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Subtractive Hybridization for the Isolation of Differentially Expressed Genes Using Magnetic Beads

Hans-Christian Aasheim, Ton Logtenberg, and Frank Larsen

1. Introduction

Subtractive hybridization methods provide a means to isolate genes that are specifically expressed in a cell type or tissue *(1–3)*, genes that are differentially regulated during activation or differentiation of cells *(4–7)*, or genes that are involved in pathological conditions such as cancer *(8–10)*. Such methods are suitable for the isolation of low-expression genes. The principle of these approaches is to remove the mRNA species common to different cell types or tissues by subtraction, leaving the cell type/tissue-specific mRNAs for further manipulation and analysis. Alternatively, mRNA from both the subtractor and target population can be reverse transcribed and amplified by PCR followed by subtractive hybridization and isolation of specific sequences *(9)*. In the last 20 yr, several subtractive strategies have been described *(2–5,9)*, but subtraction hybridization/cloning still remains a technically demanding, time-consuming, and labor-intensive technology, including the need for large amounts of mRNA or highly purified single-stranded DNA. Several subtractive hybridization strategies based on solid-phase hybridization on magnetic Dynabeads have previously been described *(11–14)*. In this chapter, we present one such solid-phase strategy developed by us *(12)* that is an improved version of an already published method *(12)*. Our approach takes advantage of the properties of magnetic Dynabeads allowing simple and rapid buffer changes required for optimal hybridization and enzymatic reactions. The main principles of the method presented in this chapter are outlined in Fig. 1. Purified mRNA from the subtractor cell or tissue population is isolated using magnetic Dynabeads

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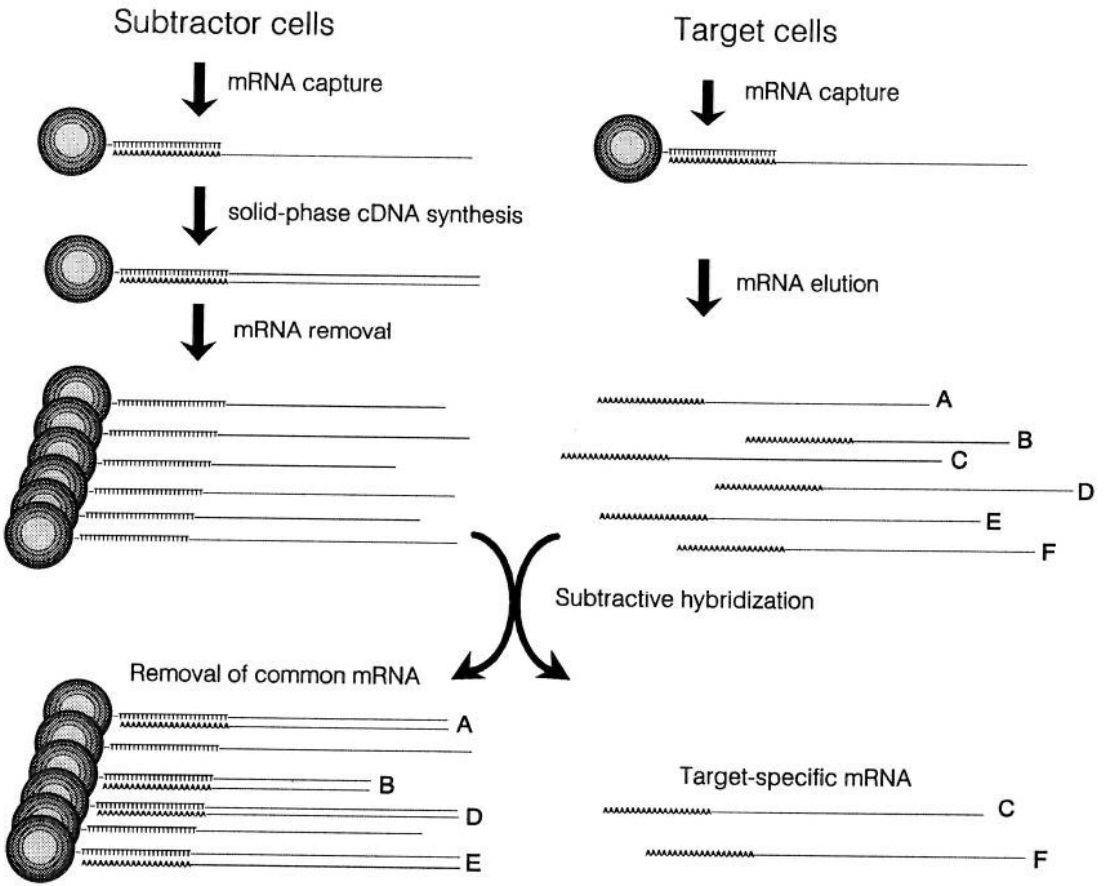
Subtractor cells
mRNA capture
solid-phase cDNA synthesis
mRNA removal
Target cells
mRNA capture
mRNA elution
A
B
C
D
E
F
Subtractive hybridization
Removal of common mRNA
A
B
D
E
Target-specific mRNA
C
F

