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cDNA AMPLIFICATION BY SMART PCR AND SUPPRESSIVE-SUBTRACTIVE HYBRIDIZATION (SSH)-PCR

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SUBTRACTED cDNA

SUMMARY

The comparison of two RNA populations that differ from the effects of a single independent variable, such as a drug treatment or a specific genetic defect, can identify differences in the abundance of specific transcripts that vary in a population dependent manner. There are a variety of methods for identifying differentially expressed genes, including microarray, SAGE, qRT-PCR and DDGE. This protocol describes a potentially less sensitive yet relatively easy and cost effective alternative that does not require prior knowledge of the transcriptomes under investigation and is particularly applicable when minimal levels of starting material (RNA) are available. RNA input can often be a limiting factor when analyzing RNA from, for example, rigorously purified blood cells. This protocol describes the use of SMART-PCR to amplify cDNA from sub-microgram levels of RNA. The amplified cDNA populations under comparison are then subjected to Suppression Subtractive Hybridization (SSH-PCR), a technique that couples subtractive hybridization with suppression PCR to selectively amplify fragments of differentially expressed genes. The final products are cDNA populations enriched for significantly over-represented transcripts in either of the two input RNA preparations. These cDNA populations may then be cloned to make subtracted cDNA libraries and/or used as probes to screen subtracted cDNA, global cDNA or genomic DNA libraries.

1. INTRODUCTION

SMART-PCR and SSH-PCR are independent but compatible methods commercially developed and marketed by Clontech Laboratories, Inc. SMART-PCR[1] amplification of cDNA begins with conversion of mRNA to cDNA using MMLV-RT, mutated in the RNase H domain, in the first strand synthesis reaction (figure 1.1). This enzyme possesses RT activity, terminal deoxynucleotidyl transferase (TdT) activity, and DNA-dependent DNA polymerase activity. During first strand synthesis, an anchored oligo-d(T)₃₀VN primer (3' cDNA synthesis primer) anneals to the 5'-end of poly(A) tails, i.e., near the 3'-end of the RNA Pol II transcribed sequences. The 5'-end of this 3' cDNA synthesis primer contains a defined sequence that serves as a PCR primer target site in the subsequent amplification step. When the first strand has been extended to the end of an mRNA template, the TdT activity of the reverse transcriptase adds several un-templated deoxycytosine (d(C)) residues to the 3'-end of first strand cDNAs (in a fashion analogous to the addition of single un-templated d(A) residues to the end of PCR products generated with Taq DNA polymerase). The 5'cDNA synthesis primer contains three guanosine residues at its 3' end that anneal to these un-templated d(C) "tails" and then serve as a template for the DNA-dependent DNA polymerase activity of the MMLV-RT. This "Switching Mechanism At RNA Termini" (SMART) allows for MMLV-RT-mediated 3'-extension of all first strand cDNA products to include a defined, contiguous sequence copied from the 5'cDNA synthesis primer. The terminal transferase and template switching activities occur during the first strand cDNA synthesis. Regions of sequence in the 5'cDNA synthesis primer and the 3'cDNA synthesis primer are identical, allowing for uniform amplification of all first strand cDNAs with a single PCR primer[2].

SSH-PCR relies on the kinetics of DNA hybridization[3]. The efficiency of identifying a particular differentially expressed gene is a function of both its initial abundance in the transcriptome and its relative difference with that of the population to which it is being compared[4]. cDNAs prepared from the two mRNA populations being

compared are first digested with a four base cutting restriction enzyme to generate short blunt-ended fragments which are more favorable to the suppressive PCR effect described below. One cDNA population, believed to contain an over-representation of some unknown genes, is denoted the “*tester*”. The *tester* cDNA is split to two aliquots and each is ligated to a different adaptor (figure 1.2). The other cDNA population is denoted the “*driver*” and is mixed with the two adaptor-ligated *tester* cDNAs separately. The two mixtures are heat denatured and then allowed to re-anneal (figure 1.3). In these complexes, the *driver* is in excess and hybridizations do not go to completion. Sequences over-represented in the *tester* cDNA pool remain single stranded and sequences common to both *tester* and *driver* reanneal as they nucleate more frequently due to their higher relative concentrations. In a second hybridization step (figure 1.4), the single stranded *tester* sequences remaining in the two first hybridization mixes, which bear different adaptor sequences at their ends, are allowed to fully re-anneal in the presence of additional denatured *driver* cDNA. In this second hybridization, there is further subtraction of sequences common to both *tester* and *driver* cDNAs. As these hybridizations go to completion, single stranded fragments unique to the *tester* cDNA form hybrid molecules with different adaptor sequences at either end (*starred* in figure 1.4).

Two successive PCR reactions are then performed to enrich for the differentially expressed sequences in the subtracted cDNA populations[5]. The first PCR reaction (figure 1.5) relies on the sequence-suppressive PCR effect to prevent amplification of hybrid molecules with identical adaptors at either end, formed during subtractive hybridization. These fragments form strong secondary structures that prevent primer annealing and extension during thermal cycling (figure 1.5). Double stranded molecules containing only one adaptor-ligated strand (arising from adaptor-ligated sequences annealing to *driver* cDNA) are only linearly amplified. Re-annealed *driver* cDNAs are not amplified. Exponential amplification is favored only from molecules with different adaptor sequences at either end, arising from sequences present only in the *tester* cDNA population. The first PCR products are diluted and used as input for the second PCR reaction. The use of nested, adaptor-targeted primers in the second PCR reaction further increases the fraction of differentially expressed sequences. Because the subtractive hybridizations and suppression PCRs enrich for genes **over**-represented in the *tester* cDNAs, the method is performed in two parallel processes. One of the two cDNA populations is arbitrarily designated as *tester* and subtracted with the other as *driver* in a FORWARD subtraction. In parallel, the *tester* and *driver* designations are switched and a REVERSE subtraction is performed to enrich for genes **over**-represented in the other sample. Control reactions are included for both FORWARD and REVERSE subtractions that result in unsubtracted cDNA pools.

The final products of the entire procedure are thus FORWARD and REVERSE subtracted and un-subtracted cDNA populations. The subtracted cDNAs can be cloned into plasmid vectors to produce subtracted cDNA libraries[6]. The subtracted cDNAs also provide excellent probes for differential screening of these or any relevant gene library, especially if the un-subtracted cDNAs are used as control probes. Clontech Laboratories, Inc. offer kits for subtracted cDNA library construction and screening, as well as for both SMART-PCR and SSH-PCR (PCR-Select). These kits also provide positive control materials which are not described here. The reader will find it useful to refer to the Clontech User Manuals for additional information on amplifying and analyzing subtracted cDNAs prepared from limited amounts of cellular RNA[7, 8]. Ji *et al*[4] describe a theoretically and experimentally tested model of the method which is also recommended reading when considering this method for your particular application.

2 MATERIALS

All materials should be stored at -20°C unless otherwise stated. Working stocks should be kept on ice unless otherwise stated.

2.1. Preparation and amplification of cDNA.

1. M-MLV-Reverse Transcriptase, RNase H Minus. (e.g. PowerScript from Clontech or Superscript II from InVitrogen)
2. 5x first strand buffer (supplied with RT)
3. 3' cDNA synthesis primer (N=G, A, T, C. V=G, A or C):
5' -AAGCAGTGGTATCAACGCAGAG**TACT**₍₃₀₎ VN
4. 5' cDNA synthesis primer. (store at -80°C)
5' -AAGCAGTGGTATCAACGCAGAG**TAC**GCrGrGrG (see note 1)
5. PCR primer (see also 2.3.7):
5' -AAGCAGTGGTATCAACGCAGAGT

(Rsa I sites are in **BOLD** and identical sequences are UNDERLINED).
6. Thermo-stable DNA Polymerase Mix (e.g. from Clontech, Advantage 2 PCR kit) (see note 2)
7. 10X PCR buffer (supplied with the polymerase)
8. STE buffer: 10mM NaCl, 10mM Tris (pH 7.8), 0.1 mM EDTA. Store at room temperature.
9. CHROMA SPIN 1000 purification columns (Clontech. Cat. no. 636056). Store at room temperature.

