Chapter 2

Differential Gene Expression in the Uterus and Blastocyst During the Reactivation of Embryo Development in a Model of Delayed Implantation

Pavine L.C. Lefèvre and Bruce D. Murphy

Abstract

Delayed implantation, a reversible arrest in embryo development while the embryo is at the blastocyst stage, provides an interesting window for observation of the preparation for implantation on both the embryonic and maternal sides. The American mink (*Mustela vison*) is a species in which delayed implantation is a normal aspect of embryo development as it consistently occurs at each breeding season. We used a transcriptome-wide approach to screen global gene expression and to identify new key genes expressed in the uterus and in the endometrium at the resumption of the embryo development, after delayed implantation. By using the suppressive subtractive hybridization (SSH) technique, two libraries of differentially expressed cDNAs, one at the uterine level and a second one at the blastocyst level, were successfully generated. Candidate genes from those two libraries were selected and their differentially expressed pattern of expression between reactivation and delayed implantation was investigated by real-time PCR and immunolocalization.

Key words: Blastocyst, uterus, embryo implantation, delayed implantation, embryonic diapause, gene expression, suppressive subtraction hybridization (SSH).

1. Introduction

Embryo implantation represents a critical step in the human reproductive process when the blastocyst becomes intimately connected to the maternal endometrium and begins to form the placenta that will provide an interface between the growing fetus and the maternal circulation. Successful implantation requires a receptive endometrium, a normal and functional embryo at

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the blastocyst developmental stage, and a synchronized dialogue between maternal and embryonic tissues. Implantation failure is considered as a major cause of infertility in healthy women *(1)*. Investigations of human embryo implantation are constrained for practical and ethical reasons. Consequently, many animal models of implantation, such as primates, rodents (mice and rats), pigs, and ruminants (sheep and cows) and carnivores (minks and ferrets) have been used to investigate implantation *(2)*. Given the variation in placentation among species, each provides different insight into the nidatory process.

Delayed implantation consists in a reversible arrest in embryo development while the embryo is at the blastocyst stage during the preimplantation period. It therefore provides an interesting window for observation of molecular and cellular events associated with the preparation for implantation on both the embryonic and maternal sides. Mechanisms underlying delayed implantation in the mice and rats have been investigated. Ovariectomy on day 4 morning of pregnancy before ovarian estrogen secretion initiates blastocyst dormancy which can last for many days if the animal is treated with progesterone *(3)*. An estrogen injection rapidly activates blastocysts and initiates their implantation. Although many studies adopted a "one by one" candidate approach to investigate gene expression in experimentally induced delayed implantation *(4–7)*, a transcriptome-wide approach is nevertheless a powerful tool to screen global gene expression and to identify new key genes in the process. Hamatani et al. *(8)* and Reese et al. *(9)* determined global gene expression by microarray analysis in mice during and after experimentally induced delayed implantation in embryo and uterus, respectively. Even though those studies generated valuable data on gene expression during preimplantation period in the embryo and in the uterus, they are subject to bias due to the experimental manipulation of embryo development. Further, restriction of investigation to a single species may not provide a global picture of the regulation of implantation. To address this issue, we used a carnivore animal model, the American mink (*Mustela vison*), a species in which delayed implantation, or embryonic diapause, is a normal aspect of embryo development, as it consistently occurs at each breeding season *(10)*. An increased photoperiod at the vernal equinox is the principal environmental signal that reactivates embryos *(11)*. Longer day photoperiod induces the secretion of prolactin, which then activates the ovary, resulting in the secretion of progesterone and other factors that act on the uterus to reactivate the embryo and initiate embryo implantation *(12, 13)*. Embryo reactivation is associated with an increase in the endometrial secretion into the uterine lumen *(14)*. Thus, our working hypothesis is that uterine factor(s) actively secreted by the endometrium into the uterine lumen act on blastocysts in diapause to stimulate the resumption

of development. The aim of our study was to identify key genes expressed in the uterus and embryo, essential for termination of embryonic diapause. We therefore collected endometria and embryos from mink females during diapause and 3, 5 and 7 days after reactivation. Using the suppressive subtractive hybridization (SSH) technique, we successfully generated two libraries of differentially expressed cDNA between the diapause state and the reactivation of the embryo development: one from the blastocyst and a second from the uterus. The analysis of the two libraries allowed us to generate data on global gene expression analysis, and to identify potential key regulatory genes.

Of the different strategies available to study differential gene expression, SSH *(15)* is an efficient and widely used PCR-based method to obtain subtracted libraries and to isolate differentially expressed genes between two populations of mRNA: the tester, or cDNA that contains specific transcripts of interest and the driver, the reference cDNA. The protocol involves normalization and subtraction in a single procedure. The normalization step (hybridization) equalizes the abundance of cDNAs within the tester population and the subtraction step excludes the common sequences between the target and driver populations (**Fig. 2.1**). Moreover, the SSH technique enriches rare sequences over 1,000-fold in one round of subtractive hybridization. Because SSH can be initiated using PCR-amplified cDNAs, it seems particularly well-suited to mammal preimplantation stage embryos which contain only a tens of picograms of mRNAs. Furthermore, because SSH does not require previous knowledge of gene sequences, it may also be suitable for species where only a small number of sequences are available in databases. The SSH technique has also the advantage of generating unknown cDNA fragments or previously unknown genes and expressed sequence tags (ESTs).

Following the SSH, expressed cDNA fragments are submitted to a differential screening to remove cDNAs common to both the tester and the driver samples from the subtracted sample (*16* and **Fig. 2.2**). The cDNA fragments are then sequenced and the

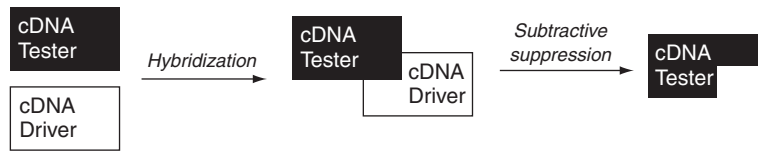


Fig. 2.1 Flowchart summarizing the two major steps of the SSH technique. First, both mRNA populations are converted into cDNA: Tester and driver cDNAs are hybridized and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent sequences that are expressed in the tester yet absent from the driver mRNA population.

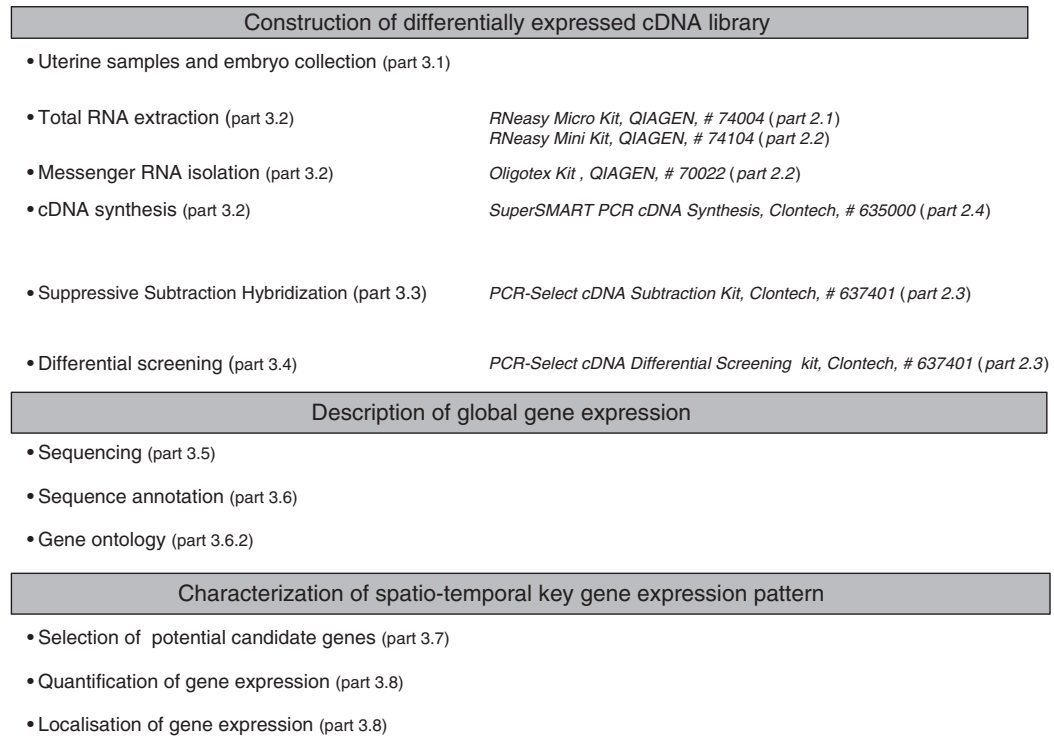


Fig. 2.2 Flowchart summarizing the steps of the methodology of global gene expression analysis and characterization of spatio-temporal key gene expression pattern following the use of the SSH technique to construct a differentially expressed cDNA library. The part number of manuscript refers to each mentioned steps, as well as the company and catalog number of the required kits.

sequences are classified according to their degree of homology with sequences listed in gene databases. The cDNA sequences that exhibit a significant degree of homology to known sequences are classified according to their gene ontology, providing a global gene expression perspective. Frequency of copies of the known sequence in the library, its implication in the preimplantation period suggested in the literature for other species and its biological function are taken into account in selecting potential candidate genes. Finally, the spatio-temporal pattern of these gene expression-selected genes is investigated by real-time polymerase chain reaction (qPCR), in situ hybridization, and immunolocalization. The last step allows validation of the SSH by identifying the authentic targets of biological significance.

In this chapter, we describe the multiple steps of the method to approach a global gene expression analysis and to identify key regulatory genes (**Fig. 2.2**). We first list the materials required (*see* **Section 2**) followed by a detailed description of the method itself (*see* **Section 3**).

2. Materials

2.1. Embryo and Uterus Sample Collection

1. Flushing medium: 500 mL TC-199 medium (Gibco, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS; Gibco) and 2.5 mL penicillin/streptomycin (Gibco). Solution is prepared under a sterile hood, aliquoted, and stored at 4°C. Stable at 4°C for 1 month.
2. 1× Phosphate buffer saline (PBS) stored at 4°C.
3. Paraformaldehyde (PAF) 4% (Sigma). Carcinogenic, corrosive. Safety glasses, gloves, and effective ventilation are required while manipulating the solution.
4. Liquid nitrogen. Store in cryogenic containers and use with adequate ventilation. Use safety glasses and gloves.

2.2. Uterine Samples Total RNA Extraction and mRNA Isolation

1. RNeasy® Mini Kit (cat. no. 74104; Qiagen, Mississauga, ON, Canada). The manufacturer provides all the required reagents except 14.3 *M* β-mercaptoethanol which has to be manipulated under a chemical hood and ethanol (70% and 96–100%).
2. Oligotex® Kit (cat. no. 70022; Qiagen). The manufacturer provides all the required reagents except 14.3 *M* β-mercaptoethanol.

2.3. Embryos Total RNA Extraction

1. RNeasy® Micro Kit (cat. no. 74004; Qiagen). All reagents are supplied in the kit expect 14.3 *M* β-mercaptoethanol and ethanol (70% and 96–100%).

2.4. Suppressive Subtraction Hybridization

1. PCR-Select™ cDNA Subtraction Kit (cat. no. 637401; Clontech, Palo Alto, CA, USA). The following reagents are required but not supplied in the kit:
   a. *Hae*III digest of bacteriophage fX174 (cat. nos. N3026S and N3026L; New England Biolabs DNA size markers).
   b. 80 and 96% ethanol.
   c. Phenol:chloroform:isoamyl alcohol (25:24:1).
   d. Chloroform:isoamyl alcohol (24:1).
   e. AdvantageTM cDNA Polymerase Mix (cat. no. 639105; Clontech).
   f. dNTP mix for PCR: 10 m*M* each dATP, dCTP, dGTP, dTTP.
   g. 50× TAE electrophoresis buffer: 242 g Tris base, 57.1 mL glacial acetic acid, 37.2 g $Na_2EDTA{\bullet}2H_2O$, add H_2O to 1 L. For 1× TAE buffer, dilute 50× stock solution 1:49 with H_2O.
2. Super SMART™ PCR cDNA Synthesis Kit (cat. no. 635000; Clontech). The following reagents are required but not supplied in the kit:

a. SMART™ MMLV Reverse Transcriptase (cat. no. PT4045-2; Clontech).
b. Advantage® 2 PCR Kit (cat. nos. 639206 and 639207; Clontech).
c. NucleoSpin® RNA II Kit (cat. no. 635990; Clontech).
d. β-Mercaptoethanol.
e. RNase Inhibitor (20 U/μL) (cat. no. 2696; Ambion's SUPERase).
f. DNA size markers (1 kb DNA ladder).
g. 50× TAE electrophoresis buffer (*see* **Section 2.4**, Step 1-g).
h. CHROMA SPIN + STE-10 Columns (cat. no. 636055; Clontech).
i. Microcentrifuge.
j. Hot-lid Thermal Cycler.
k. TNE buffer 10×: 100 m*M* Tris; 2.0 *M* NaCl; 10 m*M* EDTA; pH 7.4: provide with the Super SMART™ PCR cDNA Synthesis Kit.
l. UV spectrophotometer.

2.5. Differential Screening

1. PCR-Select Differential Screening Kit (cat. no. 637403; Clontech). The following reagents are required but not supplied in the kit:
   a. Advantage® 2 Polymerase Mix (cat. no. 639201; Clontech).
   b. dNTP mix for PCR: 10 m*M* each dATP, dCTP, dGTP, dTTP.
   c. T/A Cloning Kit Dual Promoter (cat. no. K2060-01; Invitrogen, Carlsbad, CA, USA).
   d. SOC: Max Efficiency DH5α Competent Cells (cat. no. 18258-012; Invitrogen).
   e. Luria Broth (LB) medium: 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl, add H_2O to 900 mL. Adjust pH to 7.0 with 5 *N* NaOH, then bring up to a 1-L volume with H_2O. Autoclave.
   f. Ampicillin, 50 mg/mL stock solution; store at −20°C.
   g. Isopropyl β-D-1-thiogalactopyranoside (IPTG), final concentration 1 m*M*.
   h. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), final concentration 50 μg/mL.
   i. 0.6 *N* NaOH.
   j. 0.5 *M* Tris–HCl (pH 7.5).
   k. Denaturing solution: 0.5 *M* NaOH, 1.5 *M* NaCl, make fresh each time.
   l. Neutralizing solution: 0.5 *M* Tris–HCl pH 7.5, 1.5 *M* NaCl.
   m. Nylon membrane.
   n. NucleoSpin® Extraction Kit (cat. no. 635961; Clontech).

o. [α-32P]dCTP or [α-32P]dATP (3,000 Ci/mmol).
p. Illustra^{TM} MicroSpin Columns (cat. no. S-200 27-5120-01; GE Health Care, Buckinghamshire, UK).
q. ExpressHybTM Hybridization Solution (cat. nos. 636831 and 636832; Clontech).
r. 20× SSC: 175.3 g NaCl, 88.2 g $Na_3Citrate{\bullet}2H_2O$. Adjust pH to 7.0 with 1 *M* HCl, add H_2O to 1 L. Store at room temperature.
s. 20% SDS (200 g SDS; add H_2O to 1 L. Heat to 65°C to dissolve. Store at room temperature).
t. Low-stringency washing solution: 2× SSC, 0.5% SDS.
u. High-stringency washing solution: 0.2× SSC, 0.5% SDS.
v. Ethanol.
w. Sterile, deionized H_2O.
x. 50× TAE electrophoresis buffer (*see* **Section 2.4**, Step 1-g).
y. Thermal Cycler 480 (Roche) and PCR System 2400 or 9600.
z. Cyclophilin and G3PDH primers.
aa. Milli-Q Water Ultrapure system (Millipore).
bb. ImageQuant Software (Applied BioSystem).

3. Methods

3.1. Embryo and Uterine Sample Collection

All procedures involving live animals were approved by the *Comité de déontologie de la Faculté de Médecine Vétérinaire, Université de Montréal*, which is accredited by the Canadian Council on Animal Care.

1. Remove the uterine horns from the euthanized animal.
2. Rinse the uterus in PBS 1× in a 100-mm Petri dish.
3. Excise as much the adipose tissue as possible.
4. Transfer the uterus to a 50-mm Petri dish.
5. Flush each uterine horn with 2.5 mL flushing medium prewarmed at 37°C using a syringe and a 21G1/2 in. needle (**Fig. 2.3**).
6. Search for embryos under a microscope and collect by aspirating them with a mouth pipette.
7. Rinse the embryos in PBS 1× prewarmed at 37°C in a fresh 50-mm Petri dish. Carefully use the mouth pipette to manipulate the embryos under the microscope.
8. Transfer the embryos in 1.5-mL tube into as minimum as possible (a drop or less) of PBS 1×.
9. Snap-freeze the 1.5-mL tube containing embryos in liquid nitrogen and store the samples at −80°C until use.