Fig. 1.

oligo(dT)25, and directly converted to immobilized first-strand cDNA (subtractor beads). mRNA from the target cell/tissue population is also isolated using Dynabeads oligo(dT)25 beads, eluted from the beads, dissolved in hybridization buffer, and mixed with subtractor beads. The target mRNA population is hybridized to the cDNA subtractor beads at 68°C for 20–24 h. Two additional hybridization rounds, using the same subtractor beads, are recommended to ensure optimal subtraction and enrichment of cell-specific sequences. After each hybridization step, the beads are regenerated by elution of the mRNA from the first-strand cDNA. After the final hybridization step, the specific mRNA left in the solution is captured with oligo(dT)25 beads and converted to a radioactive cDNA probe for the screening of cDNA libraries. Alternatively, this material can be used for the generation of a subtractive cDNA library or can be used as a source for the amplification of members of known gene families using degenerated primers directed against the conserved areas. The latter approach facilitates a directed search for differentially expressed genes. In our hands, this system provides a fast and reliable way of generating subtractive probes for the isolation of cell/tissue-specific genes. An additional advantage of this method is that the subtractor beads can readily be regenerated and used for at least three different subtractions, each involving three hybridization steps.

2. Materials

2.1. mRNA Isolation

1. Sigmacote (Sigma, St Louis, MO, cat. no. SL-2).
2. Magnetic particle concentrator (MPC-E) (Dynal, Oslo, Norway)
3. Dynabeads oligo(dT)25 (Dynal)
4. Phosphate-buffered saline (PBS), pH 7.4. 137 m*M* NaCl, 2 7 m*M* KCl, 4 3 m*M* Na_2HPO_4 $7H_2O$, 1.4 m*M* KH_2PO_4.
5. TE buffer, pH 8.0: 10 m*M* Tris-HCl, pH 8 0, 1 m*M* EDTA, pH 8.0.
6. Lysis buffer: PBS with 0.5% Nonidet-P40 (Sigma).
7. 2X Lysis/binding buffer. 100 m*M* Tris-HCl, pH 8 0, 500 m*M* LiCl, 10 m*M* EDTA, pH 8.0, 0.2% sodium dodecyl sulfate (SDS), 5 m*M* dithiothretiol (DTT).
8. Wash buffer 1: 10 m*M* Tris-HCl, pH 7.5, 0.15*M* LiCl, 1 m*M* EDTA, 0 1% SDS
9. Wash buffer 2: 10 m*M* Tris-HCl, pH 7.5, 0.15*M* NaCl.
10. Avian Myeloblastosis Virus (AMV) reverse transcriptase buffer· 50 m*M* Tris-HCl, pH 8.5 (20°C), 8 m*M* $MgCl_2$, 30 m*M* KCl, 1 m*M* DTT.

Fig. 1. *(previous page)* Subtractive hybridization. Schematic presentation of the method. mRNA is isolated from the subtractor cells and converted to solid-phase first-strand cDNA. mRNA is isolated from the target cells and hybridized to the subtractor beads. After hybridization, the subtractor beads with bound target mRNA are collected with a magnet, and the target-specific mRNAs are left in the supernatant. The target-specific mRNAs can be converted to radioactive cDNA probes for the screening of cDNA libraries.