2.2 Restriction digest of cDNA

10. Rsa I (10 Units/μl)
11. 10X Rsa I buffer (supplied with the enzyme, e.g. from New England Biolabs)
12. Silica slurry purification resin and associated buffers (e.g. Clontech NucleoTrap, Cat. no. 636020, Qiagen Qiaex II, Cat no. 20902, or equivalent). Store components according manufacturers specification

2.3. Adaptor ligation

1. T4 DNA ligase (400 u/μl) (containing ATP) (e.g. from BRL, Clontech or InVitrogen)
2. 5X T4 DNA Ligase buffer (supplied with the ligase)
3. Adaptor 1: (10 pmol/μl)
5' -CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3'
GGCCCGTCCA-5'
4. Adaptor 2R: (10 pmol/μl)
5' -CTAATACGACTCACTATAGGGCAGCGTGGTTCGCGGCCGAGGT-3'
GCCGGCTCCA-5'

Underlined bases match the PCR primer sequence

Double underlined bases mach the nested (secondary) PCR primer sequences (see 2.5.2-3).

5. 5' G3PDH primer (10 pmol/μl): 5' -ACCACAGTCCATGCCATCAC
6. 3' G3PDH primer (10 pmol/μl): 5' -TCCACCACCCTGTTGCTGTA

These primers will amplify human, mouse and rat G3PDH.

7. PCR primer (see also 2.5.1): 5' -CTAATACGACTCACTATAGGGC

2.4. Subtractive Hybridizations:

1. 4X Hybridization Buffer:
 - 50 mM Hepes (pH 8.3)
 - 0.5 M NaCl
 - 0.02 mM EDTA (pH 8.0)
 - 10% (wt/vol) PEG 8000.
2. Dilution buffer:
 - 20 mM Hepes (pH 6.6)
 - 20 mM NaCl
 - 0.2 mM EDTA (pH 8.0)

2.5 Suppressive and Nested PCR primers

1. PCR primer: 5' -CTAATACGACTCACTATAGGGC
2. Nested (secondary) primer 1: 5' -TCGAGCGGCCCGCCGGGCAGGT
3. Nested primer 2R: 5' -AGCGTGGTCGCGGCCGAGGT

2.6 General reagents.

General reagents should be stored and used at room temperature unless otherwise stated.

1. 10 mM dNTP's (10 mM each, dGTP, dATP, dTTP, dCTP). Store at -20°C. Keep working stocks on ice.
2. Dithiothreitol (DTT), 20 mM
3. EDTA/glycogen Mix: 0.2 M EDTA, 1mg/ml glycogen)
4. Phenol: Chloroform:Isoamyl alcohol (25:24:1) (see note 3). Store at 4°C. Keep working stock at room temperature.
5. *n*-Butanol
6. 4 M ammonium acetate (pH 7.0)
7. Absolute Ethanol. Store at -20°C
8. 80% EtOH (80 parts absolute ethanol, 20 parts sterile, deionized water)
9. TE buffer: 10mM Tris (pH 7.8), 1 mM EDTA.
10. 0.5 M EDTA (pH 8.0)
11. sterile, deionized H₂O
12. Agarose gel electrophoresis system
13. molecular weight markers

3. METHODS

3.1 Amplification of cDNA.

3.1.1 First-Strand cDNA Synthesis.

1. For each RNA sample, combine the following reagents in a clean, nuclease-free 0.5 ml reaction tube:
 - 1–3- μ l RNA sample (50-1000 ng of total RNA). (See note 4)
 - 1 μ l 3' cDNA synthesis primer (12 pmol/ μ l)
 - 1 μ l 5' cDNA synthesis primer (12 pmol/ μ l)
 - x μ l nuclease-free H₂O
 - 5 μ l Total volume
2. Mix well and centrifuge briefly to collect reaction mix at the bottom of the tube
3. Incubate at 70°C for 2 minutes.
4. Place reactions on ice for 2 minutes.

5. For each sample, prepare the following RT Mix:
 - 2 μ l 5X RT Buffer
 - 1 μ l DTT (20 mM)
 - 1 μ l dNTP Mix (10 mM of each dNTP)
 - 1 μ l MMLV RNase H minus Reverse Transcriptase
 - 5 μ l
6. Add 5 μ l RT Mix (for a 10 μ l total volume) to each sample, mix well and centrifuge briefly.
7. Incubate the tubes at 42°C for 1 hour in an air incubator. (see note 5)
8. Terminate the reactions by adding 40 μ l of TE and heat denature at 70°C for 10 minutes.
9. Each sample should now contain 50 μ l of first strand cDNA.

3.1.2 cDNA amplification by SMART PCR:

Over-cycling of the cDNA amplification reactions can lead to distortion of the relative representation of cDNAs in the amplified preparations. Therefore, it is necessary to determine the optimal cycle number for each sample that generates sufficient amplified cDNA without saturating the PCR reaction. Three identical PCR reactions are set up for each sample – one optimization reaction and two preparative reactions. All reactions are subjected to 15 cycles of PCR, after which the two preparative tubes are temporarily transferred to 4°C. An aliquot is removed from the third (optimization) tube which is then subjected to further cycles of PCR. Additional aliquots are removed from the optimization tubes after every three cycles. These aliquots, collected at 15, 18, 21 and 24 cycles, are then analyzed by electrophoresis. The two preparative tubes held at 4°C are then returned to the thermal cycler and subjected to the remainder of the optimal cycle number. The optimal cycle number is defined as one less than the number of cycles required to achieve the PCR saturation point.

The amount of first strand cDNA input used for amplification is dependent on the amount of RNA used in the first strand synthesis reaction. Use the suggested volumes of first strand cDNA below.

<u>RNA input in first strand synthesis reaction</u>	<u>Volume first strand cDNA used in PCR reactions</u>
50-100 ng	10 μ l
100-250 ng	4 μ l
250-500 ng	2 μ l
500-1,000 ng	1 μ l

1. Dispense the appropriate volume of each cDNA (from the table above) into a clean PCR tube and adjust to 10 μ l with H₂O.
2. Prepare enough of the following PCR mix for three PCR reactions for each cDNA sample:
 - Per reaction:
 - 74 μ l high quality H₂O
 - 10 μ l 10X PCR Buffer
 - 2 μ l dNTP Mix (10 mM of each dNTP)
 - 2 μ l cDNA PCR primer (12 pmol/ μ l)
 - 2 μ l Taq Polymerase Mix
 - 90 μ l
3. Mix well and centrifuge briefly to collect the mix at the bottom of the tube
4. Program your thermal cycler as follows and allow it to reach 95°C.
 - 95°C 2 minute (to denature the template and the Hot start antibody)

95°C, 15 sec }
 65°C, 30 sec. } 24 cycles
 68°C, 6 minutes }

5. Add 90µl of the PCR Mix to each 10µl cDNA sample, mix well and centrifuge briefly.
6. Place all tubes (three identical reactions for each sample) in the preheated thermal cycler.
7. Perform 15 cycles of PCR on all samples.
8. At the end of the 15th cycle, remove the two preparative reaction tubes of each sample and place at 4°C.
9. Remove a 15 µl aliquot from each optimization reaction, place it in a fresh tube and store at 4°C. (See note 6).
10. Return the optimization reaction tubes to the thermal cycler and continue the cycling program.
11. At the end of 18th cycle, remove another 15 µl aliquot from each optimization reaction tube and place at 4°C.
12. At the end of the 21st cycle, remove another 15 µl aliquot from each optimization reaction tube and place at 4°C.
13. At the end of the 24th cycle, remove another 15 µl aliquot from each optimization reaction tube and place at 4°C.
14. You should now have 15 µl aliquots of each PCR reaction cycled for 15, 18, 21 and 24 cycles. Electrophorese these aliquots on a 1.2% agarose gel using a 1 kb ladder as a marker.
15. Determine the optimal number of cycles for cDNA amplification, corresponding to one cycle less than the saturation point of the PCR reaction. (See figure 3.1).
16. Retrieve the preparative PCR reaction tubes from 4°C storage and subject these to the remaining number of cycles required to reach the optimal cycle number for each sample.
17. Add 2µl of 0.5M EDTA to each tube.
18. Combine the contents of both preparative tubes for each amplified cDNA.
19. Confirm that the PCR reactions were successful by electrophoresing 10-15µl on a 1.2% agarose gel. (See figure 3.1 B).

You should now have optimally amplified, double stranded cDNA from the two mRNA populations being compared. These samples will now be purified and digested prior to linker ligation and subtractive hybridization.

