INTRODUCTION

This method uses PCR to amplify and display many cDNAs derived from the mRNAs of a given cell or tissue type. The method relies on two different types of synthetic oligonucleotides: anchored antisense primers and arbitrary sense primers. A typical anchored primer is complementary to approx. 13 nucleotides of the poly(A) tail of mRNA and the adjacent two nucleotides of the transcribed sequence. Anchored primers therefore anneal to the junction between the poly(A) tail and the 3'-untranslated region of mRNA templates, from where they can prime synthesis of first-strand cDNA. A second primer, an arbitrary sequence of approx. 10 nucleotides, is then added to the reaction mixture, and double-stranded cDNAs are produced by conventional PCR, carried out at low stringency. The products of the amplification reaction are separated by electrophoresis through a denaturing polyacrylamide gel and visualized by autoradiography. By comparing the banding patterns of cDNA products derived from two different cell types, or from the same cell type grown under different conditions, it is sometimes possible to identify the products of differentially expressed genes. Bands of interest can then be recovered from the gel, amplified further, and cloned and/or used as probes to screen northern blots, cDNA libraries, etc. To reduce the chance of contamination with exogenous DNAs, prepare and use a special set of reagents and solutions for PCR only. Bake all glassware for 6 hours at 150°C and autoclave all plasticware.

MATERIALS

- 10x Amplification buffer
  Include 0.01% (w/v) gelatin in the buffer.
- 5x DD-PCR reverse transcriptase buffer
- 5x Formamide loading buffer
- Anchoring 3' oligonucleotide primers (300 µg/ml) in 10 mM Tris-Cl (pH 7.6), 0.1 mM EDTA
  The anchoring 3' oligonucleotides are a family of 12 primers with the general structure 5'-d(T)_{12}V\text{-}N-3', where V is either C, A, or G, and N is C, T, A, or G. For example, one primer in the series is 5'-d(T)_{12}CC-3', the next is 5'-d(T)_{12}CT-3', etc.
- Arbitrary 5' oligodeoxynucleotide primers (50 µg/ml) in 10 mM Tris-Cl (pH 7.6), 0.1 mM EDTA
  Sixteen arbitrary 5' oligonucleotide primers are required, each ten nucleotides in length. The sequence of each primer is chosen at random, but it should contain approximately equal numbers of the four bases, with a balanced distribution of G and C residues, and a low propensity to form stable secondary structures.
- DTT (dithiothreitol) (100 mM)
- Placental RNase inhibitor (20 units/ml)
- Radiolabeled dATP (10 µCi/µl, sp. act. 3000 Ci/mmole)
DNA labeled with $\alpha^{-35}S$ or $\alpha^{-33}P$ generates sharper bands on autoradiographs than does DNA labeled with $\alpha^{-32}P$.

Reverse transcriptase (RNA-dependent DNA polymerase).

For DD-PCR, a reverse transcriptase deficient in RNase H is required (e.g., Superscript from Life Technologies or StrataScript from Stratagene).

Thermostable DNA polymerase

Taq DNA polymerase is the standard and appropriate enzyme for the amplification stage of most forms of DD-PCR.

Total RNA (100 µg/ml)

Total RNA extracted from cells with chaotropic agents is generally the template of choice for DD-PCR. RNAs to be compared by DD-PCR should be prepared in an identical fashion. Poly(A)$^+$ RNA is not ideal as a template in differential display.

dNTP solution (20 mM) containing all four dNTPs (pH 8.0)

**METHOD**

1. In sterile 0.5-ml microcentrifuge tubes, set up a series of trial reactions to establish the optimum concentrations of "control" and "test" RNAs required to produce a pattern of 100-300 amplified cDNA bands after gel electrophoresis and autoradiography. Make fivefold serial dilutions in H$_2$O of the RNA preparations to produce concentrations of between 1 µg/ml and 100 µg/ml.