2.2. First-Strand cDNA Synthesis

1. 5X AMV reverse transcriptase buffer: 250 m*M* Tris-HCl, pH 8.5 (20°C), 40 m*M* $MgCl_2$, 150 m*M* KCl, 5 m*M* DTT
2. AMV reverse transcriptase (Boehringer Mannheim, Mannheim, Germany)
3. Thermus thermophilus (rTth) heat-stable polymerase (Perkin-Elmer, Branchburg, NJ, or Epicentre Technology, Madison, WI).
4. RNasin (Promega, Madison, WI).
5. First-strand cDNA synthesis reaction mix: 20 µL 5X first-strand buffer, 10 µL 10 m*M* dNTP, 100 U AMV reverse transcriptase (25 U/µL, Boehringer Mannheim) and H_2O to 100 µL.

2.3. Subtractive Hybridization

1. 20X SSPE 3*M* NaCl, 0.15*M* NaH_2PO_4, 0.02*M* EDTA, pH 7.4
2. Hybridization buffer: 4 5X SSPE, 0.2% SDS
3. Loading buffer: 0.25% bromophenol blue, 30% glycerol, 40 m*M* EDTA

2.4. Generation of a Subtractive Probe

2.4.1. Direct Labeling

1. cDNA synthesis kit (Promega or Amersham, Buckinghamshire, UK).
2. [α^{32}P]-dCTP (e.g., Amersham, cat. no. PB 165)

2.4.2. Amplification

1. Terminal deoxynucleotide transferase (TdT)
2. 5X TdT buffer: 0.5*M* potassium cacodylate, pH 7.2, 10 m*M* $CoCl_2$, 1 m*M* DTT
3. *Taq* polymerase mixture: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 100 µg/mL bovine serum albumin, 0.05% Triton X-100, 2.5 m*M* $MgCl_2$, 0.2 m*M* dNTPs, 2.5 U *Taq* polymerase (Perkin-Elmer)
4. T-primer: 5'-dTTGCATTGACGTCGACTATCCAGGTTTTTTTTTTTTTT-3'
5. L-primer: 5'-dTTGCATTGACGTCGACTATCCAGG-3'

3. Methods

In this section, we present a detailed description of the subtraction method starting with the isolation of mRNA from subtractor cells and the generation of a solid-phase bound subtractor cDNA library, followed by the subtractive hybridization and the generation of subtracted probes (*see* Notes 1, 2, and 3).

3.1. Preparation of Immobilized First-Strand Subtractor cDNA

Construction of a subtracted cDNA library requires high-quality mRNA from the tissue or cells of interest. The method described here is rapid and minimizes the risk of degradation of nucleic acid. mRNA can be isolated directly from crude lysate obviating the need first to isolate total RNA *(15)*.

The average yield of polyadenylated mRNA per gram of solid tissue is reported to vary between 200 and 400 μg (*see* Note 4). In this chapter, we present as an example the subtraction between cells of lymphoid origin.

3.1.1. Isolation of Subtractor mRNA (see Note 5)

1. Collect 50 million lymphoid cells (cell line) by centrifugation, and wash once in 10-mL cold RNase-free PBS
2. Lyse in 0.5 mL cold PBS with 0.5% NP-40 (lysis buffer), transfer to a microcentrifuge tube, and keep on ice for 2 min.
3. Spin the lysate for 2 min at maximum speed (10,000*g*) in a cold microcentrifuge to pellet the nuclei and transfer the supernatant to a new microfuge tube on ice, leaving the nuclei behind. (Successful lysis leaves a viscous pellet that cannot be resuspended.)
4. Wash 5 mg (1 mL) of Dynabeads oligo(dT)25 once in 0.5 mL cold 2X lysis/binding buffer, resuspend the beads in 0.5 mL of the same buffer, and combine with the cell lysate without nuclei
5. Mix the beads and lysate for 5 min at 4°C to achieve hybridization of polyadenylated mRNA to the oligo(dT) on the beads The incubation can be performed on ice, including mixing the tube twice during the incubation, or alternatively, we prefer to perform the incubation on a rolling wheel at 4°C to obtain optimal mixing.
6. After binding of mRNA, wash the beads twice in 0.5–1 mL wash buffer 1 and then twice with wash buffer 2. Transfer the suspension to a new RNase-free tube between the two last washing steps.
7. Finally, wash the mRNA/Dynabead complexes once in reverse transcriptase buffer or three times if 1% detergent was used in the lysis buffer. The extensive washing is important to remove all traces of LiCl and detergent, which will inhibit subsequent enzymatic reactions. The yield of subtractor mRNA should be approx 10 μg (*see* Note 6)