3.1.3 Purification of amplified cDNA

1. Set aside a 5 µl aliquot of each sample at -20°C as an “input” control. (for step 3.1.3.19, below)
2. Extract the amplified cDNA products with an equal volume (~190 µl) of phenol/chloroform/isoamyl alcohol (25:24:1). Vortex well.
3. Centrifuge for 2 minutes at full speed.
4. Transfer the upper aqueous phase to a clean tube.
5. Extract the aqueous phase with two to three volumes of n-butanol to concentrate the cDNAs. Vortex well.
6. Centrifuge for 1 minute at full speed.
7. The cDNA remains in the LOWER aqueous phase and should be approximately 50 µl. Transfer the aqueous phase to a fresh tube (see note 7). Discard the UPPER organic phase.
8. Prepare a CHROMA SPIN 1000 purification column for each sample by gently but thoroughly resuspending the column matrix.
9. Remove the top cap and bottom cap – in that order - and allow the buffer to drain out by gravity.
10. Equilibrate the column with 1.5 ml room temperature STE.
11. Apply the cDNA sample to the centre of the bed matrix and allow it to enter the column.
12. Add 25 µl of STE and allow the column to flow by gravity until it stops.
13. Add 150 µl STE and allow the column to flow by gravity until it stops.
14. Place a clean 1.5 ml microfuge tube under the column and apply 320 µl STE.
15. Collect the entire flow through as a single fraction. This contains the cDNA.
16. Place a clean 1.5 ml microfuge tube under the column and apply 75 µl STE.
17. Collect the entire flow through as another single fraction. This may contain additional cDNA.
18. On a 1.2% agarose gel, analyse the 5 µl “input” control (from step 3.1.3.1) and a 10 µl aliquot of the two eluted column fractions. Use a 1 kb ladder as a marker.

- The first (320 μ l) fraction should contain the majority of recovered cDNA. If the second eluate (75 μ l) contains a significant amount of cDNA, combine it with the first (320 μ l) eluate ONLY if the first fraction contains less than approximately 30% of the total input.

3.2 RsaI digestion

The purified cDNAs will now be digested with Rsa I. This generates blunt ends for linker ligation and produces smaller fragments better suited to subtractive hybridization.

- Set aside a 10 μ l aliquot of each purified cDNA sample at -20°C as an "undigested" control. (for step 4, below).
- Add an appropriate volume of 10X Rsa I buffer to bring the mixture to 1 X Rsa I buffer.
- Add 15-20 Units of Rsa I, mix well and incubate at 37°C for 3 hours (see note 8).
- On a 1.2% agarose gel, analyse the 10 μ l "undigested" controls and 10 μ l of the digestion products. (see figure 3.2)
- When digestion is complete, inactivate the enzyme by adding 5-10 μ l 0.5 M EDTA (pH 8.0).
- Purify the digestion products using a silica slurry resin (see materials, 2.2.12) according to the manufacturer's recommendations. (see note 9)
- Transfer the eluted, purified cDNA from the final pellet of silica resin to a fresh tube. (see note 10)
- Add 1/2 vol. NH_4OAc to the final elution volume containing the silica purified, Rsa I digested cDNAs and mix.
- Add 2.5 volumes EtOH to each sample and mix to precipitate the digested cDNA.
- Centrifuge at full speed ($>12,000\times g$) for 20 minutes at room temperature and discard the supernatants.
- Rinse the pellets with 500 μ l 80% EtOH, centrifuge at full speed ($>12,000\times g$) for 5 minutes at room temperature and discard the supernatants.
- Air-dry the pellets at room temperature for 10-15 minutes. Do not use a speed vac.
- Resuspend the pellets in 6.7 μ l of distilled water.
- Use 1.2 μ l to determine the concentration of the purified digested cDNA's by absorbance at 260 nm. You should recover between 2-6 μg Rsa I digested cDNA after purification. If the concentration is $>300\text{ng}/\mu\text{l}$, dilute to 300 $\text{ng}/\mu\text{l}$.
- Store the remaining 5.5 μ l of each Rsa I digested and purified cDNA at -20°C .

3.3 Preparation of tester cDNAs

3.3.1. Ligation of adaptors

Label the prepared cDNAs as number 1- and number 2-. An aliquot of each digested cDNA preparation, 1- and 2-, will be ligated to two separate adaptors; 1 and 2R, making a total of four separate ligation reactions. The ligation reactions are labeled 1-1, 1-2R and 2-1, 2-2R. Two additional ligation reactions, labeled 1-c and 2-c, will be prepared by mixing aliquots of reactions 1-1 with 1-2R and 2-1 with 2-2R. Reaction products 1-c and 2-c will later serve as unsubtracted controls.

- Prepare a ligation mix sufficient for 4 reactions:
Per reaction:
 3 μ l H_2O
 2 μ l 5X ligation buffer
 1 μ l T4 DNA ligase
 5 μ l
- Dilute 1 μ l of each digested and purified cDNA from step 3.2.13 with 5 μ l distilled water.
- Set up the following ligation reactions with the following labels:

Tube	1-1	1-2R	2-1	2-2R
diluted cDNA 1- :	2 μ l	2 μ l	-	-

diluted cDNA 2- :	-	-	2 μ l	2 μ l
Adaptor 1 :	2 μ l	-	2 μ l	-
Adaptor 2R :	-	2 μ l	-	2 μ l
Ligation mix :	<u>5 μl</u>	<u>5 μl</u>	<u>5 μl</u>	<u>5 μl</u>
	10 μ l	10 μ l	10 μ l	10 μ l

- Mix well and centrifuge briefly.
- Transfer 2 μ l from tube 1-1 and 2 μ l from tube 1-2R to a fresh tube labeled 1-c and mix.
- Transfer 2 μ l from tube 2-1 and 2 μ l from tube 2-2R to a fresh tube labeled 2-c and mix.
- Centrifuge all tubes briefly.
- Incubate all (6) ligation reactions overnight at 16°C.
- Stop the reactions by adding 1 μ l EDTA/glycogen mix to each tube.
- Denature the ligase by heating all tubes at 72°C for 5 minutes.
- Centrifuge all tubes briefly. These are your adaptor-ligated *tester* cDNAs to be used in step 3.3.2.1
- Transfer 1 μ l from tube 1-c to a fresh tube containing 1 ml of distilled H₂O. Label this tube "FORWARD unsubtracted 1-c"
- Transfer 1 μ l from tube 2-c to a fresh tube containing 1 ml of distilled H₂O. Label this tube "REVERSE unsubtracted 2-c"
- Store all tubes at -20°C.

3.3.2.ligation efficiency assay

It is important to determine if the ligation reactions were efficient by using a simple PCR assay. The two adaptors used in 3.3.1 share a region of common sequence. PCR reactions using a primer pair comprised of a G3PDH specific primer and the adaptor targeted PCR primer generate a specific product if the adaptor sequence is present (the 'test' reaction). This product is compared to PCR reaction products generated from a primer pair comprised of two G3PDH targeted primers (control reactions). If the efficiency of the test reaction is similar to that of the control, it indicates efficient ligation of the adaptors to the digested cDNAs.

- Prepare 1:200 dilutions of each of the four ligation products, 1-1, 1-2R, 2-1 and 2-1R.
- For each diluted sample, set up two PCR reactions (8 reactions in total)

<u>Reaction:</u>	<u>1 (test)</u>	<u>2 (control)</u>
diluted sample: (1-1, 1-2R, 2-1 or 2-2R)	1 μ l	1 μ l
3' G3PDH primer (10 pmol/ μ l)	1 μ l	1 μ l
5' G3PDH primer (10 pmol/ μ l)	-	1 μ l
adaptor-targeted PCR primer (10 pmol/ μ l)	<u>1 μl</u>	-
	3 μ l	3 μ l

- Prepare a PCR mix sufficient for 8 reactions:

	<u>Per reaction</u>
H ₂ O	18.5 μ l
10X PCR buffer	2.5 μ l
dNTP mix	0.5 μ l
Polymerase mix	<u>0.5 μl</u>
	22 μ l

4. Add 22 μ l of the PCR mix to each reaction tube from step 2.

5. Subject all tubes to the following thermal profile:

75°C	5 minutes	(this step fills in the ends of the adaptors to produce fully double stranded fragments)
95°C	2 minutes	(to fully inactivate the Hot Start antibody)
95°C	30 seconds	} 25 cycles (see note 11)
65°C	30 seconds	
68°C	2.5 minutes	

6. Analyze the PCR products alongside a molecular weight marker on a 2% agarose gel. For rat or mouse samples, you should observe a ~1.2 kb band in the test reactions. For human samples, you should observe a ~0.75 kb band (see note 12). For other species, you will need to design specific primers. Successful ligations will result in nearly equal band intensity from both the test and control reactions. If the band intensity of the test reaction products are less than approximately 40% that of the control, you should repeat the procedure starting from the cDNA amplification step 3.1.2.