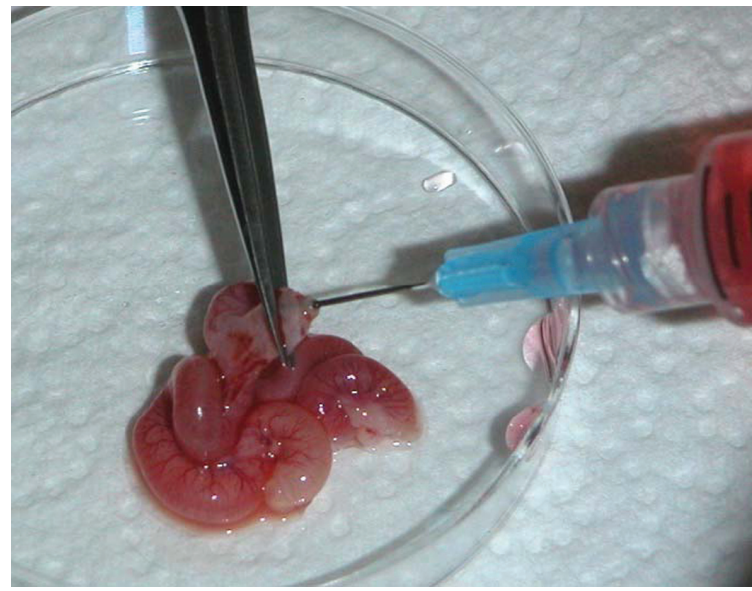


Fig. 2.3 Photography of the procedure of the embryo flushing from the uterine lumen. The needle of the syringe is introduced through the cervix into one horn and flushing medium is injected to collect the embryos on the oviductal inlet of the horn (Lefèvre, 2006, unpublished).

10. Cut on whole uterine horn, place one horn piece in a 1.5-mL tube, snap-freeze the tube in liquid nitrogen and store sample at −80°C until use.
11. Place the other uterine horn piece in 1.5-mL tube containing PAF 4%. Store the tube at 4°C for 24 h. Rinse the tissue in PBS 1×, three times and place the tissue in 70% ethanol at −20°C until use (*see* **Note 1**).

3.2. Total RNA Extraction and mRNA Isolation

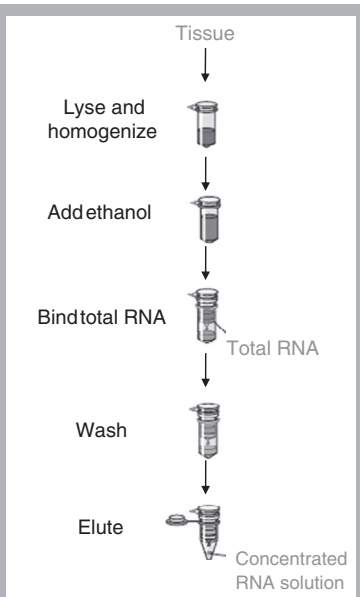
3.2.1. Total RNA Extraction from Uterine Samples (RNeasy® Mini Kit)

1. Thaw uterine sample on dry ice (*see* **Note 2**) (**Fig. 2.4**).
2. Aliquot 350 μL of Buffer RLT (RNeasy® Mini Kit) in 14-mL polypropylene tube.
3. Place the tissue in the tube with the Buffer RLT.
4. Disrupt and homogenize the tissue using a Rotor-Stator Homogenizer (Polytron): place the tip of the disposable probe into the tube containing the uterine sample. At room temperature, operate the polytron beginning at low speed and increase progressively the speed over 30 s, until the lysate is homogenous (*see* **Note 3**).
5. Transfer the lysate to 2-mL tubes. Centrifuge the lysate for 3 min at maximum speed (14,000–18,000*g*). Carefully

The RNeasy procedure represents a well-established technology for RNA purification. This technology combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate containing buffer, which immediately inactivates Rnases to ensure purification of intact RNA (see figure on the left). Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted. The table below summerizes specifications of the RNeasy Mini Kit and the RNeasy Micro kit. The latter allows purifying RNA from small amounts of tissue and is suitable for RNA extraction from embryo at blastocyst stage.

Specifications	RNeasy Mini Kit	RNeasy Micro Kit
Maximum binding capacity	100 µg	45 µg RNA
Maximum loading volume	700 µl	700 µl
RNA size distribution	RNA > 200 nucleotides	RNA > 200 nucleotides
Minimum elution volume	30 µl	10 µl

Summerization of specifications of RNeasy Micro column and RNeasy Mini column(Qiagen).



Summerization of total RNA extraction procedure using the RNeasy procedure.

(Adapted from RNeasy Micro Handbook and RNeasy Mini Handbook, Qiagen)

Fig. 2.4 RNeasy principle and procedure for RNA isolation.

transfer the supernatant to a fresh 1.5-mL tube by pipetting.

6. Add 1 volume (350 µL) of 70% ethanol to the lysate and mix well by pipetting.
7. Do not centrifuge (*see* **Note 4**).
8. Transfer the sample to an RNeasy MinElute (RNeasy® Mini Kit) spin column placed in a 2-mL collection tube. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g*. Discard the flow-through (*see* **Note 5**).
9. Add 700 µL Buffer RW1 (RNeasy® Mini Kit) to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through and the collection column.
10. Place the RNeasy MinElute spin column in a new 2-mL collection tube. Add 500 µL Buffer RPE (RNeasy® Mini Kit) to the spin column. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through.
11. Add 500 µL of Buffer RPE to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through and collection tube.

12. Place the RNeasy MinElute spin column in a new 2-mL collection tube. Close the lid gently and centrifuge at maximum speed (14,000–18,000*g*) for 1 min.
13. Place the RNeasy MinElute spin column in a new 1.5-mL collection tube. Add 30–50 μL RNase-free water directly to the spin column membrane. Close the lid gently and centrifuge for 1 min at ≥ 8,000*g* to elute the RNA (*see* **Note 6**).
14. If the expected RNA yield is >30 μg, repeat the elution step using another 30–50 μL RNase-free water or using the eluate from the first elution in case high RNA concentration is required.

3.2.2. Messenger RNA Isolation from Uterine Total RNA (Oligotex® Kit)

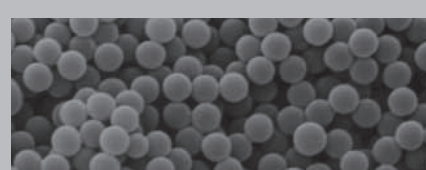
Before starting:

1. Heat Oligotex Suspension (Oligotex® Kit) to 37°C in a water bath or heating block. Mix by vortexing and then place at room temperature (**Fig. 2.5**).
2. Heat a water bath or heating block to 70°C and heat Buffer OEB (Oligotex® Kit).

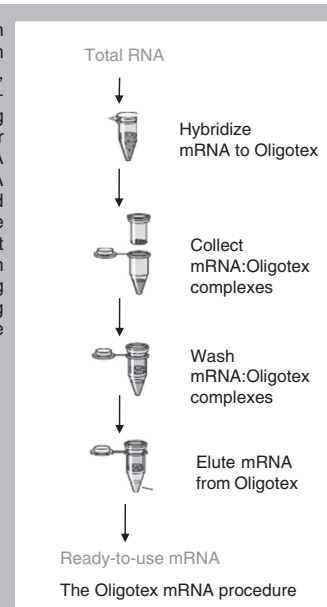
Procedure:

1. Determine the amount of starting RNA (*see* **Note 7**). Pipet total RNA into an RNase-free 1.5-mL microcentrifuge tube and adjust the volume with RNase-free water (if necessary) to the volume indicated in **Table 2.1** (*see* **Note 8**).

Oligotex Suspension consists of polystyrene–latex particles of uniform size and a perfect spherical shape (see photography below). $dC_{10}T_{30}$ oligonucleotides are covalently linked to the surface of the polystyrene–latex particles via a condensation reaction. The particles form a stable suspension that provides a large surface area for rapid and efficient binding of polyadenylic acids. The Oligotex procedure for isolation, purification, and manipulation of poly A + RNA takes advantage of the fact that most eukaryotic mRNA (and some viral RNAs) end in a poly-A tail of 20–250 adenosine nucleotides. In contrast, rRNAs and tRNAs, which account for over 95% of cellular RNAs, are not polyadenylated. Poly A+ mRNA can be purified by hybridizing the poly-A tail to a dT oligomer coupled to a solid-phase matrix. rRNA and tRNA species, without apoly-A tail, do not bind to the oligo-dT and are easily washed away. Since hybridization requires high-salt conditions, the poly A^+ mRNA can then easily be released by lowering the ionic strength and destabilizing the dT: A hybrids (see figure on the left).



Scanning electron micrograph of Oligotex particles. Magnification 7500x



The Oligotex mRNA procedure

(Adapted from Oligotex Handbook, Qiagen)

Fig. 2.5 The Oligotex principle.

Table 2.1
Buffer amounts for Oligotex mRNA spin-column protocol

Total RNA (mg)	Add RNase-free water to (μL)	Buffer OBB (μL)	Oligotex suspension (μL)
≤0.25	250	250	15
0.25–0.50	500	500	30
0.50–0.75	500	500	45
0.75–1.00	500	500	55

2. Add the appropriate volume of Buffer OBB and Oligotex Suspension (Oligotex® Kit) (**Table 2.1**). Mix the contents thoroughly by pipetting or by flicking the tube. Incubate the sample for 3 min at 70°C in a water bath or heating block to disrupt the secondary structure of the RNA.
3. Remove sample from the water bath/heating block and place at 20–30°C for 10 min. This step allows hybridization between the oligo dT30 of the Oligotex particle and the poly-A tail of the mRNA.
4. Pellet the Oligotex:mRNA complex by centrifugation for 2 min at maximum speed (14,000–18,000*g*) and carefully remove the supernatant by pipetting (*see* **Note 9**).
5. Resuspend the Oligotex:mRNA pellet in 400 μL Buffer OW2 (Oligotex® Kit) by vortexing or pipetting and pipet onto a spin column placed in a 1.5-mL microcentrifuge tube. Centrifuge for 1 min at maximum speed (14,000–18,000*g*).
6. Transfer the spin column to a new RNase-free 1.5-mL microcentrifuge tube and apply 400 μL Buffer OW2 to the column. Centrifuge for 1 min at maximum speed (14,000–18,000*g*) and discard the flow-through.
7. Transfer spin column to a new RNase-free 1.5-mL microcentrifuge tube.
8. Pipet 20–100 μL hot Buffer OEB (Oligotex® Kit) (70°C) onto the column, pipet up and down three or four times to resuspend the resin, and centrifuge for 1 min at maximum speed (14,000–18,000*g*) (*see* **Note 10**).
9. To ensure maximal yield, pipet another 20–100 μL hot Buffer OEB (70°C) onto the column. Pipet up and down three or four times to resuspend the resin and centrifuge for 1 min at maximum speed (14,000–18,000*g*) (*see* **Note 11**).

3.2.3. Total RNA Extraction from Embryos (RNeasy® Micro Kit)

1. Carefully thaw embryos on dry ice (**Fig. 2.4**).
2. Add 150 μL of Buffer RLT (RNeasy® Micro Kit) to disrupt the cells. Vortex or pipet to mix.
3. Homogenize by vortexing the tube for 1 min.

4. Add 1 volume of 70% ethanol to the lysate and mix well by pipetting (*see* **Note 4**).
5. Transfer the sample, including any precipitate that may have formed, to an RNeasy MinElute (RNeasy® Micro Kit) spin column placed in a 2-mL collection tube. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g*. Discard the flow-through (*see* **Note 5**).
6. Add 150 μL Buffer RW1 (RNeasy® Micro Kit) to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through.
7. Add 700 μL Buffer RW1 instead, centrifuge for 15 s at ≥ 8,000*g*, and discard the flow-through and collection tube.
8. Place the RNeasy MinElute spin column in a new 2-mL collection tube.
9. Add 500 μL Buffer RPE (RNeasy® Micro Kit) to the spin column. Close the lid gently and centrifuge for 15 s at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through.
10. Add 500 μL of 80% ethanol to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥ 8,000*g* to wash the spin column membrane. Discard the flow-through and collection tube.
11. Place the RNeasy MinElute spin column in a new 2-mL collection tube. Open the lid of the spin column and centrifuge at full speed for 5 min to insure that the entire ethanol residues are evaporated. Discard the flow-through and collection tube.
12. Place the RNeasy MinElute spin column in a new 1.5-mL collection tube. Add 14 μL RNase-free water directly into the center of the spin column membrane. Close the lid gently and centrifuge for 1 min at maximum speed (14,000–18,000*g*) to elute the RNA.
13. Quantify the amounts and purity of the extracted total RNA (*see* **Note 7**).

3.3. Suppressive Subtraction Hybridization (PCR-Select™ cDNA Subtraction Kit)

The Subtractive Subtraction Hybridization (SSH) is performed with the PCR-Select™ cDNA Subtraction Kit for both the uterine samples and the embryos to generate differentially expressed cDNAs between diapause and embryo reactivation. **Figure 2.6** details the molecular events that occur during PCR-Select cDNA subtraction. As the amount of total RNA extracted from embryos is too meager, an alternative is to introduce a cDNA preamplification step by means of the Super SMART™ PCR cDNA Synthesis Kit (*see* **Section 3.3.1**) With slight modifications to the standard protocol until the *Rsa*I digestion step, Super SMART cDNA can be used directly for the adaptor ligation step of the PCR-Select cDNA subtraction (*see* **Section 3.3.4**).

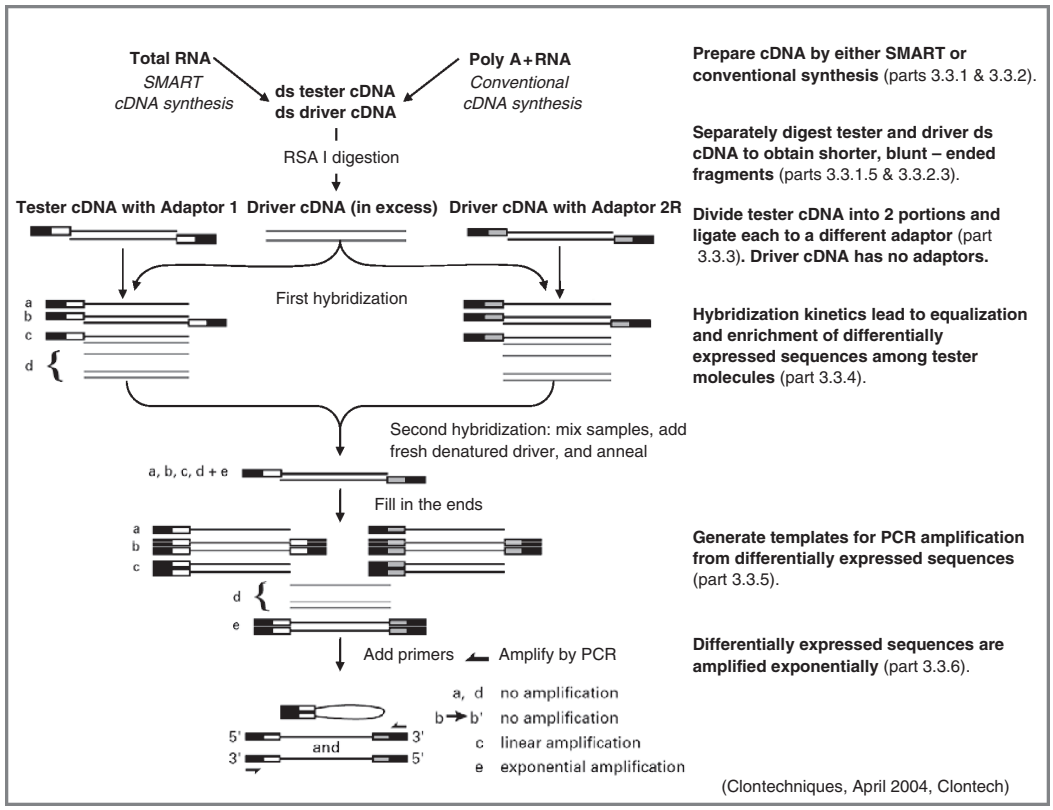


Fig. 2.6 The PCR-Select cDNA subtraction technique.