3.1.2. Solid-Phase First-Strand cDNA Synthesis

The oligo(dT) sequences covalently bound the to bead surfaces are used both to capture the polyadenylated mRNA and as primers for a reverse transcriptase reaction to synthesize the first-strand cDNA. This results in a covalently immobilized first-strand cDNA library. The mRNA bound to the oligo-dT Dynabeads is directly subjected to first-strand cDNA synthesis.

1. Remove the last washing buffer and resuspend the beads from Section 3.1.1 in 100 μL of a first-strand cDNA reaction solution composed of: 20 μL 5X first-strand buffer (Boehringer Mannheim), 10 μL 10 m*M* dNTP, 100 U AMV reverse transcriptase (Boehringer Mannheim), RNase inhibitor (RNasin, Promega), and H_2O to 100 μL
2. Place the microfuge tube in a horizontal position in an incubator with a rotating wheel at 42°C, and incubate for 1 h to ensure efficient enzymatic reactions.

3 After incubation at 42°C, add 5 µL of 10 m*M* $MnCl_2$ and 2.5 U rTth heat-stable polymerase to the mixture, and incubate for 10 min at 70°C (*see* Note 7).
4 After completion of the first-strand cDNA synthesis, wash the subtractor beads carefully twice in 500 µL TE (pH 8.0). The subtractor beads can then be stored in TE (pH 8.0) at 4°C for at least 1 mo (*see* Note 8).
5 Before use in hybridization procedures, resuspend the subtractor beads in 100 µL H_2O, and incubate at 70°C for 5 min, and then remove the H_2O to ensure removal of subtractor mRNA. Do not let the beads dry out, but store them in TE or hybridization buffer

3.2. mRNA Isolation from Target Cells (see Note 9)

1. Collect approx 2 million cells (lymphoid cell lines), and wash once in ice-cold PBS.
2 Lyse cells in 100 µL PBS containing 0 5% NP-40 for 2 min on ice.
3. Remove the nuclei by centrifugation at 10,000*g* in a cold microcentrifuge for 2 min
4 Prewash 250 µg (50 µL) of Dynabeads oligo(dT)25 in 200 µL 2X lysis/binding buffer, resuspend the beads in 100 µL of the same buffer, and combine with the cell lysate without nuclei. Mix the beads with the lysate by constant rolling at 4°C for 5 min as described in Section 3.1.1
5. After binding, wash the mRNA/Dynabead complexes twice in 200 µL wash buffer 1 It is not necessary here to perform the extensive wash step described under Section 3.1.1 , since the target mRNA is not subjected to any enzymatic reactions at this stage
6. Elute the mRNA from the beads in 30 µL H_2O for 5 min at 70°C The beads can be immediately collected by a magnet
7. Transfer the mRNA-containing supernatant to a new microcentrifuge tube on ice, and prepare for the subtraction by adding 400 µL hybridization buffer. The final concentration of the hybridization buffer should be 4.5X SSPE and 0 1% SDS (*see* Note 10)

3.3. Subtractive Hybridization (see Note 11)

To prevent evaporation, we recommend to use microcentrifuge tubes with screw caps for subtractive hybridization. As described, isolated mRNA from the target population is dissolved in 400 µL hybridization buffer (4.5X SSPE, 0.1% SDS).