3.4 Subtractive hybridization:

Two subtractions will be performed; a FORWARD and a REVERSE subtraction. For each subtraction, the adaptor-ligated products are designated the *tester* cDNAs and the un-ligated cDNAs (with no adaptors) are designated *driver* cDNAs. Adaptor-ligated products 1-1 and 1-2R *tester* cDNAs will be independently hybridized to an excess of *driver* cDNA 2 in two primary, FORWARD subtraction hybridizations (one for each adaptor-ligated preparation, 1-1 and 1-2R). Likewise, adaptor-ligated products 2-1 and 2-2R *tester* cDNAs will be independently hybridized to excess *driver* cDNA 1 in two primary REVERSE subtraction hybridizations.

3.4.1 Primary Hybridization reaction:

1. Set up the following First Hybridization reactions using the adaptor-ligated *tester* cDNAs from step 3.3.1.11 and Rsa I digested and purified *driver* cDNAs from step 3.2.15:

First Hybridization Number:	<u>FORWARD</u>		<u>REVERSE</u>	
	(1)	(2)	(3)	(4)
Rsa I digested driver cDNA 1	-	-	1.5 μ l	1.5 μ l
Rsa I digested driver cDNA 2	1.5 μ l	1.5 μ l	-	-
Adaptor ligation cDNA tester 1-1	1.5 μ l	-	-	-
Adaptor-ligated cDNA tester 1-2R	-	1.5 μ l	-	-
Adaptor ligation cDNA tester 2-1	-	-	1.5 μ l	-
Adaptor-ligated cDNA tester 2-2R	-	-	-	1.5 μ l
4X Hybridization Buffer	1.0 μ l	1.0 μ l	1.0 μ l	1.0 μ l
	4.0 μ l	4.0 μ l	4.0 μ l	4.0 μ l

2. Heat denature the hybridization reactions in a thermal cycler at 98°C for 1.5 minutes.
3. Hybridize the denatured cDNAs in a thermal cycler at 68°C for 8 hours. Hybridization times over 12 hours will allow differentially expressed cDNAs to re-anneal.
4. After hybridization, centrifuge briefly to collect the reaction mixes at the bottom of the tube and return to 68°C briefly while preparing the secondary hybridization reaction mix.

3.4.2 Secondary Hybridization reactions:

1. Prepare fresh denatured drivers for the second hybridization as follows:

	<u>FORWARD</u>	<u>REVERSE</u>
Rsa I digested driver cDNA 1	-	1 µl
Rsa I digested driver cDNA 2	1 µl	-
4X Hybridization buffer	1 µl	1 µl
Sterile H2O	<u>2 µl</u>	<u>2 µl</u>
	4 µl	4 µl

2. Heat denature these driver cDNA solutions in a separate thermal cycler or heat block. (See note 13).

The First Hybridization products (1) and (2) will now be mixed with freshly denatured driver cDNA 2 in the FORWARD subtraction. Likewise, the First Hybridization products (3) and (4) will be mixed with freshly denatured driver cDNA 1 in the REVERSE subtraction.

3. Remove 1 µl of freshly denatured driver cDNA 2 and place it on the inside upper wall of the tube containing First Hybridization Number (1). Do not let the solutions mix at this point. Set the tube down horizontally on the thermal cyclers heat block momentarily.
4. Working quickly, transfer the contents of First Hybridization Number (2) to the tube containing First Hybridization Number (1) and the freshly denatured driver cDNA 2. As you dispense the mixture, push the denatured driver cDNA down the inside surface of the tube so that all three solutions are mixed simultaneously and return the mixture to the thermal cycler at 68°C. This is your FORWARD subtractive hybridization. Label this tube FORWARD subtracted cDNA.
5. Repeat this process with 1 µl freshly denatured driver cDNA 1 and First Hybridization (3) and (4) and return the mixture to the thermal cycler at 68°C. This is your REVERSE subtractive hybridization. Label this tube REVERSE subtracted cDNA.
6. Incubate the FORWARD and REVERSE subtractive hybridization mixes at 68°C overnight. These hybridizations go to completion.
7. When the second hybridization is complete, add 200 µl of dilution buffer to each subtraction mix.
8. Return the diluted hybridization products to the 68°C thermal cycler for 7 minutes.
9. Store the “FORWARD” and “REVERSE” subtraction mixes at -20°C.

3.5 Suppressive and Nested PCR

The unsubtracted cDNAs prepared in steps 3.3.1.12 and 3.3.1.13 are used as controls the next steps of suppressive and nested PCRs.

3.5.1 Primary (suppressive) PCR:

1. Prepare enough of the following PCR mix for four reactions:

	<u>Per reaction</u>
H ₂ O	19.5 µl
10X PCR buffer	2.5 µl
10 mM dNTP mix	0.5 µl
PCR primer (10 pmol/µl)	1.0 µl
50X DNA Polymerase Mix	<u>0.5 µl</u>
	24.0 µl

- Aliquot 1 µl of each of the following into separate PCR reaction tubes, labeled s1, s2, s3 and s4 (s = “suppressive”).

- FORWARD subtracted cDNA (from step 3.4.2.9)
- FORWARD unsorted 1-c (from step 3.3.1.12)
- REVERSE subtracted cDNA (from step 3.4.2.9)
- REVERSE unsorted 2-c (from step 3.3.1.13)

- Add 24.0 µl of the PCR mix to each tube (to make 25 µl reaction volumes), mix and centrifuge briefly to collect contents of each at the bottom of the tube.

- Program your thermal cycler as follows and subject all reaction mixes to this PCR program:

75°C for 5 minutes*	} 27 cycles
94°C 30 seconds	
66°C 30 seconds	
72°C 1.5 minutes	

* This step extends the adaptors to create a complementary target for the PCR primer.

- These are your primary (suppressive) PCR products, s1-s4. When cycling is complete, transfer 8 µl of each reaction product to a fresh, appropriately labeled tube and store at -20°C for later gel analysis.

3.5.2 Secondary (nested) PCR:

- Prepare 1:10 dilutions of each primary PCR product s1–s4 with water (e.g. 3 µl PCR product and 27 µl water).
- Aliquot 1 µl of each diluted primary PCR product into separate PCR reaction tubes labeled n1, n2, n3 and n4 (n = “nested”)
- Prepare enough of the following PCR mix for four reactions:

	<u>Per reaction</u>
H ₂ O	18.5 µl
10X PCR buffer	2.5 µl
10 mM dNTP mix	0.5 µl
Nested PCR primer 1 (10 pmol/µl)	1.0 µl
Nested PCR primer 2R (10 pmol/µl)	1.0 µl
50X DNA Polymerase Mix	<u>0.5 µl</u>
	24.0 µl

- Add 24.0 µl of the PCR mix to each tube (to make 25 µl reaction volumes), mix and centrifuge briefly to collect contents of each at the bottom of the tube.
- Subject all reaction mixes to the following PCR program:

94°C	30 seconds	} 12 cycles
68°C	30 seconds	
72°C	1.5 minutes	

6. When cycling is complete, transfer 8 µl of each reaction product to an appropriately labeled tube for gel analysis.

7. The remaining 17µl of the secondary (nested) PCR products are your final products. They comprise:

- n1) FORWARD subtracted cDNA
- n2) FORWARD unsubtracted cDNA
- n3) REVERSE subtracted cDNA
- n4) REVERSE unsubtracted cDNA

8. Electrophorese the 8 µl aliquots of each suppressive and nested PCR product, s1-s4 from step 3.5.1.5, and n1-n4 from step 6, respectively, on a 2% agarose gel. Both primary and secondary PCR products should appear as a smear, with or without discrete bands. (See figure 3.3)

9. The final products (n1-n4) can now be cloned to make subtracted cDNA libraries and/or used as probes to screen subtracted cDNA, global cDNA or genomic DNA libraries.

3.5.3 Assay for subtraction efficiency:

1. In fresh tubes, prepare 1:10 dilutions of each secondary PCR product n1-n4 with water (e.g. add 3 µl PCR product to 27 µl water as before).