Complementary DNA is synthesized from 0.5 to 2 μg of poly A+ RNA generated from the uterine samples in diapause and after reactivation (*see* **Section 3.3.3**). In the PCR-Select™ cDNA Subtraction Kit, cDNA that contains specific (differentially expressed) transcripts is referred to as the tester and the reference cDNA as the driver. In the present study, samples collected in diapause are considered as the driver while those collected after reactivation represents the tester. The tester and driver cDNAs are digested with *Rsa*I (*see* **Sections 3.3.2** and **3.3.3**), a four-base-cutting restriction enzyme that yields blunt ends. The tester cDNA is then subdivided into two portions and each is ligated with a different cDNA adaptor (*see* **Section 3.3.4**). The two adaptors have stretches of identical sequence to allow annealing of the PCR primer once the recessed ends have been filled in (**Table 2.2**). Two hybridizations are then performed:

1. In the first (*see* **Section 3.3.5**), an excess of driver is added to each sample of tester. The samples are then heat denatured and allowed to anneal, generating the type "**a**," "**b**," "**c**," and "**d**" molecules in each sample. The concentration of high- and low-abundance sequences is equalized among the type "**a**" molecules because reannealing is faster for the

Table 2.2
Sequences of the primers and adaptors used in the PCR-Select™ cDNA Subtraction Kit and in the Super SMART™ PCR cDNA Synthesis Kit (Clontech). The sequence of the PCR primer 1 (in bold italic) is complementary to the 5′-end sequence of adaptor 1 (in bold) and adaptor 2R (in bold italic) and Nested Primer 1 (in grey italic) and Nested Primer 2 (in underline) are, respectively, complementary to the 3′-end sequence of adaptor 1 (in grey italic) and adaptor 2R (in underline)

Sequence name	Sequences	Length
cDNA synthesis primer	**5′**-TTTTGTACAAGCTT $_{30}N_1N$-3′	45 nt
Adaptor 1	**5′-CTAATACGACTCACTATAGGGC** *TCGAGCGGCCGCCCGGGCAGGT -3′* 3′-GGGCCCGTCCA-5′	44 nt
Nested primer 1	5′-*TCGAGCGGCCGCCCGGGCAGGT-3′*	22 nt
Adaptor 2R	**5′-CTAATACGACTCACTATAGGGC** AGCGTGGTCGCGGCCGAGGT-3′ 3′-GCCGGCTCCA-5′	42 nt
Nested primer 2	5′-AGCGTGGTCGCGGCCGAGGT -**3′**	20nt
PCR primer 1	**5′-CTAATACGACTCATATAGGGC-3′**	22 nt
G3PDH 5′ primer	5′-ACCACAGTCCATGCCATCAC-3′	20 nt
G3PDH 3′ primer	5′-TCCACCACCCTGTTGCTGTA-3′	20 nt
SMART II A	5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′	30 nt
SMART CDS Primer II A	5′-AAGCAGTGGTATCAACGCAGAGTAC$T_{(30)}$N-1N-3′ (N = A, C, G, or T; N -1 = A, G, or C)	57 nt
5′ PCR Primer II A	5′-AAGCAGTGGTATCAACGCAGAGT-3′	23 nt

more abundant molecules due to the second-order kinetics of hybridization. At the same time, type "**a**" molecules are significantly enriched for differentially expressed sequences while cDNAs that are not differentially expressed form type "**c**" molecules with the driver.

2. During the second hybridization (*see* **Section 3.3.6**), the two primary hybridization samples are mixed together without denaturing. Only the remaining equalized and subtracted single-strand (ss) tester cDNAs can reassociate and form new type "**e**" hybrids. These new hybrids are double-strand (ds) tester molecules with different ends, which correspond to the sequences of Adaptors 1 and 2R. Fresh denatured driver cDNA is added to further enrich fraction "**e**" for differentially expressed sequences.

After filling in the ends by DNA polymerase, the type "**e**" molecules – the differentially expressed tester sequences – have different annealing sites for the nested primers on their 5′ and 3′ ends. The entire population of molecules is then subjected to

PCR to amplify the desired differentially expressed sequences (*see* **Section 3.3.7**). During this PCR, type "**a**" and "**d**" molecules are missing primer annealing sites and thus cannot be amplified. Due to the suppression PCR effect, most type "**b**" molecules form a pan-like structure that prevents their exponential amplification. Type "**c**" molecules have only one primer annealing site and amplify linearly. Only type "**e**" molecules – the equalized, differentially expressed sequences with two different adaptors – amplify exponentially. Next, a secondary PCR amplification is performed using nested primers to further reduce any background PCR products and enrich for differentially expressed sequences.

3.3.1. Complementary DNA Synthesis from Total Embryonic RNA and cDNA Preamplification (Super SMART™ PCR cDNA Synthesis Kit)

This protocol has been optimized for total RNA (*see* **Note 12**) (**Fig. 2.7**).

3.3.1.1. First-Strand cDNA Synthesis

1. For each sample and control human placenta RNA, combine the following reagents in a sterile 0.5-mL reaction tube: 1–50 μL RNA sample (2–1,000 ng of total RNA) (*see* **Note 13**), 7 μL 3′ SMART CDS Primer II A (Super SMART™ PCR cDNA Synthesis Kit) (12 μ*M*), 7 μL SMART II A

All commonly used cDNA synthesis methods rely on the ability of reverse transcriptase (RT) to transcribe mRNA into single-stranded (ss) DNA in the first-strand reaction. However, because RT cannot always transcribe the entire mRNA sequence, the 5' ends of genes tend to be under represented in cDNA populations. With Clontech's patented SMART cDNA Technology, high yields of full-length and double-stranded cDNA from small amounts of RNA can be generated. SMART stands for **S**witching **M**echanism **A**t 5' end of **R**NA **T**emplate.

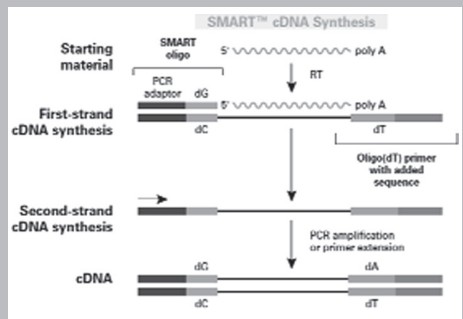
The SMART method SMART technology is based on two specific features of Moloney murine leukemia virus reverse transcriptase (MMLVRT):

1. The addition of non-template nucleotides to the 3' end of the newly synthesized cDNA strand, up on reaching the 5' end of them RNA template (terminal transferase activity).
2. The ability to switch to a second template.

First-strand cDNA synthesis is primed by a modified oligo(dT) primer that contains additional sequence at the 3' end (the 3' SMART CDS Primer II A) (see figure on the left). When the MMLVRT reaches the 5' end of the mRNA, the enzyme's terminal transferase activity attaches several additional nucleotides, primarily deoxycytidine, onto the newly synthesized strand of cDNA.

The SMART Oligonucleotide (patent pending), which has an oligo(G) sequence at its 3'end, base-pairs with the deoxycytidine stretch, creating an extended template. RT then switches templates and continues replicating to the end of the oligonucleotide (17).

The SMART anchor sequence and the poly A sequence serve as universal priming sites for end-to-end cDNA amplification. In contrast, cDNA without these sequences such as prematurely terminated cDNAs, contaminating genomic DNA, or cDNA transcribed from poly A–RNA, will not be exponentially amplified.



Overview of the SMART cDNA synthesis method.

(Adapted from the SuperSMARTPCR cDNA synthesis Kit User Manual and the FL772387_SMARTcDNA Brochure, Clontech)

Fig. 2.7 The SMART cDNA synthesis technology.

Oligonucleotide (Super SMART™ PCR cDNA Synthesis Kit) (12 μ*M*), x μL deionized H_2O (total volume = 64 μL).
2. Mix contents and spin for 5 s the tube in a microcentrifuge.
3. Incubate the tube at 65°C in a hot-lid thermal cycler for 2 min and then reduce the temperature to 42°C.
4. Add the following to each reaction tube (Super SMART™ PCR cDNA Synthesis Kit): 20 μL 5× First-Strand Buffer, 2 μL DTT (100 m*M*), 10 μL 50× dNTP (10 m*M*), 5 μL RNase Inhibitor (20 U/μL), 5 μL MMLV Reverse Transcriptase (42 mL total added per reaction).
5. Gently pipet up and down to mix, then spin the tubes briefly in a microcentrifuge.
6. Incubate the tubes at 42°C for 90 min in a hot-lid thermal cycler.
7. Add 2 μL of 0.5 *M* EDTA to stop the reaction (*see* **Note 14**).

3.3.1.2. Column Chromatography

To purify the SMART cDNA from unincorporated nucleotides and small (<0.1 kb) cDNA fragments, follow this procedure for each reaction tube:

1. Add 212 μL of Buffer NT (Super SMART™ PCR cDNA Synthesis Kit) to each cDNA synthesis reaction; mix well by pipetting.
2. Place a NucleoSpin Extract II Column (Super SMART™ PCR cDNA Synthesis Kit) into a 2-mL collection tube. Pipet the sample into the column. Centrifuge at 11,200*g* for 1 min. Discard the flow-through.
3. Add 600 mL of Wash Buffer NT3 (Super SMART™ PCR cDNA Synthesis Kit) to the column. Centrifuge at 11,200*g* for 1 min. Discard the flow-through.
4. Place the column back into the collection tube. Centrifuge at 11,200*g* for 2 min to remove any residual Wash Buffer NT3 (Super SMART™ PCR cDNA Synthesis Kit).
5. Transfer the NucleoSpin Columns into a fresh 1.5-mL microcentrifuge tube. Add 50 μL of sterile Milli-Q H_2O (Super SMART™ PCR cDNA Synthesis Kit) to the column. Allow the column to stand for 2 min with the caps open.
6. Close the tube and centrifuge at 11,200*g* for 1 min to elute the sample.
7. Repeat elution with 35 μL of sterile Milli-Q H_2O in the same 1.5-mL microcentrifuge tube. The total recovered elution volume should be 80–85 μL per sample. If necessary, add sterile Milli-Q H_2O to bring the total volume up to 80 μL (*see* **Note 15**) (**Table 2.3**).

3.3.1.3. cDNA Amplification by Long-Distance PCR (LD-PCR)

The complementary DNA amplification includes a step to determine the optimal number of PCR cycles for the amplification (**Fig. 2.8**). Guidelines for optimizing the PCR, depending on

Table 2.3
Guidelines for setting-up PCR

Total RNA (ng)	Volume of ss cDNA (μL)	Volume of H_2(μL)O	Total volume (μL)	Typical optimal number of PCR cycles
~2	80	–	80	24–28
~5	80	–	80	21–24
~25	80	–	80	17–20
~50	40	40	80	17–20
~100	25	55	80	17–20
~250	10	70	80	17–20
~500	5	75	80	17–20
~1,000	2.5	77.5	80	17–20

the amount of total RNA used in the first-strand synthesis, are provided in **Table 2.3**.

1. Set up three 100 μL PCR reactions, labeled "A," "B," and "C," for each tester and driver sample (**Fig. 2.8**).
2. Preheat the PCR thermal cycler to 95°C.
3. For each experimental sample, aliquot 80 μL ss cDNA into a labeled 1.5-mL reaction tube.
4. Prepare a Master Mix (Super SMART^{TM} PCR cDNA Synthesis Kit) for all reaction tubes, plus one additional tube. Combine per reaction (220 μL total volume): 172 μL Deionized H_2O, 30 μL 10× Advantage 2 PCR Buffer, 6 μL 50× dNTP (10 m*M*), 6 μL 5′ PCR Primer II A (12 μ*M*), 6 μL 50× Advantage 2 Polymerase Mix.
5. Mix well by vortexing and spin the tube for 5 s in a microcentrifuge.
6. Aliquot 220 μL of the PCR Master Mix into each tube containing the 80 μL ss cDNA. Mix well.
7. Aliquot 100 μL of the resulting PCR reaction mix into three reaction tubes labeled "A," "B," and "C".
8. Cap each tube and place them in the preheated thermal cycler.
9. Commence thermal cycling (with a hot-lid thermal cycler) using 95°C 1 min and 15 cycles: 95°C 5 s, 65°C 5 s, 68°C 6 min.
10. Subject each reaction tube to 15 cycles and then pause the program. Transfer 30 μL from Tube C to a second reaction tube labeled "Optimization." Store Tubes A and B and the "Experimental" tube containing the remaining 70 μL of Tube C, at 4°C.

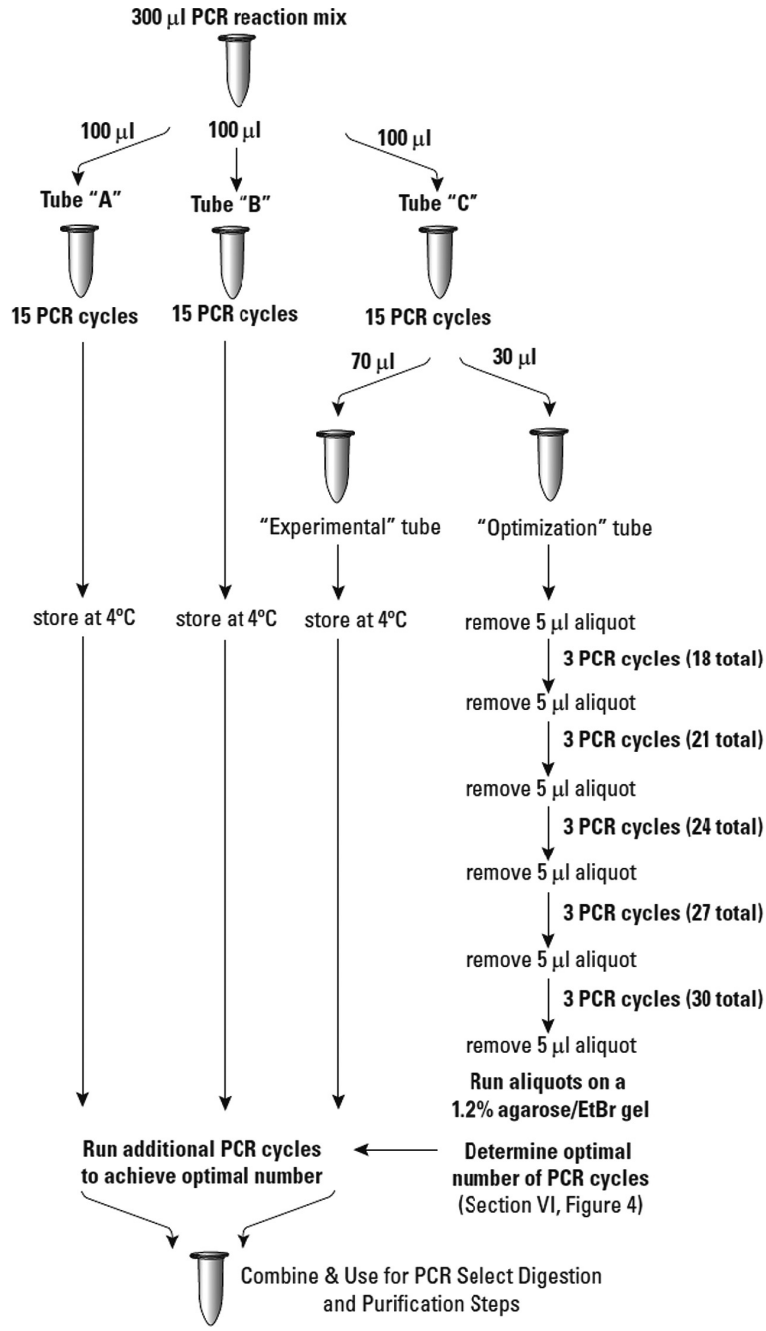


Fig. 2.8. Optimizing PCR parameters for SMART cDNA synthesis (Super SMART™ PCR cDNA Synthesis Kit User Manual, Clontech).

11. Using the Optimization PCR tube determine the optimal number of PCR cycles (**Fig. 2.8**):
    a. Set up six 100 µL PCR tubes for each experimental sample, labeled 15, 18, 21, 24, 27, and 30.

b. Transfer 5 μL from the 15 cycles PCR to a clean microcentrifuge tube.
c. Return the Optimization tubes to the thermal cycler. Run three additional cycles (for a total of 18) with the remaining 25 μL of PCR mixture. Transfer 5 μL from the 18 cycles PCR to a clean microcentrifuge tube.
d. Run three additional cycles (for a total of 21) with the remaining 20 μL of PCR mixture. Transfer 5 μL from the 21 cycles PCR to a clean microcentrifuge tube.
e. Run three additional cycles (for a total of 24) with the remaining 15 μL of PCR mixture. Transfer 5 μL from the 24 cycles PCR to a clean microcentrifuge tube.
f. Run three additional cycles (for a total of 27) with the remaining 10 μL of PCR mixture. Transfer 5 μL from the 27 cycles PCR to a clean microcentrifuge tube.
g. Run three additional cycles (for a total of 30) with the remaining 5 μL of PCR mixture.
h. Separate by Electrophoresis each 5 μL aliquot of the PCR reaction alongside 0.1 μg of 1 kb DNA size markers on a 1.2% agarose/ethidium bromide (EtBr) gel in 1× TAE buffer.
i. Determine the optimal number of cycles required for each experimental and control sample (*see* **Note 16** and **Fig. 2.9**).