1. Heat both the subtractor cDNA beads in TE and the target mRNA in hybridization buffer simultaneously to 70°C for 3 min
2. Remove the TE from the subtractor beads by capture of the beads with a magnet, and resuspend the beads in the hybridization buffer containing the target mRNA. Seal the cap of the microtube in Parafilm™ to prevent evaporation, and incubate the hybridization mixture at 68°C for 20–24 h under constant rolling (upside down) to achieve even and proper mixing of the subtractor beads with target mRNA.
3. After the first round of hybridization, transfer the tube from the 68°C incubator directly to ice, and collect the mRNA/cDNA bead complex using a magnet on ice

4 Transfer the hybridization solution containing target-specific mRNA to a new tube, and store on ice, awaiting further rounds of hybridization.
5 Regenerate the subtractor beads by adding 25 µL H_2O followed by 5 min heating at 70°C (elution of mRNA). After removing the H_2O, resuspend the beads in TE.
6 After elution of mRNA, resuspend the subtractor beads in 100 µL TE ready for further rounds of hybridization.
7. Heat the subtractor beads and the hybridization solution separately to 70°C as in step 1, and resuspend the beads in the hybridization solution containing target mRNA
8. Perform the hybridization as described in steps 1–6
9. Repeat the process once more—in total, three rounds of hybridization
10. After the last hybridization step, remove the subtractor beads from the hybridization solution by a magnet. The supernatant contains the cell-specific mRNA from the target population
11 Isolate the specific target mRNA by adding to the hybridization solution 25 µL of Dynabeads oligo(dT)25, prewashed in hybridization buffer, followed by a 5-min incubation at 25°C (room temperature) preferably on a rotating wheel.
12. Wash the oligo(dT) beads with bound specific mRNA twice with wash buffer 1 and then twice with wash buffer 2 as described in Section 3 1.1 before resuspending the beads in first-strand cDNA buffer.
13. Keep the beads on ice awaiting further processing.

3.4. Generation of Subtractive Probes

Subtractive cDNA probes can be generated for screening cDNA libraries from the specific target mRNA. Here we present two different approaches. One approach directly generates a ^{32}P-labeled double-stranded cDNA from the target-specific mRNA using high amounts of radioactivity. The second approach employs the polymerase chain reaction *(16)* followed by radioactive labeling of the material.

3.4.1. Direct Labeling of Target-Specific mRNA (see Note 14)

1. Bind the specific mRNA obtained after the final hybridization to oligo-dT beads as described in Section 3.3.
2. Elute the mRNA from the beads by incubation in 15 µL H_2O for 3 min at 65°C. The mRNA is now ready for the cDNA synthesis reaction.
3. Convert the specific mRNA to a double-stranded radiolabeled cDNA probe according to a cDNA synthesis kit protocol (e.g., Promega or Amersham) or other cDNA synthesis protocols *(17)*, except that the final concentration of dCTP in the first-strand cDNA reaction is 0.25 m*M* instead of 1 m*M*, whereas 1 mCi of [α^{32}P]dCTP (3000 Ci/m*M*; Amersham) is included in the reaction mixture. The radioisotope is shipped in EtOH and must be dried prior to use
4. After the second-strand cDNA synthesis is finished, remove free [α^{32}P]dCTP from the synthesis mixture by spinning through a Sephadex G-50 column together with 2 µg of sheared salmon sperm DNA (to avoid loss of material in the column owing to the low amount of cDNA).

5 The radiolabeled cDNA is now, after boiling, ready for use as a probe for the screening of existing cDNA libraries either in the form of plasmids (colony screening) or phages (plaque screening) according to standard protocols *(17,18)*