2. Place 1.0 µl of each diluted secondary PCR product in a fresh PCR tube.

3. Prepare enough of the following PCR mix for four reactions:

	<u>Per reaction</u>
H ₂ O	22.4 µl
10X PCR buffer	3.0 µl
10 mM dNTP mix	0.6 µl
G3PDH 5' primer (10 pmol/µl)	1.2 µl
G3PDH 3' primer (10 pmol/µl)	1.2 µl
50X DNA Polymerase Mix	<u>0.6 µl</u>
	29.0 µl

4. Add 29.0 µl of the PCR mix to each tube (to make 30 µl reaction volumes), mix and centrifuge briefly to collect contents of each at the bottom of the tube.

5. Program your thermal cycler as follows:

94°C	30 seconds	} 33 cycles
60°C	30 seconds	
68°C	1.5 minutes	

6. Place the reaction mixes in the thermal cycler and run the first 18 cycles.

7. After the 18th cycle, remove 5 µl from each tube and place in separate, fresh tubes. (See note 6).

8. Continue the program for an additional 5 cycles (for a total of 23 cycles).and again transfer 5 µl from each reaction to fresh tubes

9. Continue the program for an additional 5 cycles (for a total of 28 cycles) and again transfer 5 µl from each reaction to fresh tubes.

10. Continue the program for the final 5 cycles (for a total of 33 cycles) and again transfer 5 μ l from each reaction to fresh tubes (for a total of 33 cycles).
11. Electrophorese each of the 5 μ l aliquots collected at 18, 23, 28 and 33 cycles on a 2% agarose gel.

See figure 3.4. Efficient subtraction is indicated by depletion of G3PDH sequences from the subtracted cDNAs for both the FORWARD and REVERSE subtractions. Unsubtracted cDNAs should show an increase in G3PDH product accumulation with increasing cycle number, to the point of a plateau.

NOTES:

1. This oligonucleotide is available as part of the SMART PCR cDNA synthesis kit from Clontech. Details of their modifications are proprietary. We successfully amplify cDNA with an independently synthesized oligonucleotide, however efficiencies in subtractive hybridization and suppressive PCR have not been determined.
2. The Taq polymerase used for cDNA amplification should be nuclease deficient, formulated specifically for amplification of long fragments, and should include the Kellog antibody for automatic hot start.
3. Phenol: Chloroform:Isoamyl alcohol should be prepared with fresh (<2 weeks) phenol saturated with 50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA). Phenol appearing yellow from oxidation should not be used.
4. RNA quality is perhaps the most critical parameter for successful comparisons of two RNA populations. Determine RNA quality by, for example, an Agilent bioanalyzer or Northern blot, and determine RNA concentrations carefully by absorbance at 260 nanometers.
5. To minimize evaporation and condensation in the tube, which can significantly reduce cDNA yields, use a pre-warmed air incubator as opposed to a heat block.
6. It is important that aliquots are removed from each sample while at the 68°C extension step, before the thermal cycler begins to ramp up to the denaturing temperature. Denatured samples will not migrate correctly during electrophoresis.
7. Re-extract the aqueous phase with *n*-butanol or back-extract the organic phase with H₂O, if necessary, to reach an aqueous volume of approximately 50 μ l containing your cDNA.
8. Digestion must go to completion. Aliquots (~10 μ l) can be removed at several time points and analyzed on an agarose gel. No further decrease in molecular weight with time indicates complete digestion.
9. Alternatively, samples can be extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and ethanol precipitated, although this may lead to decreased adaptor ligation efficiency.
10. After transferring to a fresh tube, re-centrifuge the elutate at full speed for 1 minute and transfer to yet another fresh tube to eliminate possible carry over of silica resin. Alternatively, elutates may be passed through a 0.45 μ filter

11. If G3PDH is highly abundant in your cDNA populations, reduce the cycle number to avoid saturation of the PCR reactions.
12. If a band of ~1.2 kb is generated from a human G3PDH PCR product, this indicates incomplete digestion because human G3PDH contains an Rsa I site.
13. It is practical to have this heat block or thermal cycler near the thermal cycler used for the hybridizations in order to avoid premature renaturation of the freshly denatured driver cDNA's.