2. Choose one or more primers from the collection of anchored 3' oligonucleotides and set up a series of annealing reactions that contain different amounts of diluted RNA templates:

   | template RNA |     8.0 µl |
   | anchored 3' oligonucleotide primer | 2.0 µl |

   Incubate the reactions for 10 minutes at 65°C and then place them in a 37°C water bath.

   The total amount of RNA in the annealing reactions should vary between 8 ng and 800 ng.

3. Add the following to the annealing reactions:

   | 5x DD-PCR reverse transcriptase buffer | 4 µl |
   | 100 mM dithiothreitol | 2 µl |
   | 200 µM solution of four dNTPs | 2 µl |
   | approx. 25 units/µl placental RNase inhibitor | 0.25 µl |
   | 200 units/µl reverse transcriptase | 0.25 µl |
   | H$_2$O | to 20 µl |

   Incubate the tubes at 37°C for 1 hour.

   To test for contaminating genomic DNA, set up one or more control reactions that contain no reverse transcriptase enzyme and carry these through Step 10 of the protocol. If necessary, the RNA preparation can be treated with RNase-free DNase I either as a separate step during purification or in the same reaction tube that will later be used to synthesize cDNA.

4. Inactivate the reverse transcriptase by incubating the reaction mixtures for 10 minutes at 94°C.
Steps 3 and 4 can be carried out in a thermal cycler programmed with a single cycle of 37°C for 1 hour/94°C for 10 minutes, followed by a 4°C hold.

5. Set up two series of eight 0.5-ml amplification tubes. Each tube should contain:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x amplification buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>anchored 3’ oligonucleotide primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>20 mM solution of four dNTPs (pH 8.0)</td>
<td>1 µl</td>
</tr>
<tr>
<td>[α-33P]dATP or [α-35S]dATP (3000 Ci/mmole)</td>
<td>1 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>9 µl</td>
</tr>
<tr>
<td>5 units/µl thermostable DNA polymerase</td>
<td>1 unit</td>
</tr>
</tbody>
</table>

To each tube, add 2 µl of a different arbitrary 5’ primer. Mix the contents by tapping the sides of the tubes.

Wherever possible, use the 10x amplification buffer supplied by the manufacturer of the Taq DNA polymerase.

6. Into one series of eight tubes, dispense approx. 3-µl aliquots of the reverse transcriptase reaction containing the test RNA. Into the other series of eight tubes dispense approx. 3-µl aliquots of the reverse transcriptase reaction containing the preparation of control RNA. Close the tubes and mix the contents gently.

7. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in the thermal cycler.

8. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Polymerization</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 cycles</td>
<td>15 sec at 94°C</td>
<td>30 sec at 42°C</td>
<td>15 sec at 72°C</td>
</tr>
<tr>
<td>Last cycle</td>
<td>15 sec at 94°C</td>
<td>30 sec at 42°C</td>
<td>2 min at 72°C</td>
</tr>
</tbody>
</table>

Times and temperatures may need to be adapted to suit the particular reaction conditions.

9. At the end of the program, remove the tubes from the thermal cycler and add 5 µl of 5x formamide loading buffer to each.

10. Separate the radiolabeled products of the reactions by electrophoresis through an electrolyte gradient polyacrylamide gel of the type used for DNA sequencing. Electrophoresis is carried at constant electrical power until the xylene cyanol tracking dye has migrated about two thirds of the length of the gel (please see Preparation of Electrolyte Gradient Gelsand Loading and Running DNA Sequencing Gels). Dry the gel and expose it to autoradiographic film.

11. Examine the pattern of DNA bands arising from reactions containing different concentrations of control and test RNAs. A good differential display contains between 100 and 250 well-resolved bands. Select the concentration of test and control RNAs that work well with the largest number of primer pairs.

12. Repeat the annealing, reverse transcriptase, and amplification reactions using all combinations of primer pairs and the optimum amount of RNA templates. Set up the reactions in 96-well microtiter plates designed for use in a thermal cycler.

13. Separate the products of the amplification reactions by electrophoresis through polyacrylamide sequencing
gels, as in Steps 9 and 10.