3.4.2. Amplification of Target-Specific mRNA (see Note 15)

1 Prewash 25 µL oligo-dT Dynabeads in hybridization buffer, and add to the mRNA in hybridization buffer from Section 3 3 , step 11
2 Incubate at room temperature (25°C) for 5 min preferably on a rolling wheel
3 After extensive washing of the mRNA/bead complexes (Section 3.1 2), generate immobilized first-strand cDNA by adding first-strand cDNA synthesis mix 5 µL 5X reverse transcriptase buffer, 2 5 µL 10 m*M* dNTP, 15 U reverse transcriptase (Boehringer Mannheim), RNase inhibitor, and H_2O to a final volume of 25 µL Incubate at 42°C for 1 h under constant rolling
4. After completion of the cDNA synthesis, wash the cDNA/bead complexes twice in TE
5 Take the beads up in 20 µL H_2O, and incubate at 70°C for 5 min to elute the mRNA
6 Collect the first-strand cDNA beads using the magnet, and wash once in TE
7. To tail the cDNA with terminal transferase and dATP, set up a 20-µL reaction containing the immobilized first-strand cDNA with 22 U terminal transferase (Gibco), 1.5 m*M* dATP and terminal transferase buffer. Incubate for 15 min at 37°C, and then stop with the addition of 2 µL 0.5*M* EDTA
8 Wash the beads once in TE before adding 50 µL of *Taq* polymerase mix containing 29 pmol of T-primer. Extend the primer for 15 min at 30°C, 15 min at 40°C, and 15 min at 72°C in an incubator under constant rolling (tube in horizontal position).
9. Discard the supernatant, and add 50 µL of fresh *Taq* polymerase mix containing 50 pmol of L-primer and 1 pmol of T-primer, and heat at 94°C for 2 min to release the second-strand cDNA
10 Transfer the supernatant to a new tube, and add a drop of mineral oil (when thermal cyclers with heated lids are used, mineral oil can be omitted) (*see* Note 16)
11 Incubate the supernatant for 15 min at 30°C, 15 min at 40°C, and 15 min at 72°C to extend the T-primer.
12 After 2 min at 94°C, carry out 15 cycles of PCR amplification (94°C for 1 min, 72°C for 5 min) followed by a 10-min extension at 72°C.
13 Reamplify 5 µL of this reaction for 20 additional cycles (94°C for 1 min, 72°C for 5 min) in *Taq* polymerase mix containing 50 pmol of L-primer
14. Spin the product through a Sephacryl-400 spin column (Pharmacia, Uppsala, Sweden) to remove nucleotides, perform a phenol/chloroform extraction, and precipitate with $^1/_{10}$ vol 5*M* NaCl and 2.5 vol ethanol

The material is now ready for radioactive labeling either by nick translation or by random hexamer labeling using $^1/_5$ for a single-labeling reaction. The probe is boiled and used for screening cDNA libraries according to standard protocols *(17,18)*. A discussion of the applications of these techniques is given in Note 17.

4. Notes

1. To isolate genes that are differentially expressed successfully, it is important to follow the protocol in detail It is also important that all solutions are RNase-free and that the isolation procedure of mRNA works well to obtain high-quality mRNA.
2. In general, Dynabeads with immobilized mRNA or cDNA should be handled with care to avoid shearing of the nucleic acids. Always resuspend the beads carefully by pipeting slowly up and down.
3. Microcentrifuge tubes are siliconized by coating them with Sigmacote solution (Sigma) followed by air-drying to avoid loss of material by nonspecific binding of nucleic acid to plastic.
4. To use the optimal ratio of magnetic beads and RNA, it is important to determine the mRNA content of the cells or tissues by optical density measurement (OD_{260}) The capacity of the Dynabeads oligo(dT)25 is 2 μg mRNA/mg of beads In our present and previous work, we have worked with either cell lines of hematopoietic origin or normal lymphocytes The yield of isolated mRNA from 50 million subtractor cells of lymphoid origin (lymphoid cell lines) is approx 10 μg mRNA. Cultured lymphoid cells are collected by centrifugation and washed once in ice-cold PBS before mRNA isolation. Adherent cell lines can be detached from plastic by using a scraping device, treatment with PBS/0.5 m*M* EDTA, or trypsinization followed by washing in PBS prior to mRNA isolation. Subpopulations of blood cells can be isolated by immunomagnetic separation using magnetic beads coated with cell-specific antibodies *(19)* The captured cells are washed with cold PBS while attached to the beads and subsequently lysed before removal of the beads followed by mRNA isolation (*Dynal Technical Handbook*, 2nd ed.). When working with solid tissue, we recommend freezing the samples in liquid nitrogen immediately after dissection and storing them at –80°C until extraction. Up to 50 mg of animal tissue and 100 mg of plant tissue can be used per mg of Dynabeads oligo(dT)25 *(20)*.
5. The lysis of several types of hematopoietic cells can be done with a mild nonionic detergent, which will disrupt the cells, leaving the nuclei intact. The nuclei are removed by centrifugation before performing the mRNA isolation. The same lysis condition can be used for adherent cells, such as HepG2, HeLa, and COS cells. For most other cell types and all solid tissues, a more robust method of lysis is necessary. We recommend using lysis buffer with 1% SDS (2X lysis/binding buffer, 1% SDS). Solid tissues are ground to fine powder in a liquid nitrogen in a mortar before homogenization in a glass homogenizer with 2X lysis/binding buffer with 1% SDS.
6. To determine that the mRNA isolation procedure works optimally, one can elute $^1/_{10}$ of a large-scale isolation and quantify the amount by measuring the OD_{260}. One can also elute and load $^1/_{10}$ of one isolation, estimated to yield 10 μg mRNA, in a small well in a 1% agarose gel used for normal DNA separation (without formamide), and then run it shortly thereafter into the gel The mRNA (approx 1 μg) will be clearly visualized as a smear between 0.5 and 4 kb. Add cold RNase-free loading buffer to the mRNA, and load it quickly into the well of the gel Our

