligation

1. Dilute 1 µl of each *Rsa* I-digested tracer cDNA from the above section with 5 µl of TN buffer.
2. Prepare a ligation mix of the following components for each reaction:
   – 3 µl sterile water
   – 2 µl 5X ligation buffer
   – 1 µl T4 DNA ligase (3 U/µl)
   – 4 µl annealed adapter solution

Please note that ATP required for ligation is in the T4 DNA ligation buffer (300 µM final).

3. Centrifuge tubes briefly, and incubate at 16°C overnight.
4. Stop ligation reaction by adding 1 µl of 0.2 M EDTA.
5. Heat samples at 72°C for 5 min to inactivate the ligase.

6. Briefly centrifuge the tubes. Purify ligated DNA from the excess of adapter oligonucleotides using commercially available PCR product purification kit (e.g. manufactured by Promega or QiaGen). Dissolve the DNA in a final volume of 50 µl. Preparation of your experimental adapter-ligated DNA is now complete.

6.2.4 Ligation efficiency test

The following PCR experiment is recommended to verify that at least 25% of the DNA fragments obtained have adapters on both ends. However, this control experiment is not needed if there are no doubts in annealed adapter ligation efficiency. This experiment is designed to amplify fragments that span the adapter–DNA junctions by adapter-specific A1 primer and a unique gene-specific primer (if cDNA was ligated) or genomic DNA-specific primer (if genomic DNA was used). The PCR products generated using one gene-specific primer and adapter-specific primer should be about the same intensity as the PCR products amplified using two gene-specific primers. When designing primers, pay attention to the location of restriction sites for the enzyme you use (*Rsa* I in our example) in analyzing cDNA or genomic DNA; it is important that the amplified gene-specific fragment has no *Rsa* I restriction site. Here, we provide an example of using primers specific for the gene *GAPDH* (GAPDH 3′ primer 5′-*TCCACCACCCTGTTGCTGTA*-3′, *GAPDH* 5′ primer 5′-*ACCACAGTCCATGCCATCAC*-3′), which work well for human, mouse, and rat cDNA samples.

1. Dilute 1 µl of ligated cDNA into 200 µl of water.
2. Combine the reagents in two separate tubes as follows:

Component (µl)	Tube 1	2
cDNA (ligated to adapter A1A2)	1	1
GAPDH 3′ primer(10 µM)	1	1
GAPDH 5′ primer(10 µM)	0	1
PCR primer A1(10 µM)	1	0
Total volume	3	3

3. Prepare a master mix for both reaction tubes. For each reaction, combine the reagents in the following order:

Reagent	Amount per reaction tube (µl) 2	Amount for reactions (µl)
Sterile H_2O	18.5	37.0
10X PCR reaction buffer	2.5	5.0
dNTP mix (10 mM)	0.5	1.0
50X Taq polymerase mix	0.5	1.0
Total volume	22.0	44.0

4. Mix thoroughly and briefly centrifuge the tubes.
5. Aliquot 22 µl of master mix into each reaction tube from step 2.

6. Overlay with 50 µl of mineral oil. Skip this step if an oil-free thermal cycler is used.
7. Incubate the reaction mixture in a thermal cycler at 75°C for 5 min to extend the adapters. (Do not remove the samples from the thermal cycler.)
8. Immediately commence 20 cycles of:
   94°C – 30 s
   65°C – 30 s
   68°C (or 72°C, depending on the polymerase used) – 2.5 min
9. Examine the products by electrophoresis on a 2% agarose/EtBr gel.

If no products are visible after 20 cycles, perform 5 more cycles of amplification, and again analyze the product by gel electrophoresis. The number of cycles will depend on the abundance of the specific gene. The efficiency of ligation is estimated to be the ratios of the intensities of the bands corresponding to the PCR products of tube 2 to 1. Low ligation efficiency of 25% or less may substantially reduce the efficiency of the subsequent PS-based procedure. In this case, the ligation reaction should be repeated with fresh samples before proceeding to the next step.

For mouse or rat cDNAs, the PCR products amplified with the *GAPDH* 3′ primer and PCR primer A1 will be ~1.2 kb instead of the 0.75 kb band observed for human cDNA (because rat and mouse *GAPDH* cDNAs lack the *Rsa* I restriction site in 340 nt position). However, for the human cDNA (which contains the *Rsa* I site), the presence of a 1.2 kb band suggests that the cDNAs are not completely digested by *Rsa* I. If a significant amount of this longer PCR product persists, the procedure should be repeated from the step of *Rsa* I digestion.

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