Figure 1.1

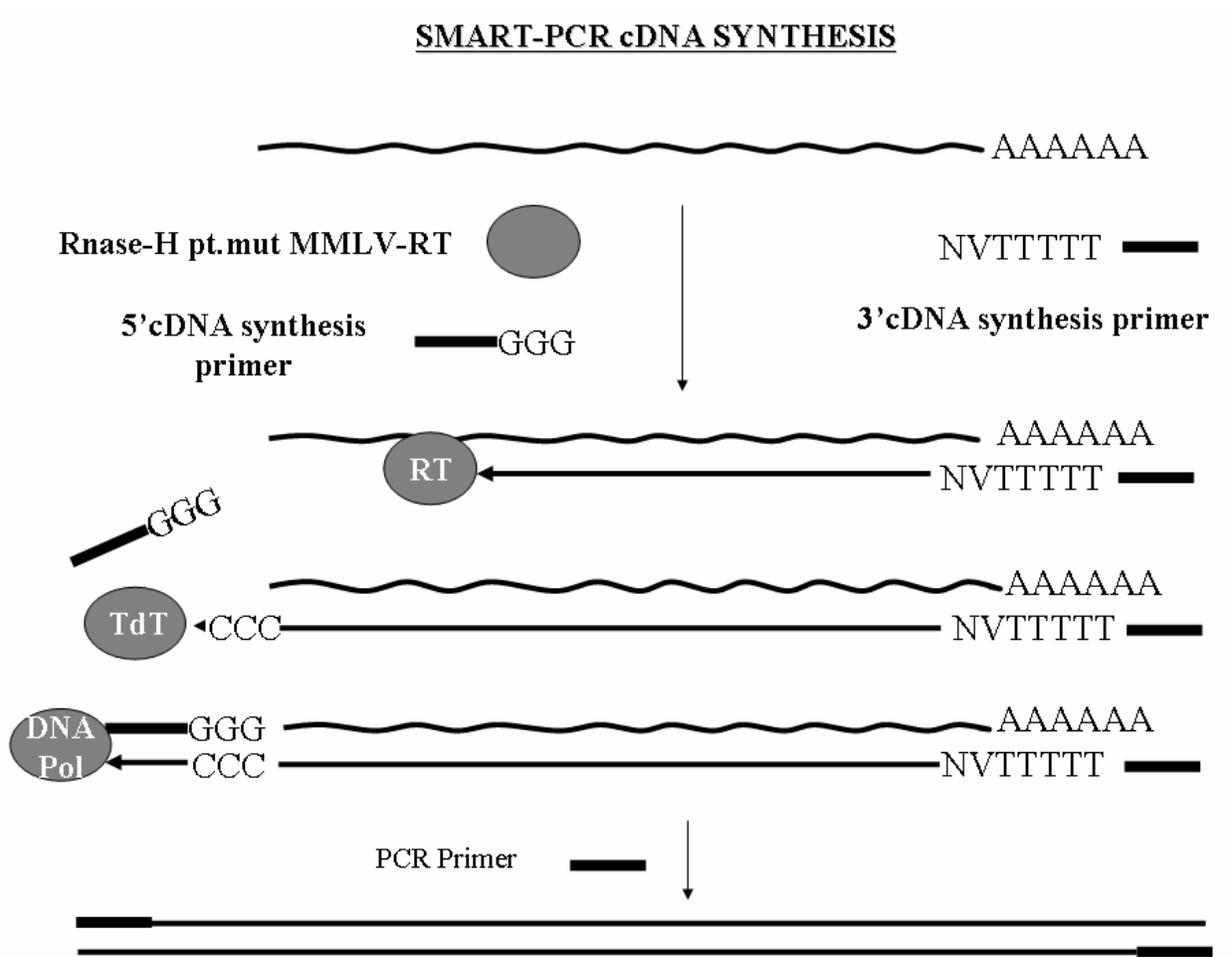


Figure 1.2

ADAPTOR LIGATION

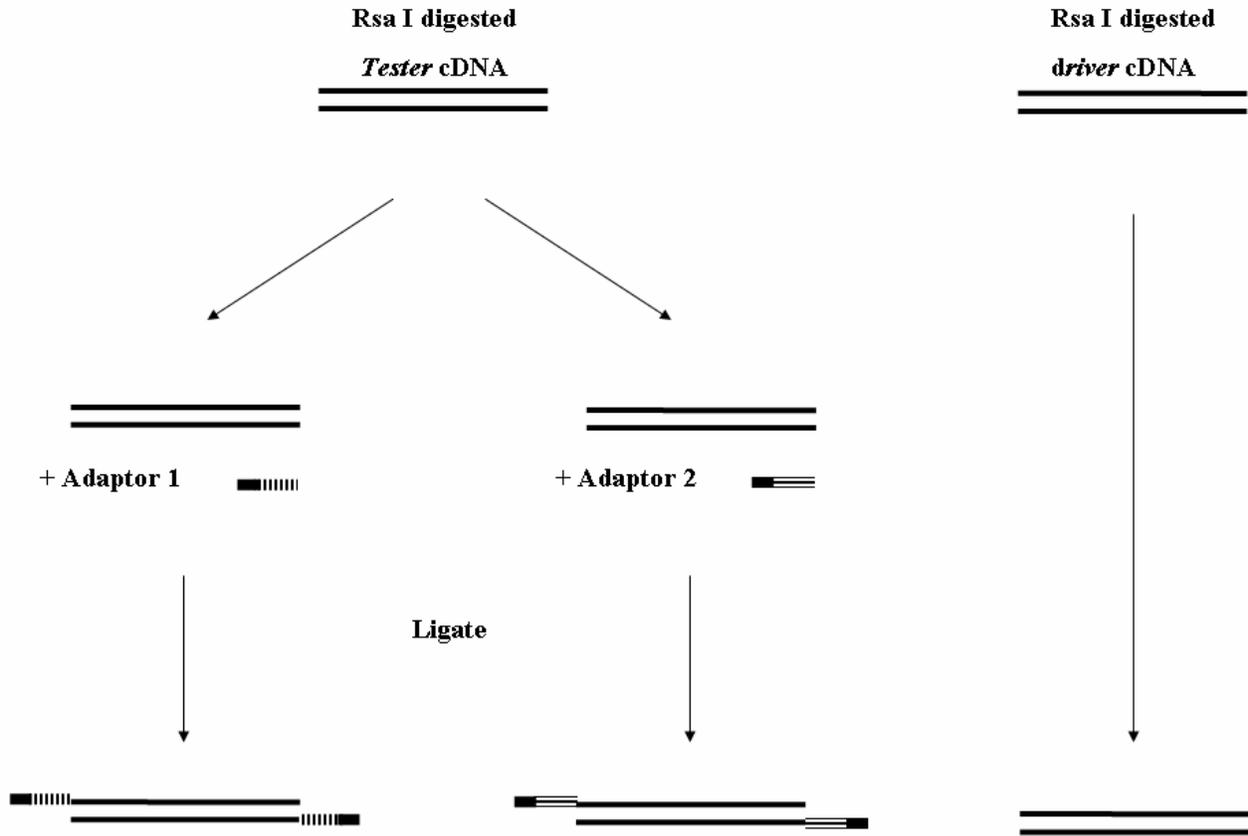


Figure 1.3

FIRST HYBRIDIZATION

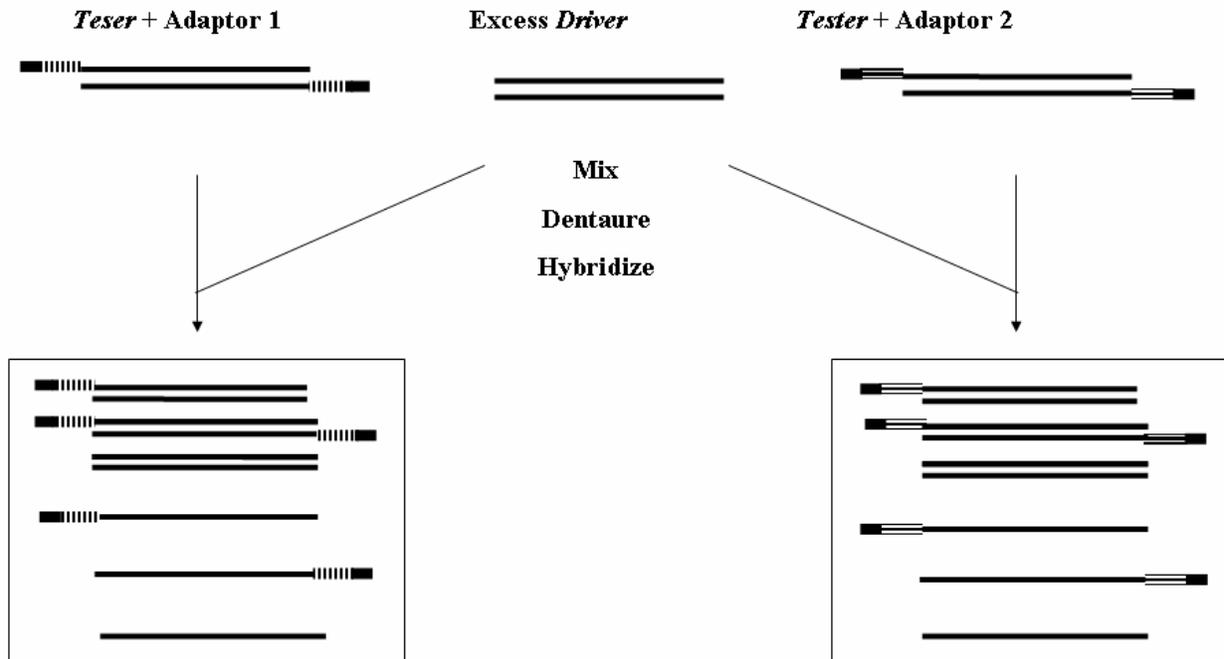


Figure 1.4

SECOND HYBRIDIZATION

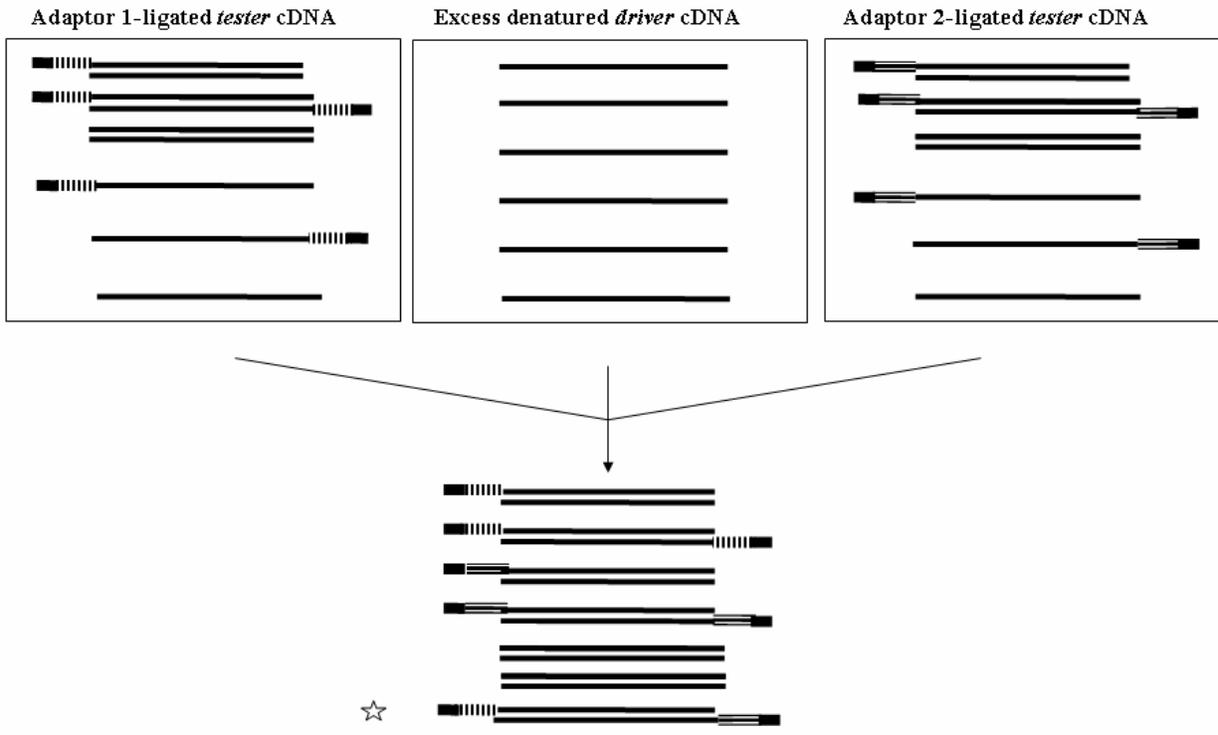


Figure 1.5

SUPPRESSIVE SUBTRACTIVE HYBRIDIZATION PCR

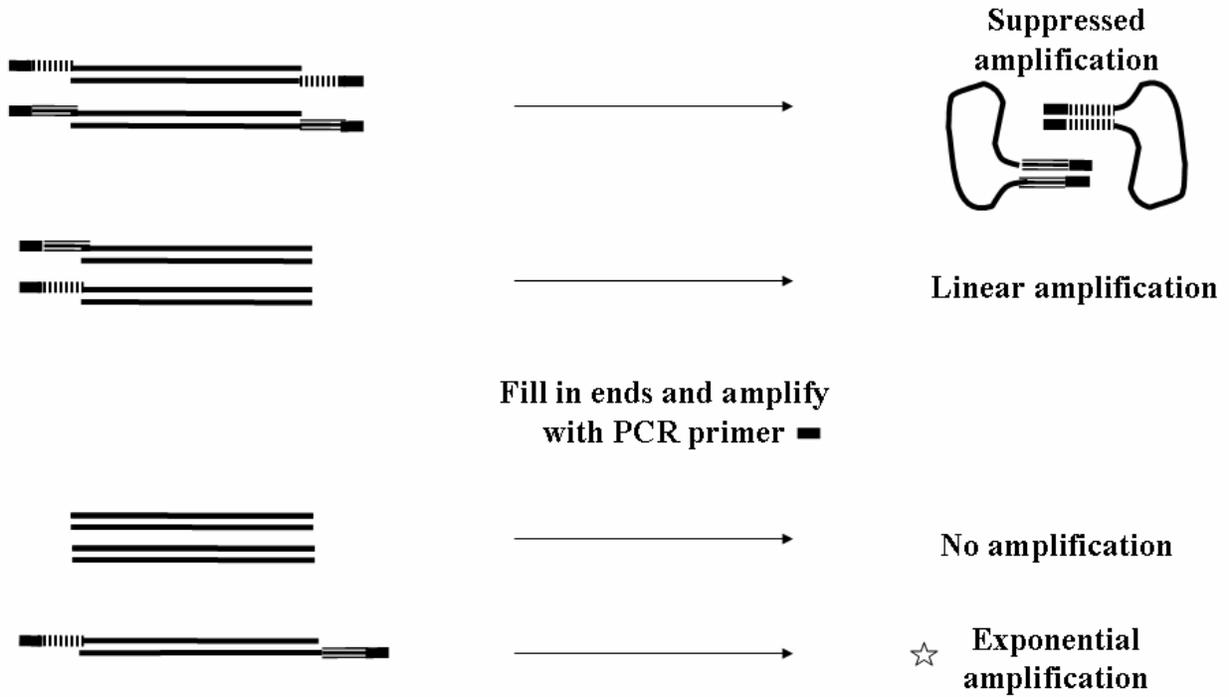


Figure 3.1

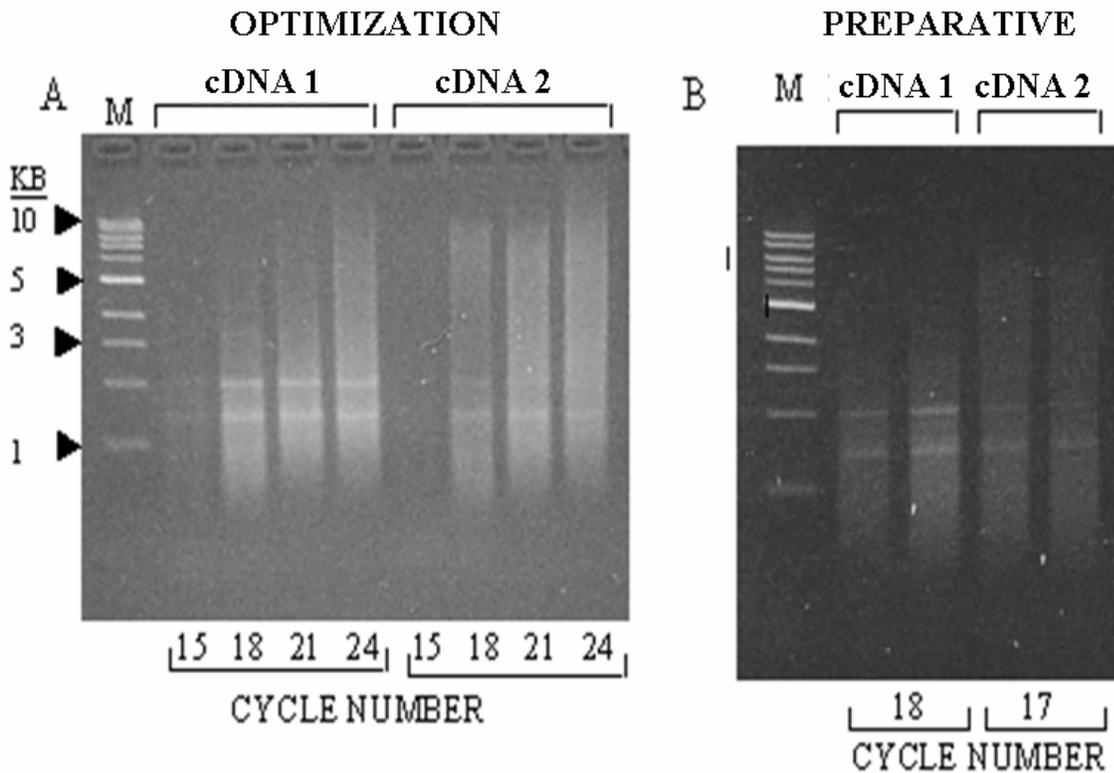


Figure 3.1 Optimization and preparation of amplified cDNA: A) cDNA's 1 and 2 were prepared as described in section 3.1.1 and used as PCR input. SMART PCR was performed for the indicated number of cycles according to the optimization protocol in section 3.1.2 and electrophoresed on a 1.2% agarose gel. Amplified cDNA accumulates with increasing cycle number until saturation, estimated as cycle 18 and 17 for cDNA 1 and 2, respectively. B) Two preparative reaction mixes for each cDNA were then subjected to the optimal number of cycles and again analyzed on a 1.2% agarose gel. M = DNA Molecular Weight marker.