experience is that <0.2 μg mRNA can be detected on a 1% agarose gel if loaded in a small well and the gel is run for a short time This means that it is possible to visualize both the first and second elution of hybridized target mRNA from the subtractor beads after the subtractive hybridizations. Alternatively, the sensitivity can be increased by staining the gel with Sybgreen II (Molecular Probes, Eugene, OR) instead of ethidium bromide. Both optical density measurement and visualization of mRNA on a gel are crude ways of assessing the quality of mRNA. Performing a Northern blot and hybridization of a probe directed toward the 5'-end of a moderate to abundantly expressed mRNA will assess more accurately the quality of the isolated mRNA.

7. The rTth DNA polymerase can act as a reverse transcriptase in the presence of Mn^{2+} In our hands, the addition of rTth polymerase increases the efficiency of first-strand cDNA synthesis by 60–80% as measured by the incorporation of radioactive nucleotides (^{32}P-dCTP). However, the addition of the rTth polymerase is considered an optional step.
8. With an estimated 25% efficiency of the first-strand cDNA synthesis reaction, approx 2.5 μg of cDNA are generated from 10 μg mRNA.
9. From the target cells, about 0.3 μg of mRNA is isolated. This gives an 8- to 10-fold excess of subtractor over target mRNA. Cultured cells, blood cells, or solid tissue can be used as a target for mRNA isolation as described for the subtractor mRNA isolation (Section 3.1.1.).
10. Alternatively, larger amounts of mRNA from the target cells can be isolated and stored in ethanol at –70°C or in the hybridization buffer at the same temperature ready for use
11. Subtraction hybridization of mRNA to immobilized cDNA is presumed to follow a second-order kinetic reaction *(21)* and is dependent on such factors as nucleic acid concentration, temperature, salt concentration, and time. It is uncertain if the presence of the Dynabeads has any direct influence on the hybridization process. During the subtraction, abundantly expressed mRNAs will be removed first. This means that if the material is not optimally subtracted, the detection of differentially expressed sequences will be difficult. We therefore chose to perform three hybridization steps on the same material to ensure optimal subtraction. The mRNA can be visualized after each round of subtraction on an agarose gel, showing a clear decrease in intensity as visualized on UV light after the third round compared with the first round of hybridization. In case no decrease is observed, we recommend proceeding to a fourth round of subtractive hybridization. The subtractive hybridization is performed in a buffer containing 0.75*M* NaCl and at 68°C. One can imagine that under more stringent salt conditions, the loss of highly homologous nucleic acids in subtractor and target population will diminish. We have observed that lowering the hybridization temperature to 65°C results in the loss of homologous nucleic acids. The subtractor beads need to be completely removed from the hybridization solution after the last hybridization step before oligo(dT)25 beads are added to bind the target-specific mRNA. This is important to avoid the cDNA on the subtractor beads giving you any problems

when generating a subtractive probe, in particular when the specific target mRNA is amplified by PCR before the generation of a probe. We therefore recommend performing a second collection of the subtractor beads from the hybridization solution. When working with low amounts of beads, a fast spin (30 s) at maximum speed in a microcentrifuge facilitates the concentration of beads, and facilitates the separation of beads from the supernatant. We normally use 70°C when eluting mRNA from the subtractor beads, but the temperature can be raised to 85–90°C to assure complete elution of mRNA.