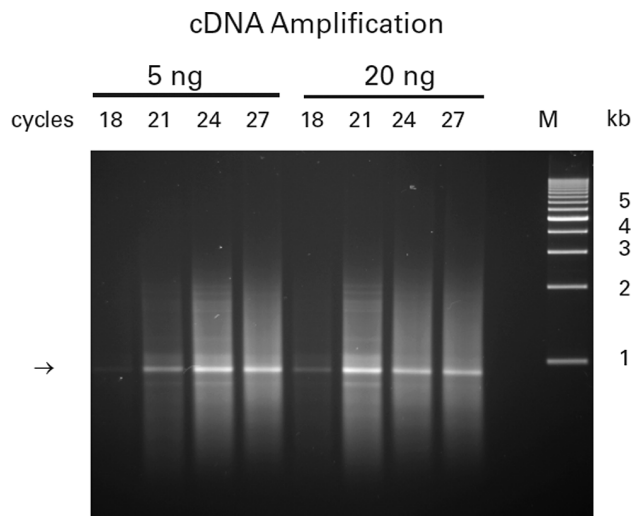


Fig. 2.9. Analysis for optimizing PCR parameters. Five or 20 ng of the control human placental total RNA was subjected to the first-strand cDNA synthesis and purification as described in the protocol. Eighty microliters was used for PCR amplification. A range of PCR cycles were performed (18, 21, 24, and 27). Five microliters of each PCR product was electrophoresed on a 1.2% agarose/EtBr gel in 1× TAE buffer following the indicated number of PCR cycles. Lane M: 1 kb DNA ladder size markers. The *arrow* indicates the strong band at 900 bp typically seen for human placenta total RNA (Super SMART™ PCR cDNA Synthesis Kit User Manuel, Clontech).

12. Retrieve the 15 cycles Experimental PCR tubes from 4°C, return them to the thermal cycler, and subject them to additional cycles, if necessary, until the optimal number is reached.
13. When the cycling is completed, analyze a 5-μL sample of each PCR product alongside 0.1 μg of 1 kb DNA size markers on a 1.2% agarose/EtBr gel in 1× TAE buffer (*see* **Note 16** and **Fig. 2.9**).
14. Add 2 μL of 0.5 *M* EDTA to each tube to terminate the reaction.

3.3.1.4. Column Chromatography

1. For every experimental sample and control, combine the three reaction tubes (A, B, and Experimental) of PCR product into a 1.5-mL microcentrifuge tube.
2. Transfer 7 μL of the raw PCR product to a clean microcentrifuge tube and label this tube as "Sample A." Store at −20°C. Sample A will be later used for analysis using column chromatography.
3. To each tube of combined PCR product, add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Vortex thoroughly.
4. Centrifuge the tubes at 11,200*g* for 10 min to separate the phases.
5. Remove the top (aqueous) layer and place it in a clean 1.5-mL tube.
6. Add 700 μL of *n*-butanol and vortex the mix thoroughly (*see* **Note 17**).
7. Centrifuge the solution at room temperature at 11,200*g* for 1 min.
8. Remove and discard the upper (*n*-butanol organic) phase (*see* **Note 18**).
9. Invert a CHROMA SPIN-1000 column several times to completely resuspend the gel matrix. Check for air bubbles in the column matrix. If bubbles are visible, resuspend the matrix in the column buffer by inverting the column again.
10. Remove the top cap from the column and then remove the bottom cap.
11. Place the column into a 1.5-mL centrifuge tube.
12. Discard any column buffer that immediately collects in the tube and add 1.5 mL of 1× TNE buffer.
13. Let the buffer drain through the column by gravity flow until the surface of the gel beads in the column matrix can be seen (*see* **Note 19**).
14. Discard the collected buffer and proceed with purification.
15. Carefully and slowly apply the sample to the center of the gel bed's flat surface. Do not allow any sample to flow along the inner wall of the column.
16. Apply 25 μL of 1× TNE buffer and allow the buffer to completely drain out of the column.

17. Apply 150 μL of 1× TNE buffer and allow the buffer to completely drain out of the column.
18. Transfer column to a clean 1.5-mL microcentrifuge tube.
19. Apply 320 μL of 1× TNE buffer and collect the eluate as the purified ds cDNA fraction. Transfer 10 μL of this fraction to a clean microcentrifuge tube and label this tube as "Sample B." Store at −20°C. Use this aliquot for agarose/EtBr gel analysis.
20. Apply 75 μL of 1× TNE buffer and collect the eluate in a clean microcentrifuge tube. Label this tube as "Sample C" and store at −20°C. Save this fraction until after agarose/EtBr gel analysis.
21. To confirm that the PCR product is present in the purified ds cDNA fraction, perform the agarose/EtBr gel analysis (*see* **Note 20**).

3.3.2. RsaI Digestion (Super SMART™ PCR cDNA Synthesis Kit)

This step generates shorter, blunt-ended ds cDNA fragments, which are necessary for both adaptor ligation and subtraction.

1. Before proceeding with *Rsa*I digestion, set aside another 10 μL of purified ds cDNA for agarose/EtBr gel analysis to estimate the size range of the ds cDNA products. Label this tube as "Sample D."
2. Add the following reagents (provided in the Super SMART™ PCR cDNA Synthesis Kit) to the purified cDNA fraction collected from the CHROMA-SPIN column:
   a. 36 μL 10× *Rsa*I Restriction Buffer
   b. 1.5 μL *Rsa*I (10 U)
3. Mix by vortexing and spin briefly in a microcentrifuge.
4. Incubate at 37°C for 3 h.
5. To confirm that *Rsa*I digestion was successful, electrophorese 10 μL of uncut ds cDNA (Sample D) and 10 μL of *Rsa*I-digested cDNA on a 1.2% agarose/EtBr gel in 1× TAE buffer (*see* **Note 21** and **Fig. 2.10**).
6. Add 8 μL of 0.5 *M* EDTA to terminate the reaction.
7. Transfer 10 μL of the digested cDNA to a clean microcentrifuge tube, label this tube as "Sample E," and store at −20°C. Compare this sample with the PCR product after final purification.

3.3.2.1. Purification of Digested cDNA (Clontech NucleoTrap Nucleic Acid Purification Kit and Accessories provided in the Super SMART™ PCR cDNA Synthesis Kit)

1. Aliquot the *Rsa*I-digested ds cDNA into two clean 1.5-mL microcentrifuge tubes (approximately 170 μL in each tube).
2. Vortex the NucleoTrap Suspension (NucleoTrap Nucleic Acid Purification Kit) thoroughly until the beads are completely resuspended.
3. Add 680 μL of Buffer NT2 and 17 μL of NucleoTrap Suspension to each tube of digestion mixture.
4. Incubate the sample at room temperature for 10 min. Mix gently every 2–3 min during the incubation period.

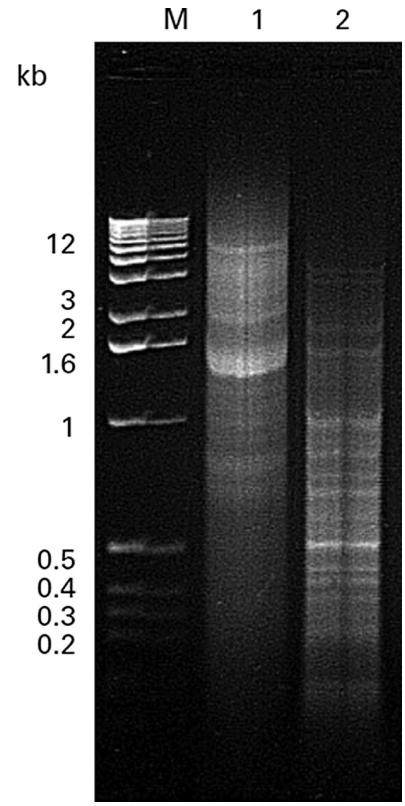


Fig. 2.10 Positive control skeletal muscle ds cDNA before (lane 1) and after (lane 2) *Rsa*I digestion. Complementary DNA was synthesized as described in the protocol (*see* **Section 3.3.3**) using the human skeletal muscle control poly A+ RNA included in the kit. Lane M: DNA size markers.

5. Centrifuge the sample at 10,000*g* for 1 min at room temperature. Discard the supernatant.
6. Add 680 μL of Buffer NT2 (NucleoTrap Nucleic Acid Purification Kit) to the pellet. Mix gently to resuspend. Centrifuge at 10,000*g* for 1 min at room temperature. Remove the supernatant completely and discard.
7. Add 680 μL of Buffer NT3 (NucleoTrap Nucleic Acid Purification Kit) to the pellet. Mix gently to resuspend. Centrifuge the sample at 10,000*g* for 1 min at room temperature. Remove the supernatant completely and discard.
8. Repeat the last step.
9. Centrifuge the pellet again at 10,000*g* for 1 min at room temperature. Air dry the pellet for 15 min at room temperature. Add 50 μL of TE buffer (10 m*M* Tris-Cl, pH 7.5; 1 m*M* EDTA provided with the NucleoTrap Nucleic Acid Purification Kit) to the pellet. Resuspend the pellet by mixing gently. Combine the resuspended pellets into one tube. Mix gently.

10. Elute the cDNA by incubating the sample at 50°C for 5 min. Gently mix the suspension two to three times during the incubation step.
11. Centrifuge the sample at 10,000*g* for 30 s at room temperature. Transfer the supernatant to a clean 1.5 mL that has been inserted into a 1.5-mL tube. Centrifuge for 5 min and discard the column.
12. Transfer 6 μL of the filtered cDNA solution to a clean 1.5-mL microcentrifuge tube containing 14 μL of deionized H_2O. Label this tube as "Sample F" and store at −20°C. Use this sample to analyze the SMART cDNA after purification.
13. To precipitate the cDNA, add 50 μL of 4 *M* ammonium acetate and 375 μL of 95% ethanol to the remaining sample.
14. Vortex the mix thoroughly and centrifuge the tubes at 11,200*g* for 20 min at room temperature. Carefully remove and discard the supernatant.
15. Overlay the pellet with 500 μL of 80% ethanol. Centrifuge the tube at 11,200*g* for 10 min. Carefully remove the supernatant and discard.
16. Air dry the pellets for 5–10 min.
17. Dissolve the pellet in 6.7 μL of 1× TNE buffer.
18. Transfer 1.2 μL to a clean 1.5-mL microcentrifuge tube containing 11 μL of deionized H_2O, label this tube as "Sample G," and store the remaining sample at −20°C.
19. Use 10 μL of the diluted cDNA to assess the yield of DNA by UV spectrophotometry (*see* **Note 22**). If DNA concentration is >300 ng/μL, dilute cDNA to a final concentration of 300 ng/μL in 1× TNE buffer and follow the adaptor ligation step in accordance with the PCR-Select cDNA subtraction protocol.

3.3.3. Conventional cDNA Synthesis and RsaI Digestion (PCR-Select™ cDNA Subtraction Kit)

3.3.3.1. First-Strand cDNA Synthesis

Perform this procedure with each experimental tester and driver poly A+ RNA and with the Control Poly A+ RNA (from human skeletal muscle) provided with the PCR-Select™ cDNA Subtraction Kit. The skeletal muscle cDNA made in this section serves as control driver cDNA in later steps.

1. For each tester, driver, and the Control Poly A+ RNA (from human skeletal muscle), combine in a sterile 0.5-mL microcentrifuge tube: 2–4 μL poly A+ RNA (2 μg) (*see* **Note 23**), 1 μL cDNA Synthesis Primer (10 μ*M*) (PCR-Select™

cDNA Subtraction Kit). Add sterile H_2O to a final volume of 5 μL if needed. Mix and spin briefly in a microcentrifuge.
2. Incubate at 70°C for 2 min in a thermal cycler.
3. Cool on ice for 2 min and briefly centrifuge.
4. Add to each reaction (final volume 10 μL): 2 μL 5× First-Strand Buffer (PCR-Select™ cDNA Subtraction Kit), 1 μL dNTP Mix (10 m*M* each) (PCR-Select™ cDNA Subtraction Kit), 1 μL sterile H_2O, 1 μL AMV Reverse Transcriptase (20 U/μL) (PCR-Select™ cDNA Subtraction Kit).
5. Gently vortex and centrifuge the tubes for 5 s.
6. Incubate the tubes at 42°C for 1.5 h in an air incubator. Do not use a water bath or thermal cycler. Evaporation can reduce the reaction mixture volume, and therefore, reaction efficiency.
7. Place on ice to terminate first-strand cDNA synthesis.

3.3.3.2. Second-Strand cDNA Synthesis

Perform the following procedure with each first-strand tester, driver, and the control skeletal muscle cDNA:

1. Add to the first-strand synthesis reaction tubes (final volume 80 μL): 48.4 μL sterile H_2O, 16.0 μL 5× Second-Strand Buffer (PCR-Select™ cDNA Subtraction Kit), 1.6 μL dNTP Mix (10 m*M*) (PCR-Select™ cDNA Subtraction Kit), 4.0 μL 20× Second-Strand Enzyme Cocktail (PCR-Select™ cDNA Subtraction Kit).
2. Mix contents and spin for 5 s.
3. Incubate at 16°C for 2 h in water bath or thermal cycler.
4. Add 2 μL (6 U) of T4 DNA Polymerase (PCR-Select™ cDNA Subtraction Kit). Mix contents well.
5. Incubate at 16°C for 30 min in a water bath or thermal cycler.
6. Add 4 μL of 20× EDTA/glycogen Mix (PCR-Select™ cDNA Subtraction Kit) to terminate second-strand synthesis.
7. Add 100 μL of phenol:chloroform:isoamyl alcohol (25:24:1).
8. Vortex thoroughly and centrifuge at 11,200*g* for 10 min at room temperature to separate phases.
9. Carefully collect the top aqueous layer and place in a fresh 0.5-mL microcentrifuge tube. Discard the inter- and lower-phases.
10. Add 100 μL of chloroform:isoamyl alcohol (24:1).
11. Vortex thoroughly and centrifuge at 11,200*g* for 10 min at room temperature to separate phases.
12. Carefully collect the top aqueous layer and place in a fresh 0.5-mL microcentrifuge tube. Discard the inter- and lower-phases.
13. Add 40 μL of 4 *M* NH_4OAc and 300 μL of 95% ethanol.
14. Vortex thoroughly and centrifuge at 11,200*g* for 20 min at room temperature.

15. Carefully collect the supernatant.
16. Overlay the pellet with 500 μL of 80% ethanol. Centrifuge at 11,200*g* for 10 min.
17. Remove the supernatant.
18. Air dry the pellet for about 10 min to evaporate residual ethanol.
19. Dissolve precipitate in 50 μL of sterile H_2O.
20. Transfer 6 μL to a fresh microcentrifuge tube. Store this sample at −20°C until after *Rsa*I digestion (for agarose gel electrophoresis) to estimate the yield and size range of ds cDNA products synthesized.

3.3.3.3. RsaI Digestion (PCR-Select™ cDNA Subtraction Kit)

Perform the following procedure with each experimental ds tester and driver cDNA, as well as with the control skeletal muscle cDNA.

1. Add per reaction (final volume 94 μL): 43.5 μL ds cDNA, 5.0 μL 10× *Rsa*I Restriction Buffer, 1.5 μL *Rsa*I (10 U/μL).
2. Mix by vortexing and centrifuge for 5 s.
3. Incubate at 37°C for 1.5 h.
4. Set aside 5 μL of the digest mixture to analyze the efficiency of *Rsa*I digestion.
5. Add 2.5 μL of 20× EDTA/glycogen.
6. Mix to terminate the reaction.
7. Add 50 μL of phenol:chloroform:isoamyl alcohol (25:24:1).
8. Vortex thoroughly and centrifuge at 11,200*g* for 10 min at room temperature to separate phases.
9. Carefully collect the top aqueous layer and place in a fresh 0.5-mL tube.
10. Add 50 μL of chloroform:isoamyl alcohol (24:1).
11. Vortex thoroughly and centrifuge at 11,200*g* for 10 min at room temperature to separate phases.
12. Carefully collect the top aqueous layer and place in a fresh 0.5-mL tube.
13. Add 25 μL of 4 *M* NH_4OAc and 187.5 μL of 95% ethanol.
14. Vortex thoroughly and centrifuge at 11,200*g* for 10 min at room temperature to separate phases.
15. Remove the supernatant.
16. Gently overlay the pellets with 200 μL of 80% ethanol.
17. Centrifuge at 11,200*g* for 5 min.
18. Carefully remove the supernatant.
19. Air dry the pellets for 5–10 min.
20. Dissolve the pellet in 5.5 μL of H_2O and store at −20°C (*see* **Note 24**).
21. Check *Rsa*I-digested cDNA using agarose/EtBr gel electrophoresis (*see* **Note 21**).

3.3.4. Adaptor Ligation (PCR-Select™ cDNA Subtraction Kit)

Subtractions should be performed in both directions for each tester/driver cDNA pair (forward and reverse subtraction) (**Fig. 2.11**), in preparation of the differential screening step. To perform subtractions in both directions, tester cDNA corresponding to each of the poly A+ RNA samples is required. A control subtraction is also performed with the control skeletal muscle cDNA with φX174/*Hae*III DNA. As illustrated in **Fig. 2.12**, three separate adaptor ligations must be performed for each experimental tester cDNA and the control skeletal muscle tester cDNA. Each cDNA (cDNA 1, cDNA 2, and cDNA 3 from the control) is aliquoted into two separate tubes: one aliquot is ligated with Adaptor 1 (Testers 1-1, 2-1, and 3-1) and the second is ligated with Adaptor 2R (Testers 1-2, 2-2, and 3-2). After the ligation reactions are set up, portions of each tester tube are combined so that the cDNA is ligated with both adaptors (unsubtracted tester controls 1-c, 2-c, and 3-c). Each unsubtracted tester control cDNA serves as a positive control for ligation and later serves as a negative control for subtraction.