Figure 3.2

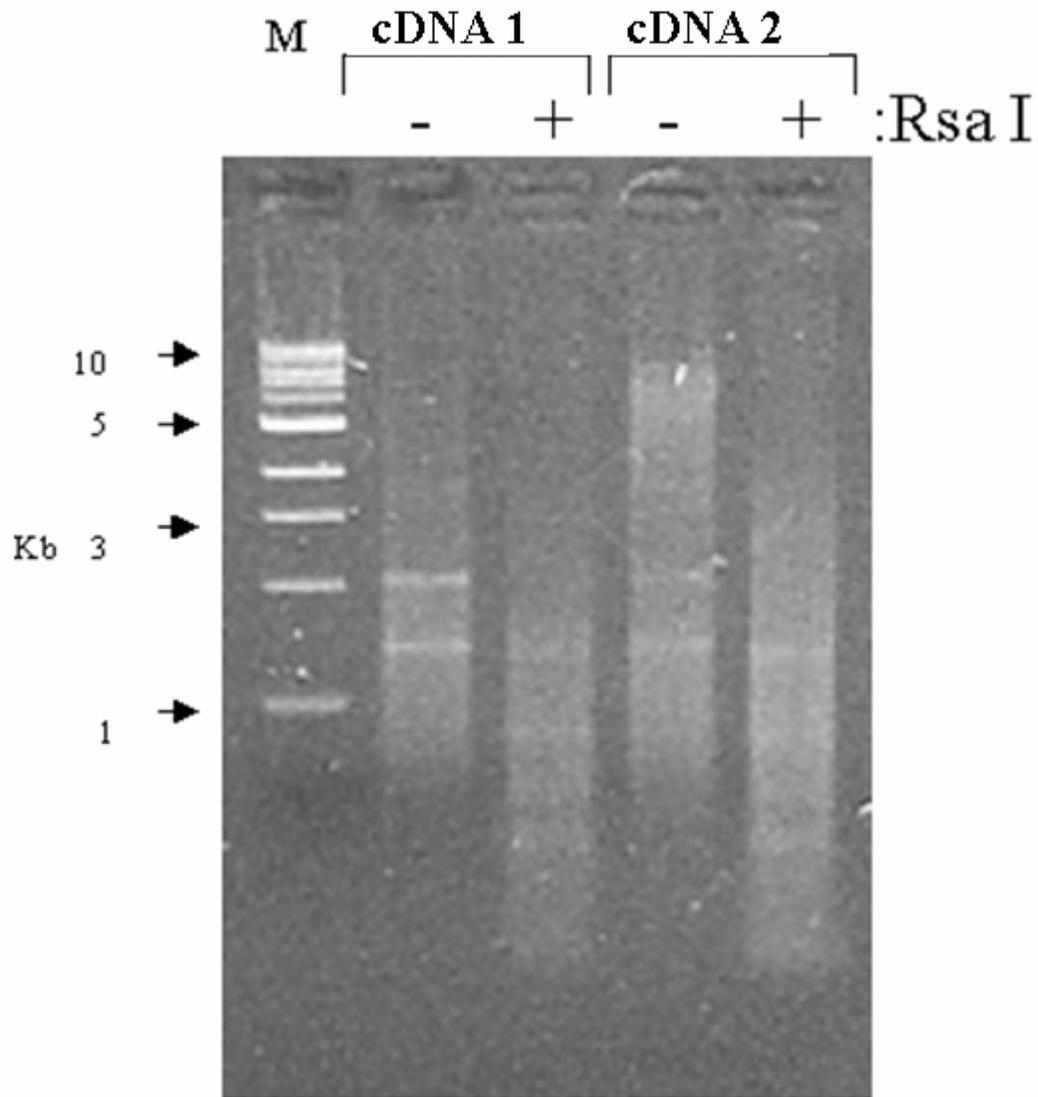


Figure 3.2: Restriction digestion of amplified cDNA's: The amplified and purified cDNA preparations were digested with the four-base cutter RsaI as described in section 3.1 and analyzed alongside undigested cDNA on a 1.2% agarose gel. Complete digestion is confirmed when no further decrease in molecular weight of the digestion products is observed with increased time of digestion.

Figure 3.3

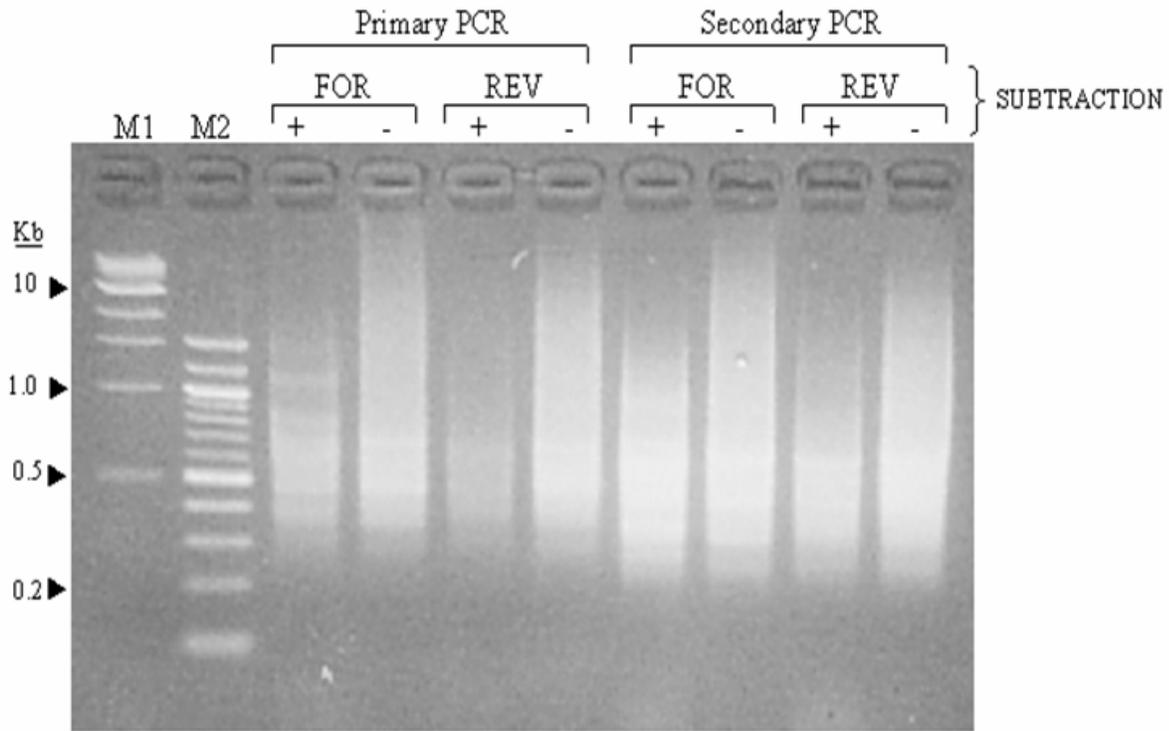


Figure 3.3: Amplification of subtracted and unsubtracted cDNA's: Primary (suppressive) and Secondary (nested) PCR reactions were performed as described in section 3.5.1 and 3.5.2, respectively, and analyzed on a 2% agarose gel. The input cDNA's were from both FORWARD and REVERSE subtractions as indicated. Lanes labeled "+" are subtracted and lanes labeled "-" are unsubtracted. The four secondary PCR products (right) are the final FORWARD and REVERSE subtracted and unsubtracted products (referred to as samples n1-n4 in section 3.5.2, step 7). M1 and M2 are high and low Molecular Weight DNA markers, respectively. Kb=kilobase pairs

Figure 3.4

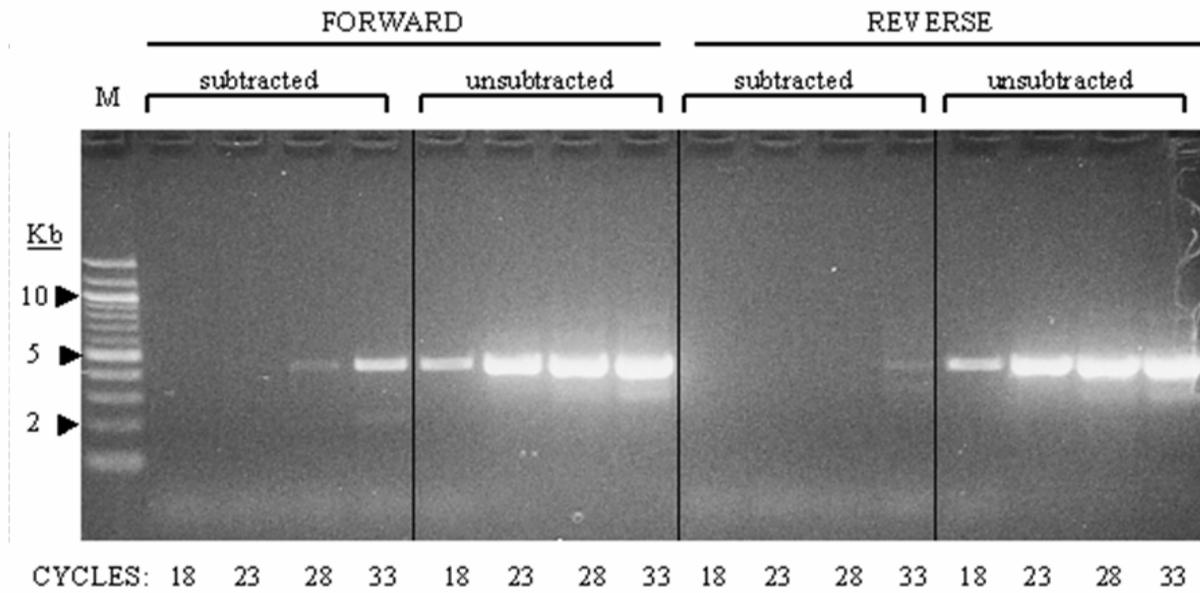


Figure 3.4: Subtraction efficiency assay: The final FORWARD and REVERSE subtracted and un-subtracted cDNA preparations were each diluted 1:10 and used as inputs for the G3PDH-specific PCR reaction described in section 3.5.3, step 7. Equal aliquots of these reactions were removed during the extension step of the indicated cycle number and analyzed on a 2% agarose gel. Note, for example, in the FORWARD subtraction, 33 cycles were required to achieve G3PDH product yield similar to 18 cycles from the un-subtracted cDNA; a difference of 15 logarithmic cycles. The efficiency of subtraction is indicated by the increase in cycle number needed to generate a similar level of PCR product.

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