12 At this point, the mRNA eluted from the subtractor beads can be visualized on a normal 1% agarose gel used for DNA separation. Add 3 µL of 6X RNase-free loading buffer to the eluted mRNA and load directly in a small well of the agarose gel. Perform a short electrophoresis, and visualize the mRNA on UV light. The mRNA should now be seen as a smear between 0.5 and 4 kb in size.

13. We add 250 ng of yeast tRNA to the hybridization solution prior to the last round of subtraction to avoid loss of the low amount of specific mRNA through nonspecific binding to the beads or to the microcentrifuge tube

14. As mentioned in Section 3.4.1., the generation of a probe from specific target mRNA left after the third round of hybridization may be difficult owing to the limited amount of material. In those instances, it is recommended either to scale up the protocol (starting with 20 µg of mRNA from the subtractor cells and 0.6 µg of the target population) or pool material from two or three independent subtractions. The PCR-based approach for the generation of a radiolabeled probe allows a qualitative assessment of the subtraction procedure One-tenth of the PCR-amplified material is subjected to Southern blotting *(17)* and the filter probed with an abundantly expressed gene, like actin. If no signal or greatly diminished signal is obtained with an actin probe compared to nonsubtracted material, we consider the material to be enriched and proceed to the screening of a cDNA library using this probe. All cDNA clones isolated from the cDNA library should be used as probes in Northern blots to assure their specific expression pattern.

15. We have slightly modified a protocol recently described by Lambert and Williamson *(16)* that is suitable for the amplification of mRNA from small amounts of cDNA. This method was originally described for the generation of cDNA libraries from small amounts of mRNA. The principle of this method is to generate solid-phase first-strand cDNA on Dynabeads from the enriched target mRNA. This cDNA is polyA-tailed by terminal transferase, and a second-strand cDNA is synthesized with a T-primer containing an anchor (*see* Section 2 4.2) Subsequently, the material is amplified with the T-primer and its anchor, L-primer (*see* Section 2.4.2).

16. The beads are stored in TE at 4°C and can be reused to generate another second-strand cDNA.

17. A directed search for differentially expressed genes may be performed using degenerate primers to highly conserved regions of members of the gene family of interest as has been described for protein tyrosine kinases *(22)*. In this case, the

solid-phase bound first-strand cDNA from the enriched target mRNA serves as a template in the PCR reaction Although the amount of specific target mRNA may be limited, up to 50 cycles in the PCR allows the selective amplification of members of, e g , the protein tyrosine kinase family, including novel members The removal of most of the common sequences by subtractive hybridization and reduction of the complexity of the mRNA population left results in less problems with background when performing low stringency degenerate PCR One can overcome the bias in this type of PCR owing to differences in expression level of the genes and/or more favorable usage of the degenerated primers for some sequences of the gene family than for others By this approach, subtraction and enrichment can lead to the discovery of novel and differentially expressed sequences, e.g., the usage of PTK1 and PTK2 primers *(22)* to isolate protein tyrosine kinases has been suggested to have reached the extent of its usefulness, since the sequences amplified using this primer set tend to be the same using different tissues *(23)*. Using the same primer set, we have isolated several novel tyrosine kinase sequences that we are now in the process of characterizing (Aasheim et al., unpublished). In addition, immobilized cDNA gives less background problems in the PCR than cDNA generated in solution without beads One reason for this is that the first-strand cDNA beads can be washed after cDNA synthesis to remove contaminants (e.g , free oligo[dT] primers, enzymes, buffer differences) from the reverse transcriptase reaction These contaminants will still be present after cDNA synthesis in solution and may generate a background that appears as a smear on an agarose gel. Low background also makes it easier to clone the material in the vector of choice for further sequencing

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