1. Dilute 1 μL of each *Rsa*I-digested experimental cDNA (from conventional cDNA synthesis (*see* **Section** 3.3.3.3) and from the Super SMART cDNA synthesis (*see* **Section** 3.3.2) with 5 μL of sterile H_2O.
2. Prepare the control skeletal muscle tester cDNA: dilute the φX174/*Hae*III Control DNA with sterile H_2O to a final concentration of 150 ng/mL, mix 1 μL of control skeletal muscle cDNA (PCR-Select™ cDNA Subtraction Kit) with 5 μL of the diluted φX174/*Hae*III Control DNA (*see* **Note 25**).
3. Prepare the human placenta cDNA from the Super SMART™ PCR cDNA Synthesis Kit procedure by mixing

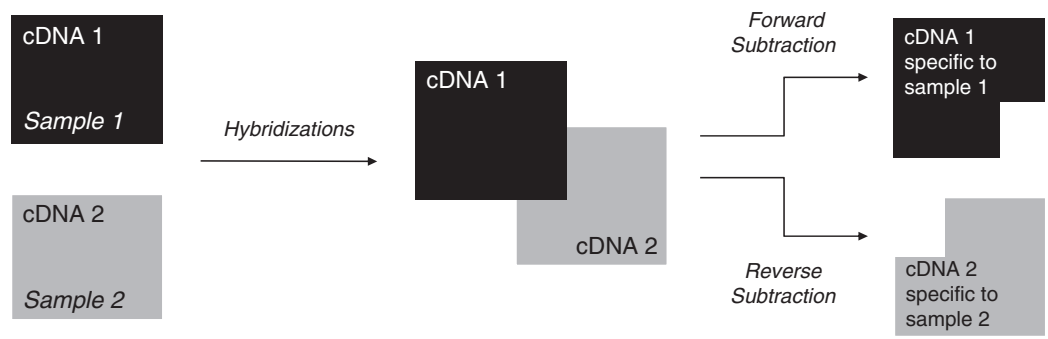


Fig. 2.11 Forward and reverse subtraction. The forward subtraction experiment is designed to enrich differentially expressed sequences present in poly A+ RNA sample 1 (cDNA 1, tester) but not poly A+ RNA sample 2 (cDNA 2, driver). However, in the reverse subtraction, cDNA 2 serves as a tester and cDNA 1 serves as a driver. The result is two subtracted cDNA populations: the forward-subtracted cDNA contains sequences that are specific to Sample 1 and the reverse-subtracted cDNA contains sequences that are specific to Sample 2.

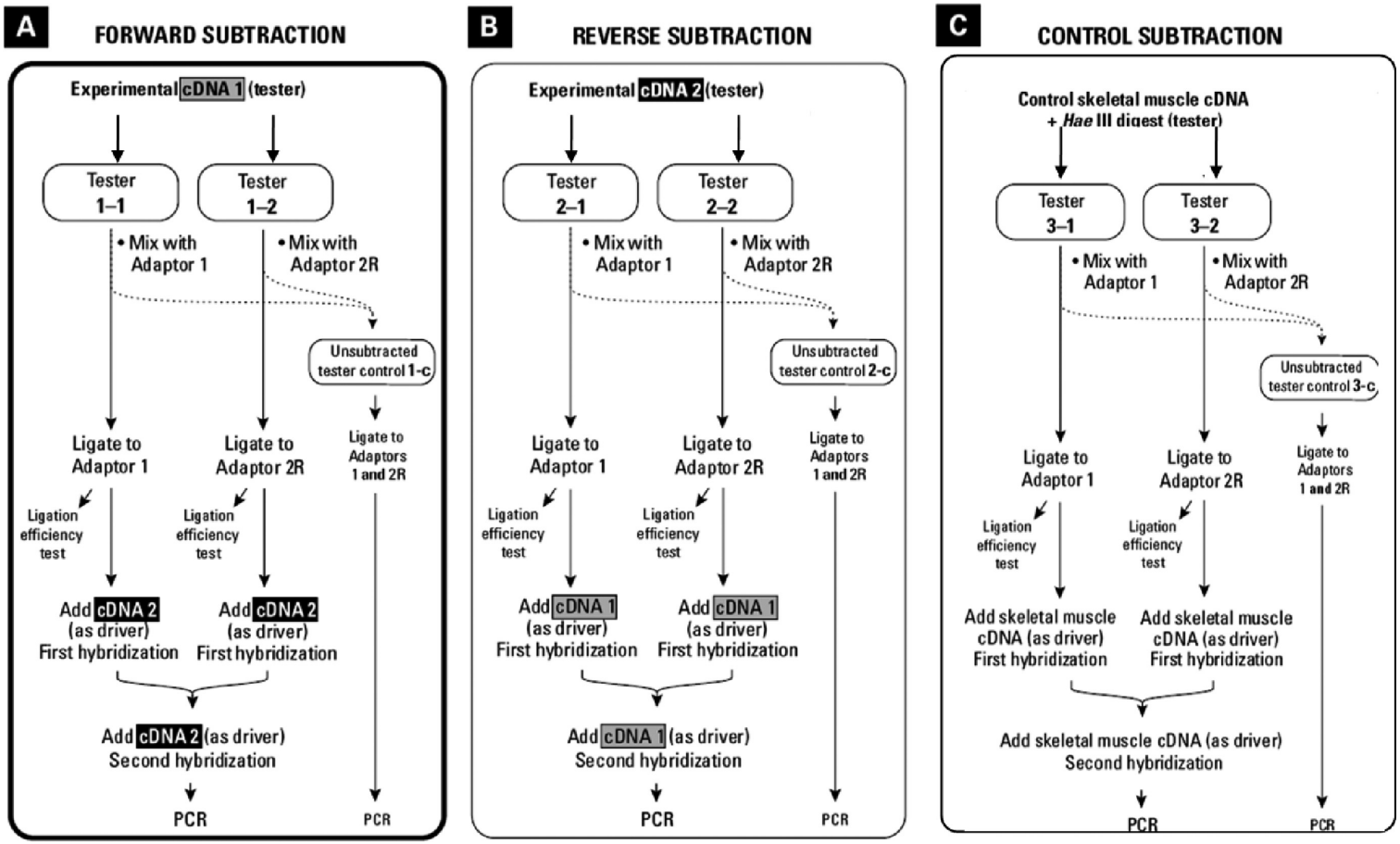


Fig. 2.12 Preparing adaptor-ligated tester cDNAs for hybridization and PCR. Each tester cDNA (i.e., each different experimental cDNA and the control skeletal muscle tester cDNA) must be ligated to the appropriate adaptors, as shown above. **Panel A**. The forward subtraction is the intended experiment. **Panel B**. A second subtraction in reverse (i.e., tester as driver, driver as tester) is required for differential screening of the subtracted cDNA library. **Panel C**. Control subtraction with skeletal muscle cDNA. (Adapted from the PCR-Select cDNA Subtraction Kit User Manual, Clontech.)

Table 2.4
Setting up the ligation reactions

Component	Tester 1-1 (μL)	Tester 2-2 (μL)
Diluted tested cDNA	2	2
Adaptor 1 (10 μ*M*)	2	–
Adaptor 2R (10 μ*M*)	–	2
Master Mix	6	6
Final volume	**10**	**10**

it with φX174/*Hae*III Control DNA as for the control skeletal muscle tester cDNA (*see* **Note 26**).

4. Prepare a ligation Master Mix by combining in a 0.5-mL microcentrifuge tube: 3 μL sterile H_2O, 2 μL 5× Ligation Buffer (PCR-Select™ cDNA Subtraction Kit), 1 μL T4 DNA Ligase (400 U/μL) (PCR-Select™ cDNA Subtraction Kit) (*see* **Note 27**).
5. For each experimental tester cDNA and for the control skeletal muscle tester cDNA, combine the reagents in **Table 2.4** in the order shown in 0.5-mL microcentrifuge tubes. Pipet mixture up and down to mix thoroughly.
6. In a fresh microcentrifuge tube, mix 2 μL of Tester 1-1 and 2 μL of Tester 1-2 (PCR-Select™ cDNA Subtraction Kit). After ligation is complete, this will be the unsubtracted tester control 1-c. Do the same for each additional experimental tester cDNA and the control skeletal muscle tester cDNA. After ligation, approximately 1/3 of the cDNA molecules in each unsubtracted tester control tube will bear two different adaptors.
7. Centrifuge briefly and incubate at 16°C overnight.
8. Add 1 μL of EDTA/glycogen (provided with the PCR-Select™ cDNA Subtraction Kit). Mix to stop ligation reaction.
9. Heat samples at 72°C for 5 min to inactivate the ligase.
10. Centrifuge the tubes for 5 s.
11. Remove 1 μL from each unsubtracted tester control (1-c, 2-c, and 3-c) and dilute into 1 mL of H_2O.
12. Store samples at −20°C.

3.3.4.1. Ligation Efficiency Analysis (PCR-Select™ cDNA Subtraction Kit)

The following PCR experiment allows verification that at least 25% of the cDNAs have adaptors on both ends. This experiment is designed to amplify fragments that span the adaptor/cDNA junctions of Testers 1-1 and 1-2, of second experimental tester cDNA (Testers 2-1 and 2-2) from the reverse subtraction, on the adaptor-ligated control skeletal muscle cDNA (Testers 3-1 and

3-2) and adaptor-ligated control human placenta cDNA (Testers 4-1 and 4-2).

1. Dilute 1 μL of each ligated cDNA (e.g., the Testers 1-1 and 1-2) into 200 μL of H_2O.
2. Combine the reagents in **Table 2.5** in four separate tubes for each experimental cDNA samples and controls.
3. Prepare a Master Mix for all of the reaction tubes. For each reaction planned, combine the reagents in **Table 2.6** in the order shown.
4. Mix well by vortexing and centrifuging the tubes for 5 s.
5. Aliquot 22 μL of Master Mix into each of the reactions.
6. Mix well by vortexing and centrifuging the tubes for 5 s.
7. Overlay with 50 μL of mineral oil.
8. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors thus creating binding sites for the PCR primers. Do not remove the samples from the thermal cycler.
9. Immediately commence thermal cycling:

Thermal Cycler 480	PCR Systems 2400 or 9600
20 cycles	1 cycle
94°C, 30 sec	94°C, 10 sec
65°C, 30 sec	20 cycles
68°C, 2.5 min	94°C, 10 sec
	65°C, 30 sec
	68°C, 2.5 min

10. Analyze 5 μL from each reaction on a 2.0% agarose/EtBr gel run in 1× TAE buffer (*see* **Note 28** and **Fig. 2.13**).

3.3.5. First Hybridization (PCR-Select™ cDNA Subtraction Kit)

1. Allow the 4× Hybridization Buffer to warm up to room temperature for at least 15–20 min. Verify that there is no

Table 2.5
Setting up the ligation efficiency analysis

Tube	1	2	3	4
Component (μL)				
Tester 1-1 (ligated to Adaptor 1)	1	1	–	–
Tester 1-2 (ligated to Adaptor 2R)	–	–	1	1
G3PDH 3′ Primer (10 μ*M*)	1	1	1	1
G3PDH 5′ Primer (10 μ*M*)	–	1	–	1
PCR Primer 1 (10 μ*M*)	1	–	1	–
Total volume (μL)	**3**	**3**	**3**	**3**

Table 2.6
Preparation of the ligation efficiency analysis, PCR Master Mix

Component	Per reaction
Sterile H_2O	18.5 μL
10× PCR reaction buffer	2.5 μL
dNTP Mix (10 m*M*)	0.5 μL
50× Advantage cDNA Polymerase Mix	0.5 μL
Total volume	**22.0 μL**

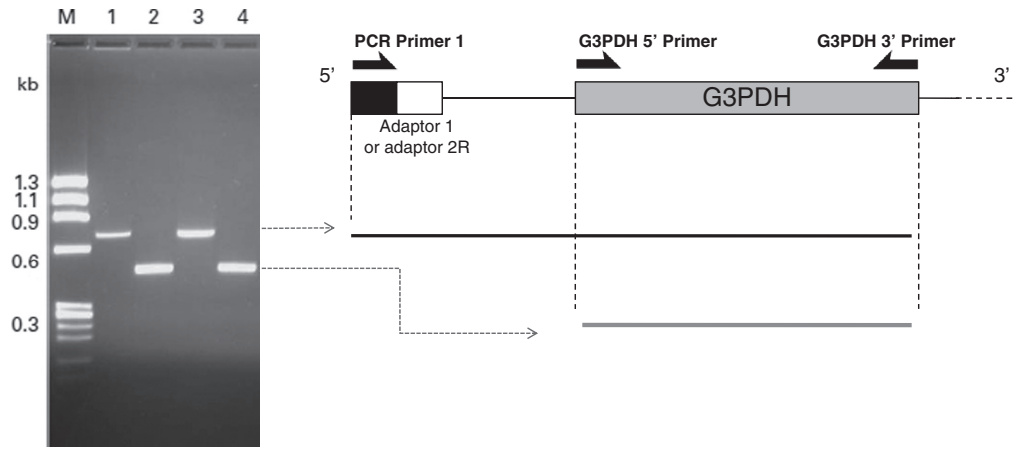


Fig. 2.13 Typical results of ligation efficiency analysis. The results shown here are for human samples; lane 1: PCR products using Tester 1-1 (Adaptor 1-ligated) as the template and the G3PDH 3′ Primer and PCR Primer 1. Lane 2: PCR products using Tester 1-1 (Adaptor 1-ligated) as the template and the G3PDH 3′ and 5′ Primers. Lane 3: PCR products using Tester 1-2 (Adaptor 2R-ligated) as the template and the G3PDH 3′ Primer and PCR Primer 1. Lane 4: PCR products using Tester 1-2 (Adaptor 2R-ligated) as the template and the G3PDH 3′ and 5′ Primers, 2% agarose/EtBr gel. Lane M: φX174 DNA/*Hae*III digest size markers. (Adapted from PCR-Select™ cDNA Subtraction Kit User Manual, Clontech.)

visible pellet or precipitate before using the buffer. If necessary, heat the buffer at 37°C for ~10 min to dissolve any precipitate.

2. For each of the experimental and skeletal muscle subtractions, combine the reagents in **Table 2.7** in 0.5-mL tubes in the order shown.
3. Overlay samples with one drop of mineral oil and centrifuge briefly.
4. Incubate samples at 98°C for 1.5 min in a thermal cycler.
5. Incubate samples at 68°C for 8 h. Samples may hybridize for 6–12 h. Do not let the incubation exceed 12 h.

3.3.6. Second Hybridization (PCR-Select™ cDNA Subtraction Kit)

Do not remove the hybridization samples from the thermal cycler for longer than is necessary to add fresh driver.

1. Add into a sterile tube for each experimental tester cDNA and for the control skeletal muscle cDNA (final volume 4

Table 2.7
Setting up the first hybridization

Hybridization samples	1 Tester 1-1	2 Tester 1-2
Component		
*Rsa*I-digested Driver cDNA	1.5 μL	1.5 μL
Adaptor 1-ligated Tester 1-1	1.5 μL	–
Adaptor 2R-ligated Tester 1-2	–	1.5 μL
4× Hybridization Buffer	1.0 μL	1.0 μL
Final volume	4.0 μL	4.0 μL

μL): 1 μL Driver cDNA, 1 μL 4× Hybridization Buffer, 2 μL sterile H_2O.

2. Place 1 μL of this mixture in a 0.5-mL microcentrifuge tube and overlay it with one drop of mineral oil.
3. Incubate at 98°C for 1.5 min in a thermal cycler.
4. Remove the tube of freshly denatured driver from the thermal cycler.
5. Use the following procedure to simultaneously mix the driver with hybridization samples 1 and 2 (**Table 2.7**). This ensures that the two hybridization samples mix together only in the presence of freshly denatured driver:
   a. Set a micropipettor at 15 μL.
   b. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing hybridization sample 2.
   c. Carefully draw the entire sample partially into the pipette tip. Do not be concerned if a small amount of mineral oil is transferred with the sample.
   d. Remove the pipette tip from the tube and draw a small amount of air into the tip, creating a slight air space below the droplet of sample.
   e. Gently touch the pipette tip to the mineral oil/sample interface of the tube containing the freshly denatured driver sample.
   f. Carefully draw the entire sample partially into the pipette tip. Do not be concerned if a small amount of mineral oil is transferred with the sample. The pipette tip should now contain both samples (hybridization sample 2 and denatured driver) separated by a small air pocket.
   g. Transfer the entire mixture to the tube containing hybridization sample 1.
   h. Mix by pipetting up and down.
   i. Incubate reaction at 68°C overnight.
   j. Add 200 μL of dilution buffer (PCR-Select™ cDNA Subtraction Kit) and mix by pipetting.

k. Heat at 68°C for 7 min in a thermal cycler.
l. Store at −20°C.

3.3.7. PCR Amplification (PCR-Select™ cDNA Subtraction Kit)

Seven PCR reactions are recommended as described in **Fig. 2.12**:

1. Forward-subtracted experimental cDNA.
2. Unsubtracted tester control (1-c).
3. Reverse-subtracted experimental cDNA.
4. Unsubtracted tester control for the reverse subtraction (2-c).
5. Subtracted control skeletal muscle cDNA.
6. Unsubtracted tester control for the control subtraction (3-c).
7. PCR control-subtracted cDNA. The PCR control-subtracted cDNA provides a positive PCR control and contains a successfully subtracted mixture of X174 DNA-*Hae*III/Digest

3.3.7.1. Primary Amplification

Prepare the PCR templates:

1. Aliquot 1 μL of each diluted cDNA from subtracted experimental sample and the corresponding diluted unsubtracted sample into an appropriately labeled tube.
2. Aliquot 1 μL of the PCR control subtracted cDNA into an appropriately labeled tube.
3. Prepare a Master Mix for all of the primary PCR tubes. Combine in order (total volume 24 μL): 19.5 μL sterile H_2O, 2.5 μL 10× PCR reaction buffer (PCR-Select™ cDNA Subtraction Kit), 0.5 μL dNTP Mix (10 m*M*) (PCR-Select™ cDNA Subtraction Kit), 1.0 μL PCR Primer 1 (10 μ*M*) (PCR-Select™ cDNA Subtraction Kit), 0.5 μL 50× Advantage cDNA Polymerase Mix.
4. Mix well by vortexing and centrifuge the tube for 5 s.
5. Aliquot 24 μL of Master Mix into each of the reaction tubes containing 1 μL of the diluted cDNA.
6. Overlay with 50 μL of mineral oil.
7. Incubate the reaction mix at 75°C for 5 min in a thermal cycler to extend the adaptors thus creating binding sites for the PCR primers.
8. Immediately commence thermal cycling:

Thermal Cycler 480	PCR Systems 2400 or 9600
27 cycles	1 cycle:
94°C, 30 sec	94°C, 25 sec
66°C, 30 sec	27 cycles
72°C, 1.5 min	94°C, 10 sec
	66°C, 30 sec
	72°C, 1.5 min

9. Set 8 μL aliquots aside from each tube for gel electrophoresis of PCR amplification.

3.3.7.2. Secondary Amplification (Nested PCR)

1. Dilute 3 μL of each primary PCR mixture in 27 μL of H_2O.
2. Aliquot 1 μL of each diluted primary PCR product mixture into an appropriately labeled tube.
3. Prepare Master Mix for the secondary PCR reactions by combining in order (total volume 24 μL): 18.5 μL sterile H_2O, 2.5 μL 10× PCR reaction buffer (PCR-Select™ cDNA Subtraction Kit), 1.0 μL Nested PCR Primer 1 (10 μ*M*) (PCR-Select™ cDNA Subtraction Kit), 1.0 μL Nested PCR Primer 2R (10 μ*M*) (PCR-Select™ cDNA Subtraction Kit), 0.5 μL dNTP Mix (10 m*M*) (PCR-Select™ cDNA Subtraction Kit), 0.5 μL 50× Advantage cDNA Polymerase Mix.
4. Mix well by vortexing and centrifuge the tube for 5 s.
5. Aliquot 24 μL of Master Mix into the tubes containing the diluted primary PCR product.
6. Overlay with one drop of mineral oil.
7. Immediately commence thermal cycling:

Thermal Cycler 480	PCR Systems 2400 or 9600
10–12 cycles:	10–12 cycles:
94°C, 30 sec	94°C, 10 sec
68°C, 30 sec	68°C, 30 sec
72°C, 1.5 min	72°C, 1.5 min

8. Analyze 8 μL from each reaction on a 2.0% agarose/EtBr gel run in 1× TAE buffer (*see* **Note 29** and **Figs. 2.14**, **2.15**).
9. Store reaction products at −20°C.

3.3.8. PCR Analysis of Subtraction Efficiency (PCR-Select™ cDNA Subtraction Kit)

Amplification by PCR can be used to estimate the efficiency of subtraction by comparing the abundance of known cDNAs before and after subtraction. Ideally this is done with both a nondifferentially expressed gene (e.g., housekeeping gene) and with a gene known to be differentially expressed between the two RNA sources being compared. The test described below uses the G3PDH primers provided with the PCR-Select™ cDNA Subtraction Kit and cyclophilin primers to confirm the reduced relative abundance of G3PDH and cyclophilin following the PCR-Select procedure (*see* **Note 30** and **Figs. 2.11**, **2.12**).

1. Dilute the subtracted and unsubtracted (unsubtracted tester control 1-c and 2-c) secondary PCR products 10× in H_2O. The concentration of subtracted and unsubtracted product should be roughly equal.
2. Combine in 0.5-mL microcentrifuge tubes in order:
   a. For the skeletal muscle cDNA control (total volume 30 μL): 1 μL diluted subtracted cDNA or diluted unsubtracted tester control 1-c, 1.2 μL Cyclophilin 3′ Primer

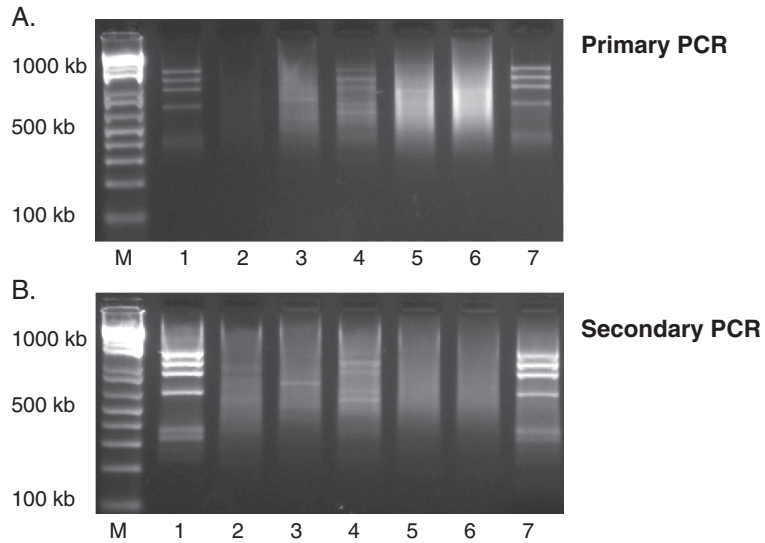


Fig. 2.14 Amplification of results for reactivated uterus versus uterus in diapause subtraction analysis. Lane M: 100 bp size markers. **Panel A**: Primary PCR. **Panel B**: Secondary PCR. Lane 1: PCR products of subtracted skeletal muscle tester cDNA with 0.2% φX174/*Hae* III-digested DNA. Lane 2: PCR products of forward subtraction cDNA. Lane 3: PCR products of reverse subtraction cDNA. Lane 4: PCR products of unsubtracted skeletal muscle tester cDNA with 0.2% φX174/*Hae* III-digested DNA. Lane 5: PCR products of forward unsubtracted cDNA. Lane 6: PCR products of reverse unsubtracted cDNA. Lane 7: PCR control subtracted cDNA. Samples are electrophoresed on a 2% agarose/EtBr gel (Lefèvre, 2005, unpublished).

(10 μ*M*), 1.2 μL Cyclophilin 5′ Primer (10 μ*M*), 3.0 μL 10× PCR reaction buffer (PCR-Select^{TM} cDNA Subtraction Kit), 22.4 μL sterile H_2O, 0.6 μL dNTP Mix (10 m*M*) (PCR-Select^{TM} cDNA Subtraction Kit), 0.6 μL 50× Advantage cDNA Polymerase Mix.

b. For the experimental cDNA samples (total volume 30 μL): 1 μL diluted subtracted cDNA or diluted unsubtracted tester control 1-c, 1.2 μL G3PDH 3′ Primer (10 μ*M*), 1.2 μL G3PDH 5′ Primer (10 μ*M*) (PCR-Select^{TM} cDNA Subtraction Kit), 3.0 μL 10× PCR reaction buffer, 22.4 μL sterile H_2O, 0.6 μL dNTP Mix (10 m*M*), 0.6 μL 50× Advantage cDNA Polymerase Mix.

3. Mix by vortexing and briefly centrifuging.
4. Overlay with one drop of mineral oil.
5. Use the following thermal cycling program; 18 cycles: 94°C 30 s, 60°C 30 s, 68°C 2 min.
6. Remove 5 μL from each reaction and place it in a clean tube. Put the rest of the reaction mixture back into the thermal cycler for five additional cycles.
7. Repeat the last step twice (i.e., remove 5 μL after 28 and 33 cycles).

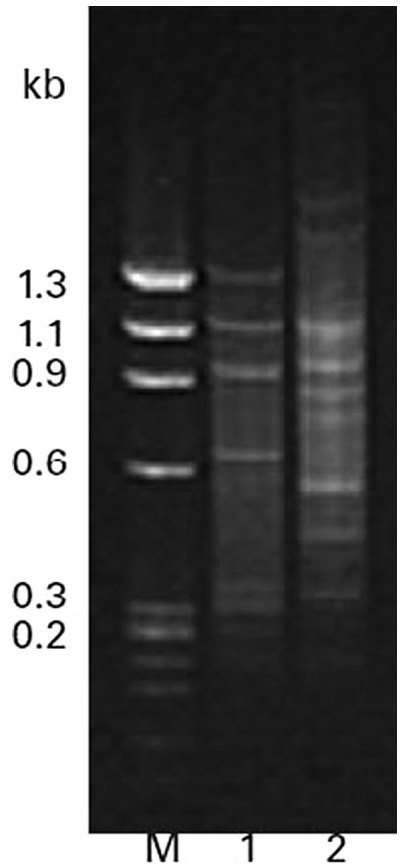


Fig. 2.15 Typical results of control skeletal muscle subtraction analysis. The secondary PCR product of the subtracted skeletal muscle sample contains mostly DNA fragments corresponding to the φX174/*Hae*III digest. The adaptor sequences on both ends of DNA fragments cause the mobility shift of these PCR products in comparison with original, digested φX174 DNA. Lane M: φX174 DNA/*Hae*III digest size markers. Lane 1: Secondary PCR products of subtracted skeletal muscle tester cDNA with 0.2% φX174/*Hae*III-digested DNA. Lane 2: Secondary PCR products of unsubtracted skeletal muscle tester cDNA ligated with both Adaptors 1 and 2R and containing 0.2% φX174/*Hae*III-digested DNA. Samples are electrophoresed on a 2% agarose/EtBr gel (from PCR-Select™ cDNA Subtraction Kit User Manual, Clontech).

8. Examine the 5 μL samples (i.e., the aliquots that were removed from each reaction after 18, 23, 28, and 33 cycles) on a 2.0% agarose/EtBr gel (*see* **Note 31** and **Fig. 2.16**).

3.4. Differential Screening (PCR-Select Differential Screening Kit)

The PCR-Select Differential Screening Kit allows to screen your PCR-Select subtracted library for differentially expressed cDNAs and to eliminate nondifferentially expressed transcripts. The procedure involves a first step of cloning cDNA sequences into a cloning vector to isolate from each other and amplify subtracted cDNA fragments. Clones from the subtracted library are then arrayed on nylon membranes and hybridized with four

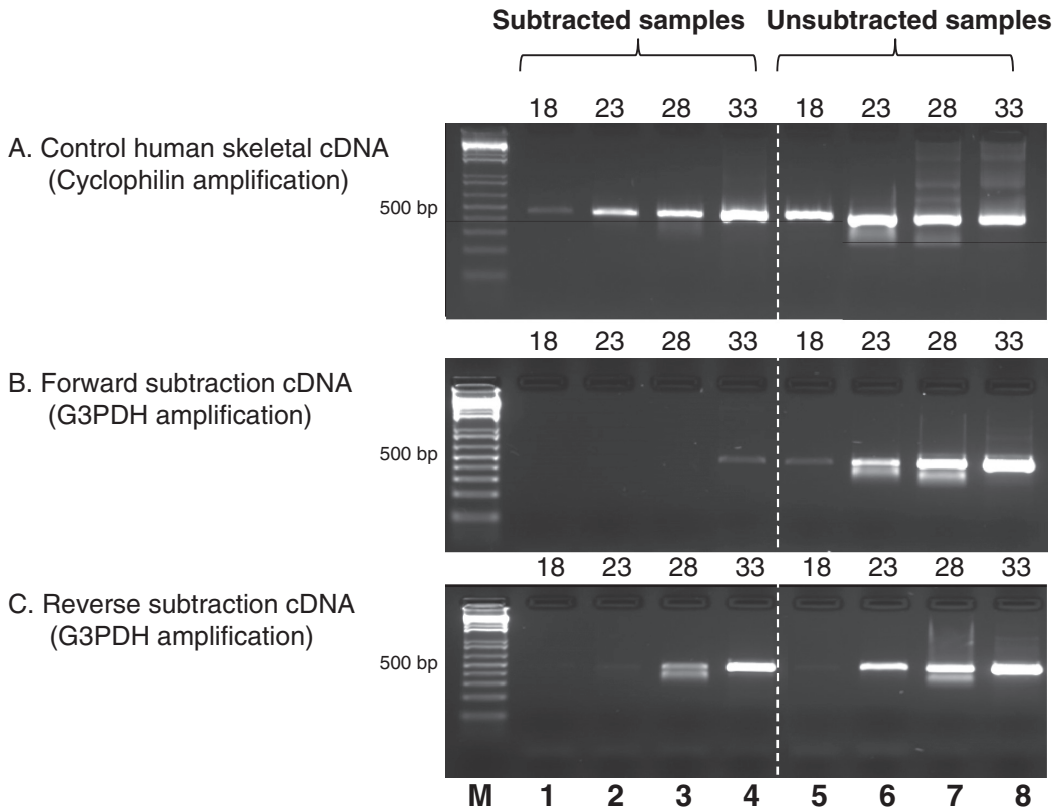


Fig. 2.16 Amplification results for the efficiency of the reactivated uterine cDNA versus cDNA of uterus during diapause subtraction, in the mink. PCR was performed on the subtracted (lanes 1–4) or unsubtracted (lanes 5–8) secondary PCR product using housekeeping gene primers. Lanes 1 and 5: 18 cycles; lanes 2 and 6: 23 cycles; lanes 3 and 7: 28 cycles; lanes 4 and 8: 33 cycles. Lane M: marker, 100 bp. **Panel A**: Cyclophilin reduction in control human skeletal cDNA subtraction. **Panels B and C**: G3PDH reduction in the forward subtraction cDNA and in the reverse subtraction cDNA, respectively. Housekeeping gene abundance is significantly reduced by PCR-Select subtraction. The three subtractions were successful. (Lefèvre, 2005, unpublished).

different probes (**Fig. 2.17**): a probe made from the subtracted cDNA, another probe made from reverse-subtracted cDNA, and nonsubtracted probes synthesized directly from tester and driver cDNAs. Clones hybridizing to tester but not to driver are differentially expressed; however, nonsubtracted probes are not sensitive enough to detect rare messages. Subtracted probes are greatly enriched for rare differentially expressed sequences and can detect rare transcripts, but they may give false positive results. Thus, using subtracted and nonsubtracted probes together provides the most effective way to identify potentially differentially expressed genes.

3.4.1. Secondary PCR of Subtracted cDNA

1. Label sterile 0.5-mL reaction tubes for PCR. Prepare two secondary PCR tubes for each subtracted and/or unsubtracted probe.

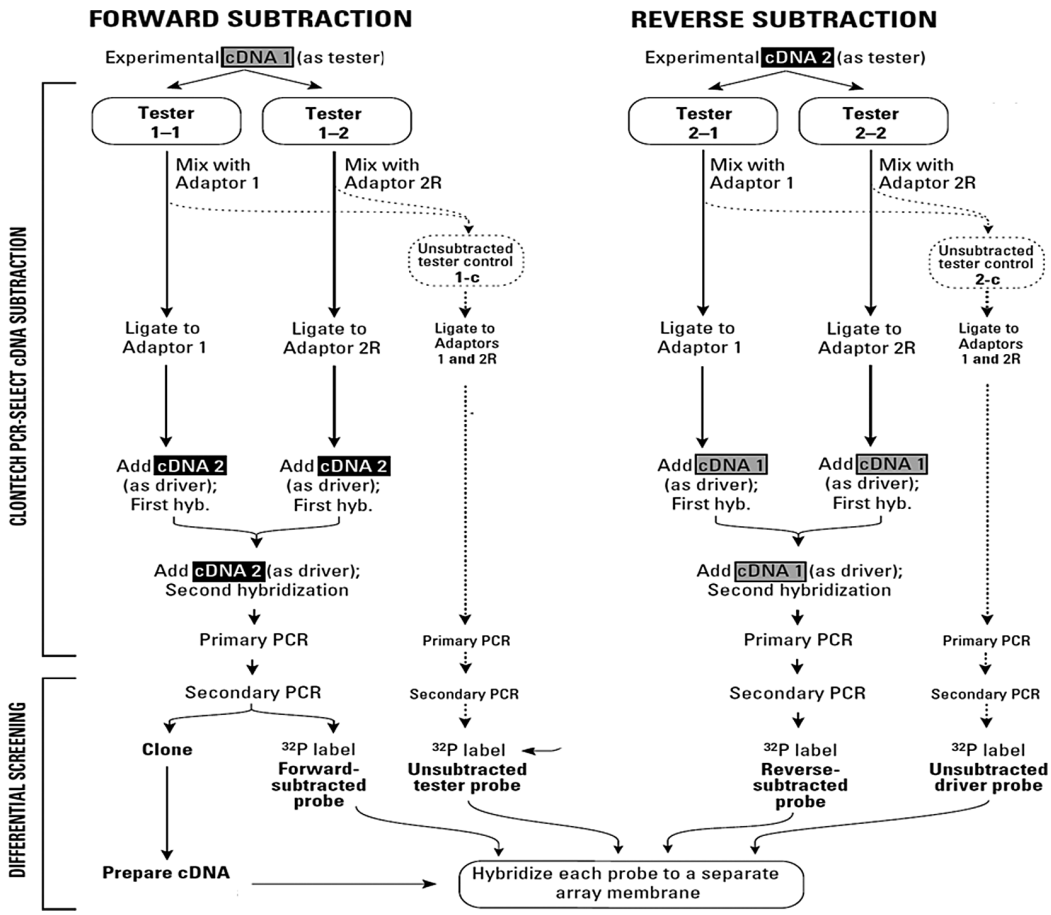


Fig. 2.17 Experimental set-up for PCR-Select Differential Screening following the PCR-Select cDNA subtraction. This flow chart indicates the procedure for preparing both subtracted and unsubtracted probes. Differential screening with subtracted probes is more sensitive. However, using both subtracted and unsubtracted probes is recommended. Secondary PCR products are cloned to construct the subtracted cDNA library. Complementary DNA clones are hybridized on nylon membranes that are arrayed by four different probes: the forward and reverse subtracted probes and the unsubtracted tester and driver probes.

2. Prepare a PCR Master Mix in a 1.5-mL microcentrifuge tube. Combine in order (total volume 24 μL): 18.5 μL sterile H_2O, 2.5 μL 10× PCR reaction buffer (PCR-Select Differential Screening Kit), 1.0 μL Nested PCR Primer 1 (10 μ*M*) (PCR-Select Differential Screening Kit), 1.0 μL Nested PCR Primer 2R (10 μ*M*) (PCR-Select Differential Screening Kit), 0.5 μL dNTP Mix (10 m*M*) (PCR-Select Differential Screening Kit), 0.5 μL 50× Advantage cDNA Polymerase Mix.
3. Mix well by vortexing. Spin the tube for 5 s in a microcentrifuge to collect contents at the bottom.

4. Aliquot 24 μL of Master Mix into each reaction tube labeled.
5. Into each tube, aliquot 1 μL of the appropriate template:
   a. For the forward- and reverse-subtracted probes, use the diluted products of *primary* PCR amplification from the PCR-Select cDNA subtraction.
   b. To amplify tester and driver cDNAs to make unsubtracted probes: For the tester probe, use the *primary* PCR product of the unsubtracted tester control from the *forward* subtraction as the template. For the driver probe, use the *primary* PCR product of the unsubtracted tester control from the *reverse* subtraction as the template.
6. Immediately commence thermal cycling:

Thermal Cycler 480	PCR Systems 2400 or 9600
10–12 cycles:	10–12 cycles:
94°C, 30 sec	94°C, 10 sec
68°C, 30 sec	68°C, 30 sec
72°C, 1.5 min	72°C, 1.5 min
1 cycle:	1 cycle:
72°C, 5 min	72°C, 5 min

7. Electrophorese 8 μL from each reaction on a 2.0% agarose/EtBr gel in 1× TAE buffer (*see* **Note 29**).
8. Store reactions at −20°C. *See* **Section 3.4.4** for next step.

3.4.2. Subtracted cDNA Library Construction

3.4.2.1. T/A Cloning (Invitrogen T/A Cloning Kit Dual Promoter)

The TA Cloning Kit Dual Promoter with pCRII provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector. *Taq* polymerase has a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products. The linearized vector supplied in this kit has single 3′-deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector.

1. Set up the 10 μL ligation reaction as follows (final volume 10 μL): 3 μL fresh secondary PCR product from the forward subtraction (*see* **Note 32**), 1 μL 10× Ligation Buffer (T/A Cloning Kit Dual Promoter), 2 μL pCRII vector (25 ng/μL) (T/A Cloning Kit Dual Promoter), sterile water to a total volume of 9 μL, 1 μL T4 DNA Ligase (4.0 Weiss units) (T/A Cloning Kit Dual Promoter).
2. Incubate the ligation reaction at 14°C for a minimum of 4 h (preferably overnight).

3. Ligation reaction may be stored at −20°C until cell transformation.

3.4.2.2. Transformation of MAX Efficiency DH5α Competent Cells (Invitrogen T/A Cloning Kit Dual Promoter and Invitrogen Max Efficiency DH5α Competent Cells)

The ϕ80d*lac*ZΔM15 marker of those competent cells provides α-complementation of the β-galactosidase gene from the pCRII vector and, therefore, can be used for blue/white screening of colonies on bacterial plates containing X-gal.

1. Thaw competent cells on wet ice.
2. Gently mix cells, then aliquot 100 μL of competent cells into chilled polypropylene tubes. Refreeze any unused cells in the dry ice/ethanol bath for 5 min before returning to the −70°C freezer.
3. For DNA from ligation reactions, dilute the reactions 5× TE buffer (provide with the Max Efficiency DH5α Competent Cells). Add 1 μL of the dilution to the cells (1–10 ng DNA), moving the pipette through the cells while dispensing. Gently tap tubes to mix.
4. Incubate cells on ice for 30 min.
5. Incubate the cells for 45 s in a 42°C water bath; do not shake.
6. Place on ice for 2 min.
7. Add 0.9 mL of Super Optimal Catabolite repression medium (SOC; Max Efficiency DH5α Competent Cells).
8. Shake vigorously at 37°C for 1 h.
9. Dilute the experimental reactions 1/1000, 1/100, 1/10 or no dilutions and spread 100–200 μL of this dilution on LB medium with 100 μg/mL ampicillin (LB-amp) and 40 μL 0.1 *M* IPTG and 40 μL X-Gal 20 mg/mL.
10. Incubate overnight at 37°C.
11. Analyze the presence, the number, and the color of the colonies grown on the plate for each different dilution.
12. Spread 100 μL of the optimal dilution per LB plate with 100 μg/mL ampicillin for the remaining experimental reactions.
13. Incubate overnight at 37°C.

3.4.2.3. Complementary DNA Amplification from Vector Inserts (PCR-Select Differential Screening Kit)

After growth of individual bacterial colonies, the unique presence of the insert is verified by PCR using the Nested Primer 1 and the Nested Primer 2R which have binding site on the adaptors localized at the end of subtracted cDNA inserts. The amplified cDNA is then used to be arrayed on nylon membrane.

1. Randomly pick 1,000 white bacterial colonies on LB plates.
2. Grow each colony in 100 μL of LB-amp medium at 37°C overnight with vigorous shaking.
3. Prepare a Master Mix for the clones to be amplified. Combine in a clean microcentrifuge tube (total volume 19 μL): 2.0 μL 10× PCR reaction buffer, 0.6 μL Nested Primer 1 (PCR-Select Differential Screening Kit), 0.6 μL Nested

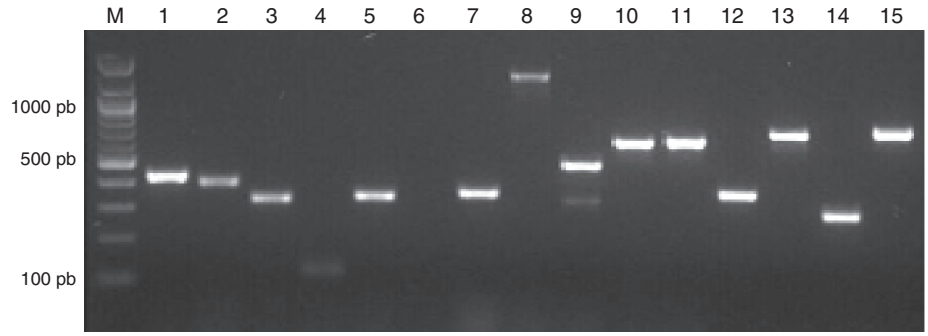


Fig. 2.18 Amplification of 15 embryonic subtracted cDNA inserts of cloning vector after transformation of MAX Efficiency DH5α Competent Cells using the Nested Primer 1 and Nester Primer 2R. Clone 6 is excluded from the analysis because no amplification product is detected as well as clone 9 because two different amplification products are detected. In the latter case, the original competent cell may have been transformed by two vectors containing an insert at the same time. Electrophoresis in a 2% agarose/EtBr gel, 1× TAE buffer (Lefèvre, 2006, unpublished).

Primer 2R (PCR-Select Differential Screening Kit), 0.4 μL dNTP mix (10 m*M*), 15.2 μL H_2O, 0.2 μL 50× PCR enzyme mix.
4. Mix well by vortexing and spin the tube for 5 s in a microcentrifuge.
5. Aliquot 19 μL of the Master Mix into each tube.
6. Transfer 1 μL of each bacterial culture to each tube.
7. Begin thermal cycling: 94°C 30 s and 23 cycles: 95°C 10 s, 68°C 3 min.
8. Electrophorese 5 μL from each reaction on a 2.0% agarose/EtBr gel in 1× TAE buffer. Each PCR product should correspond to the cDNA insert (*see* **Note 33** and **Fig. 2.18**).

3.4.3. Preparation of cDNA Dot Blots of the PCR Products

1. For each PCR reaction, combine 5 μL of PCR product and 5 μL of 0.6 N NaOH (freshly made or at least freshly diluted from concentrated stock) in a 96-well plate. NaOH will denature the DNA for hybridization.
2. Mix by briefly spinning the plate.
3. Using a micropipettor, transfer 1–2 μL of each mixture to a nylon membrane. Prepare four identical blots for hybridization with both subtracted and unsubtracted probes. For best results, array each cDNA in duplicate on each membrane.
4. Neutralize the blots for 2–4 min in 0.5 *M* Tris–HCl (pH 7.5) and wash in H_2O.
5. Cross-link the DNA to the membrane using a UV linking device under 120 mJ. Alternatively, bake the blots for 1–2 h at 70°C in an oven (*see* **Note 34**).

3.4.4. Random Primer Labeling of cDNA Probes

3.4.4.1. Purification of Secondary PCR Products (NucleoSpin Extract II Kit Provided with the PCR-Select Differential Screening Kit)

1. Add 34 μL of Buffer NT to each cDNA synthesis reaction (*obtained* **Section 3.4.1**); mix well by pipetting.
2. Place a NucleoSpin Extract II Column (NucleoSpin Extract II Kit) into a 2-mL collection tube. Pipet the sample into the column. Centrifuge at 11,200*g* for 1 min.
3. Discard the flow-through.
4. Return the column to the collection tube.
5. Add 600 mL of Wash Buffer NT3 (NucleoSpin Extract II Kit) to the column. Centrifuge at 11,200*g* for 1 min.
6. Discard the flow-through.
7. Place the column back into the collection tube. Centrifuge at 11,200*g* for 2 min to remove any residual Wash Buffer NT3.
8. Transfer the NucleoSpin Columns into a fresh 1.5-mL microcentrifuge tube.
9. Add 20 μL of sterile ultrapure H_2O to the column. Allow the column to stand for 2 min with the caps open.
10. Close the tube and centrifuge at 11,200*g* for 1 min to elute the sample. (Note 35)

3.4.4.2. Radiolabeling of cDNA Probes (PCR-Select Differential Screening Kit)

1. In separate 0.5-mL microcentrifuge tubes, mix 3 μL (20–90 ng) of each of the purified forward- and reverse-subtracted cDNAs and 3 μL of each of the unsubtracted tester and driver cDNAs with 6 μL of H_2O.
2. Denature by heating for 8 min at 95°C and then chill on ice.
3. Add to each tube (final volume 20 μL): 3 μL Reaction Buffer (–dCTP), 2 μL Random Primer Mix, 5 μL [α-^{32}P] dCTP (50 μCi, 3000 Ci/mmol, aqueous solution), 1 μL Klenow Enzyme (exo-) (PCR-Select Differential Screening Kit).
4. Incubate tubes at 37°C for 30 min.
5. Terminate each reaction by adding 5 μL of Stop Solution (provided with the PCR-Select Differential Screening Kit).
6. Purify probe from unincorporated dNTPs using Illustra™ MicroSpin Columns (GE Health Care).
   a. Resuspend the resin in the column by vortexing. Use columns immediately after preparation to avoid drying out of the resin.
   b. Loosen the cap one-quarter turn and snap off the bottom closure.
   c. Place the column in a collection tube.
   d. Pre-spin the column for 1 min at 735*g*.
   e. Remove the top cap.
   f. Transfer the column to a new clean microcentrifuge tube.

g. Slowly apply the sample to the centre of the resin bed. The resin will appear compacted and angled. Take care not to disturb the resin bed. Do not allow any of the sample to flow around the sides of the bed.
h. Spin the column for 2 min at 735*g*. Purified sample is collected in the bottom of the microcentrifuge tube.
i. Remove the spin column from the microcentrifuge tube and discard.

7. Determine the specific activity of each probe. More than 1×10^7 cpm per probe should be obtained.

3.4.5. Hybridization with the Subtracted cDNA

3.4.5.1. Membrane Preparation

1. Make hybridization solution for each membrane prepared and arrayed with forward subtracted cDNA:
   a. Combine 50 μL of 20× SSC and 50 μL of Blocking Solution.
   b. Mix well.
   c. Boil for 5 min and chill on ice.
   d. Combine with 5 mL of hybridization solution.
2. Place each membrane in a hybridization container and add the hybridization solution.
3. Prehybridize for 40–60 min with continuous agitation at 72°C. Continuous agitation of the membranes in a hybridization incubator with rotating bottles is necessary during all prehybridization, hybridization, and washing steps.

3.4.5.2. Preparation of the Hybridization Probes

1. Mix 50 μL of 20× SSC, 50 μL of Blocking Solution and your purified probe (at least 1×10^7 cpm per 100 ng of subtracted cDNA). Be sure to add the same number of cpm for each pair of probes.
2. Boil for 5 min and then chill on ice.
3. To each hybridization container, add the probes. Avoid adding the probe directly into the membrane.
4. Hybridize at 72°C overnight with continuous agitation.
5. Warm low-stringency (2× SSC/0.5% SDS) and high-stringency (0.2× SSC/0.5% SDS) washing solutions to 68°C. Keep buffers at 68°C during washing.
6. Wash membranes with low-stringency washing solution (4×20 min) at 68°C.
7. Wash membranes with high-stringency washing solution (2×20 min) at 68°C.
8. Seal up each membrane in a plastic envelop.

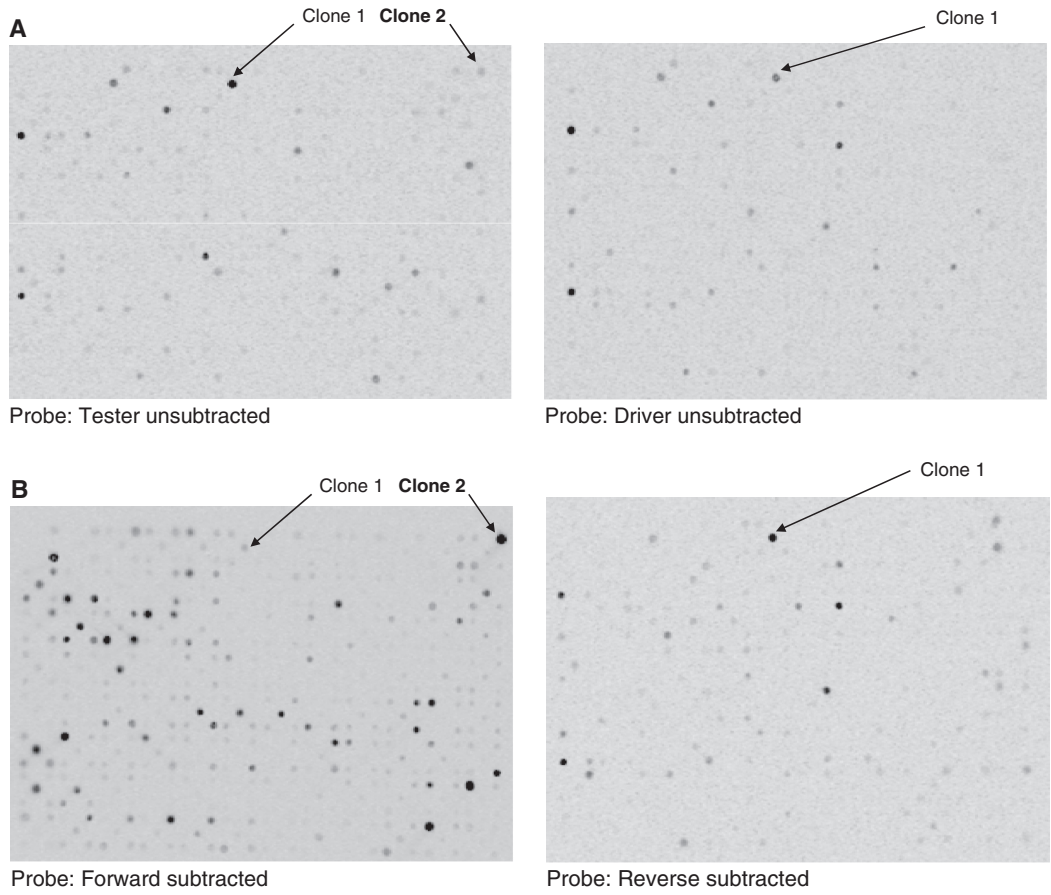


Fig. 2.19 Sample differential screening results. **Panel A**. *Dot blots* hybridized with unsubtracted cDNA probes made from tester (mink uterus after reactivation) and driver (mink uterus during diapause) RNAs. **Panel B**. *Dot blots* hybridized with cDNA probes made from forward-subtracted cDNA (mink uterus after reactivation as tester, mink uterus in diapause as driver) and reverse-subtracted cDNA (mink uterus in diapause as tester, mink uterus after reactivation as driver). As an example, a radioactive signal is detected for clone 2 only on the dot blots hybridized with unsubtracted cDNA probes made from tester and on dot blots hybridized with cDNA probes made from forward-subtracted cDNA. Consequently, clone 2 is a positive clone and is selected for the sequencing step. On the contrary, clone 1 is detected on the four dot blots hybridized with either both unsubtracted cDNA probes and both subtracted cDNA probes. As a false positive clone, clone 1 is removed from the library for the analysis (Lefèvre, 2006, unpublished).

9. Scan the membrane using a Phosphoimager-Storm system to detect the radioactive signal and quantify each signal intensity using ImageQuant Software (Applied Biosystem) (**Fig. 2.19**).
10. Analyze data and remove false positive clones (**Fig. 2.20**).
11. The membranes can be stored at −20°C or be reused: remove the probes by stripping (100°C, 7 min in 0.5% SDS). Blots can typically be probed at least five times.

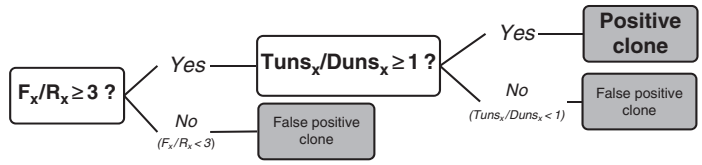


Fig. 2.20 Analysis of the differential screening *dot blots* after quantification of the signal's intensity for each blot by ImageQuant (Applied Biosystem). F_x, R_x, $Tuns_x$, and $Duns_x$ correspond to the radioactive signal's intensity measured with ImageQuant software (Applied Biosystem) for one clone *x* hybridized, respectively, with subtracted-forward cDNA probe (F_x), with subtracted-reverse cDNA probe (R_x), with unsubtracted tester cDNA probe ($Tuns_x$), and with unsubtracted driver cDNA probe ($Duns_x$). The procedure to determine whether a clone is positive or false positive is represented in the figure above and is the one recommended by the manufacturer (PCR-Select Differential cDNA Kit, Clontech). As the unsubtracted tester/driver cDNA probe do not hybridize with cDNA sequences that are less represented than 0.2% in the whole population of cDNA, the ratio F_x/R_x is analyzed before the ratio $Tuns_x/Duns_x$.

3.5. Differentially Expressed cDNA Sequence Analysis

3.5.1. Sequencing

Bacterial colonies from positive clones must be freshly grown on LB plates before the cDNA insert contained in the vector is extracted and then sent for sequencing. The primer used for the sequencing procedure can be either the Nested Primer 1 and Nested Primer 2R which are localized on the cDNA insert itself (on the adaptors, **Table 2.2**) or primers like M13, Sp6 which have hybridization sites on the pCR II vector sequence in the cloning site.

3.5.2. Sequences Annotation

3.5.2.1. Sequences Comparison with Sequences Listed in Genbank Database

The differentially expressed cDNA sequences are annotated by comparison with some sequences that are already listed in the Genbank database available on the Internet network. A classification can be established to describe the proportion of sequences that has a high degree of homology with known and characterized or uncharacterized sequences already listed in the Genbank database and the proportion of new sequences that have no significant degree of homology with already listed sequences. To characterize the degree of homology, the percentage of identity between two aligned sequences, the length of homologous sequences, and the *E*-value which reflects the probability to find a given alignment among all the possible alignments with all the listed sequences of the Genbank are taken into account.

3.5.2.2. Gene Ontology

Based on sequences that are homologous to known genes, a second classification of the gene ontology may be drawn up. Multiple softwares are available on the Internet network and provide free gene ontology analysis. This software may also identify signaling pathways involving some of the genes contained in the constructed library. That approach provides a global gene expression analysis.

3.5.3. Selection of Candidate Genes

Finally, a "one by one" analysis of each known genes expected to be of significance based on expression patterns from the literature leads to the selection of candidate genes. Clones highly represented in the library, whether known or unknown sequences, may also be selected.

3.5.4. Identification of Candidate Genes

Once a list of candidate genes have been selected, validation of the differentially expression pattern of those genes must be undertaken. Informative techniques can be applied to define the expression pattern of any single gene. Among the most reliable are real-time PCR or semi-quantitative PCR for gene expression quantification and in situ hybridization of mRNA or immunocytochemical localization of protein gene products to establish the spatial gene expression pattern.

4. Notes

1. If embedding samples in paraffin in a few days following the tissue collection, they can be held in PBS 1× at 4°C, after the three rinses.
2. It is essential to use the correct amount of starting material in order to obtain optimal RNA yield and purity. A maximum amount of 5 mg fresh or frozen tissue can generally be processed. Weighing tissue is the most accurate way to quantitate the amount of starting material. As a guide, a 1.5-mm cube (3.4 mm^3) of most animal tissues weighs 3.5–4.5 mg.
3. To avoid damage to the TissueRuptor and disposable probe during operation, make sure the tip of the probe remains submerged in the buffer. Foaming may occur during homogenization. If this happens, let the homogenate stand at room temperature for 2–3 min until the foam subsides before continuing with the procedure.
4. The volume of 70% ethanol to be added may be less if some lysate was lost during homogenization. Precipitates may be visible after the addition of ethanol, but this does not affect the procedure.

5. After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through.
6. As little as 10 μL RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%.
7. The concentration of RNA should be determined by measuring the absorbance at 260 nm ($A260$) in a spectrophotometer. The ratio of the readings at 260 nm and 280 nm ($A260/A280$) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. Do not use more than 1 mg total RNA.
8. The initial volume of the RNA solution is not important as long as the volume can be brought up to the indicated amount with RNase-free water.
9. Loss of the Oligotex resin can be avoided if approximately 50 μL of the supernatant is left in the microcentrifuge tube. The remaining solution will not affect the procedure. Save the supernatant until certain that satisfactory binding and elution of poly A+ mRNA has occurred.
10. The volume of Buffer OEB used depends on the expected or desired concentration of poly A+ mRNA. Ensure that Buffer OEB does not cool significantly during handling. With multiple samples, it may be necessary to place the entire microcentrifuge tube (with spin column, Oligotex, and sample) into a 70°C heating block to maintain the temperature while preparing the next samples.
11. To keep the elution volume low, the first eluate may be used for a second elution. Reheat the eluate to 70°C and elute in the same microcentrifuge tube.
12. The minimum amount of starting material for standard cDNA synthesis is 2 ng of total RNA. However, if the RNA sample is not limiting, it is recommended to begin with 20–1,000 ng of total RNA for cDNA synthesis. Please note that if there is >100 ng of total RNA, dilute first-strand cDNA product before proceeding with cDNA amplification.
13. For the control synthesis, add 10 ng of control human placenta total RNA.
14. If necessary, cDNA can be stored at −20°C until ready to proceed with column chromatography.
15. Samples can also be stored at −20°C for up to 3 months.
16. Choosing the optimal number of PCR cycles ensures that the ds cDNA will remain in the exponential phase of amplification. When the yield of PCR products stops increasing with more cycles, the reaction has reached its plateau. The optimal number of cycles for an experiment is one cycle fewer than is needed to reach the plateau. **Figure 2.9** shows a typical gel profile of ds cDNA synthesized using the Con-

trol Human Placenta Total RNA for SMART cDNA synthesis and amplification *(17)*. The PCR reached its plateau after 24 cycles for the 5-ng experiment and 21 cycles for the 20-ng experiment; that is, the yield of PCR products stopped increasing. After 24 and 21 cycles, a smear appeared in the high molecular weight region of the gel, indicating that the reactions were overcycled. Therefore, the optimal number of cycles would be 23 for the 5-ng experiment and 20 for the 20-ng experiment. Note that the number and position of the bands and the size of the smear observed will be different for each particular total RNA used.

17. Butanol extraction allows concentration of the PCR product to a volume of 40–70 μL. Addition of too much *n*-butanol may remove all the water and precipitate the nucleic acid. If this happens, add water to the tube and vortex until an aqueous phase reappears.
18. If volume is less than 40–70 μL, repeat the last three steps: Add 700 μL of *n*-butanol and vortex the mix thoroughly. Centrifuge the solution at room temperature at 11,200*g* for 1 min and remove and discard the upper (*n*-butanol organic) phase. If final volume is <40 μL after the second butanol concentration step, add H_2O to the aqueous phase to adjust volume to 40–70 μL.
19. The top of the column matrix should be at the 0.75 mL mark on the wall of the column. If the column contains much less matrix, discard it and use another column.
20. To analyze the ds cDNA after column chromatography, electrophorese 3 μL of the unpurified PCR product (Sample A) alongside 10 μL of the PCR product purified by column chromatography (Sample B) and 10 μL of the second fraction (Sample C) on a 1.2% agarose/EtBr gel. Compare the intensities of Sample A and Sample B and estimate the percentage of PCR product that remains after column chromatography. The yield of cDNA after column chromatography is typically 50%. If the yield is <30%, check to see if it is present in the second fraction, Sample C. If this second fraction has a higher yield of cDNA than the first, combine the fractions. Otherwise if the cDNA is not present in Sample C, repeat the PCR and column chromatography steps.
21. Electrophorese 2.5 μL of undigested ds cDNA and 5 μL of *Rsa*I-digested cDNA on a 1% agarose/EtBr gel in 1× TAE buffer side-by-side. Double-strand cDNA appears as a smear from 0.5 to 10 kb. Bright bands correspond to abundant mRNAs or rRNAs. After *Rsa*I digestion, the average cDNA size is smaller (0.1–2 kb compared to 0.5–10 kb). Typical results are shown in **Fig. 2.10**.

22. For each reaction, 1–3 μg of SMART cDNA is obtained after purification. If the yield is lower than this, perform the agarose/EtBr gel analysis: electrophorese 10 μL of *Rsa*I-digested cDNA before purification (Sample E) alongside 10 μL of purified diluted cDNA before ethanol precipitation (Sample F) and 1.8 μL of purified diluted cDNA after ethanol precipitation (Sample G) on a 1.5% agarose/EtBr gel. Compare the intensities of the samples and estimate what percentage of *Rsa*I-digested PCR product remains after purification and ethanol precipitation. The yield of cDNA after purification using the NucleoTrap PCR Kit and ethanol precipitation is typically 70%.
23. For the control synthesis, add 2 μL of the skeletal muscle control poly A+ RNA.
24. These 5.5-μL samples of *Rsa*I-digested cDNA will serve as the experimental driver cDNA and the control skeletal muscle driver cDNA.
25. This is the control skeletal muscle tester cDNA. It contains 0.2% *Hae*III-digested φX174 DNA; each fragment corresponds to about 0.02% of the total cDNA. After subtraction of the skeletal muscle tester cDNA against the skeletal muscle driver cDNA, the primary bands produced in the final PCR should correspond to these control fragments.
26. For the rest of the PCR-Select protocol, the control human placenta cDNA should be analyzed in parallel with the control skeletal muscle cDNA.
27. The ATP required for ligation is a component of the T4 DNA Ligase mix (3 m*M* initial, 300 μ*M* final).
28. Typical results for the ligation efficiency test are shown in **Fig. 2.13**. If no products are detected after 20 cycles, perform 5 additional cycles and analyze by gel electrophoresis. The PCR product using one gene-specific primer (G3PDH 3′ Primer) and PCR Primer 1 should be about the same intensity as the PCR product amplified using two gene-specific primers (G3PDH 3′ and 5′ Primers). If the band intensity for these PCR products differs by more than 4-fold, the ligation was less than 25% complete and will significantly reduce subtraction efficiency. If working with mouse or rat cDNA, the PCR product amplified using the G3PDH 3′ Primer and PCR Primer 1 will be ~1.2 kb instead of 0.75 kb for human cDNA.
29. Primary PCR: With the PCR control subtracted cDNA, the major bands appearing after 27 cycles should correspond to the φX174 DNA-*Hae*III fragments. This result should look similar to the performed skeletal muscle subtraction. The experimental primary PCR subtraction products usually appear as a smear from 0.2 to 2 kb, with or without some distinct bands (**Fig. 2.14**). Secondary PCR: The

patterns of secondary PCR products from the PCR Control Subtracted cDNA and from the skeletal muscle subtraction should resemble lanes 1, 4, and 7 in **Fig. 2.14** and lane 1 in **Fig. 2.15**. A few additional bands may appear. The experimental subtracted samples usually appear as smears with or without a number of distinct bands (**Fig. 2.14**).

30. Not all housekeeping gene transcripts are subtracted evenly. In certain instances a particular housekeeping gene is present at different levels in tester and driver poly A+ RNA. If the concentration of G3PDH message is even 2-fold higher in the tester sample, G3PDH will not be efficiently subtracted out. If subtraction is performed in both directions and there is unsubtracted tester control for both the subtraction and the reverse subtraction, the PCR analysis of subtraction efficiency will indicate if there is any difference in G3PDH abundance in the two cDNA samples being compared. Moreover, G3PDH is not efficiently subtracted in skeletal muscle cDNA, which is the positive control of the subtraction. Therefore, we used cyclophilin as a housekeeping gene to validate the subtraction of the skeletal muscle cDNA. However, in the control skeletal muscle subtraction experiment, the agarose gel banding pattern of the X174 DNA-*Hae*III/digest has already indicated whether or not subtraction was successful (**Figs. 2.11** and **2.12**).
31. **Figure 2.16** shows an example of G3PDH and cyclophilin reduction in successfully subtracted mixtures of cDNA from positive control of the subtraction and from reactivated uterus versus uterus in diapause subtraction in the mink. The difference in the number of cycles required for equal amplification of the corresponding PCR product in subtracted and unsubtracted samples indicates the efficiency of the subtraction. For the unsubtracted cDNA, the housekeeping gene product is seen after 18–23 cycles, depending on its abundance in the particular cDNA. With the subtracted samples, a product should be detected at ~5–15 cycles later.

    As a positive control for the enrichment of differentially expressed genes, repeat the procedure above using PCR primers for a gene known to be expressed in the tester RNA, but not in the driver RNA. This cDNA should become enriched during subtraction. The difference in the number of cycles required for equal amplification of the corresponding PCR product in subtracted and unsubtracted samples indicates the efficiency of the subtraction. Five cycles correspond roughly to a 20-fold cDNA enrichment. Because of the equalization that occurs during subtraction, the level of enrichment will depend on the

initial abundance of each differentially expressed gene, as well as the difference in abundance of each gene in tester and driver. Differentially expressed genes that are present in low abundance in the tester cDNA will be enriched more than differentially expressed genes that are present in high abundance.

32. For optimal ligation efficiencies, using fresh (less than 1-day-old) PCR products is recommended. The single 3′ A-overhangs on the PCR products will be degraded over time, reducing ligation efficiency. Take care when handling the pCRII vector as loss of the 3′ T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency.
33. **Figure 2.18** shows an example of cDNA amplification of insert contained in vectors after MAX Efficiency DH5α Competent Cells transformation. While the amplification generates PCR product to be arrayed on membrane for the differential screening, it also provides an elimination step of false positive clones, i.e., bacteria colonies that may have either lost the vector or been transformed by more than one vector.
34. Both techniques can be proceeded to insure an efficient cross-link of the cDNA on the membrane. First cross-link the DNA to the membrane using a UV linking device under 120 mJ and then bake the blots for 1–2 h at 70°C in an oven.
35. Yield of larger fragments (>5–10 kb) can be increased by using prewarmed elution buffer (70°C): For elution, add prewarmed elution buffer and incubate at room temperature for 1 min before collecting.

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