Load the reactions generated with each primer pair in adjacent lanes on the gel, i.e., load the reaction obtained with primer pair A + B from one RNA preparation next to the reaction obtained with primer pair A + B from the other RNA preparation.

14. Compare the patterns of bands obtained with each primer pair from the different RNA populations. When a differentially expressed band is identified, it is advisable to repeat the experiment to make sure that the initial finding is reproducible. Ideally, different batches of the two RNAs should be used, although this precaution may not always be practicable.

15. Recover target bands from the dried polyacrylamide gel. Lay the autoradiogram on top of the gel and use a soft pencil to lightly mark the position of the desired band on the autoradiogram. Cutting through the autoradiogram with a clean razor blade, excise each target band and the attached Whatman 3MM paper. Soak each sliver of dried gel/paper overnight at room temperature in a separate 0.5-ml microcentrifuge tube containing 50 µl of sterile H₂O.

16. Puncture the bottom of each 0.5-ml tube with a small-gauge needle. Place each punctured tube inside a 1.5-ml microcentrifuge tube. Centrifuge for 20 seconds to transfer the eluate to the larger tube. Discard the amplification tube containing the residue of the Whatman 3MM paper and polyacrylamide.

17. Amplify the eluted fragment in a reaction containing the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x amplification buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>DNA eluted from polyacrylamide gel</td>
<td>3 µl</td>
</tr>
<tr>
<td>arbitrary 5’ oligonucleotide primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>anchoring 3’ oligonucleotide primer</td>
<td>2 µl</td>
</tr>
<tr>
<td>20 mM solution of four dNTPs (pH 8.0)</td>
<td>1 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.5 µl</td>
</tr>
<tr>
<td>5 units/µl Taq thermostable DNA polymerase</td>
<td>2 units</td>
</tr>
</tbody>
</table>

Wherever possible, use the 10x amplification buffer supplied by the manufacturer of the Taq DNA polymerase.

18. If the thermal cycler is not fitted with a heated lid, overlay the reaction mixtures with 1 drop (approx. 50 µl) of light mineral oil. Place the tubes in a thermal cycler.

19. Amplify the nucleic acids using the denaturation, annealing, and polymerization times and temperatures listed in the table.

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<th>Cycle Number</th>
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<th>Annealing</th>
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<tr>
<td>Last cycle</td>
<td>15 sec at 94°C</td>
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<td>2 min at 72°C</td>
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</tbody>
</table>

Polymerization should be carried out for 1 minute for every 1000 bp of length of the target DNA.

20. Estimate the concentration of the reamplified DNA fragment by electrophoresis of 5-10% of the reaction through a 1% (w/v) agarose gel.

21. If mineral oil was used to overlay the reaction (Step 18), remove the oil from the sample by extraction.
with 150 µl of chloroform.

The aqueous phase, which contains the amplified DNA, will form a micelle near the meniscus. The micelle can be transferred to a fresh tube with an automatic micropipette.

IMPORTANT
Do not attempt chloroform extractions in microtiter plates. The plastic used in these plates is not resistant to organic solvents.

22. Ligate the DNA into a vector that has been tailed with dT (e.g., pGEM T vector from Promega) (please see Cloning PCR Products into T Vectors) and transform *E. coli* with aliquots of the ligation reaction.

23. Isolate plasmid DNA from six or more recombinants and compare the sizes of the inserts released by restriction enzyme digestion.

The sequence of the insert DNA can be established by using universal primers that bind to the flanking regions of the vector. These oligonucleotides can also be used as primers to check the size of the inserts by PCR (please see Rapid Characterization of DNAs Cloned in Prokaryotic Vectors).

It is important to isolate and sequence more than one plasmid recombinant from the ligation reaction. Compare the cDNA sizes and sequences to each other and to those in the various databases.

24. Confirm the differential expression of a candidate cDNA/mRNA in as many ways as possible, including northern hybridization (Northern Hybridization), RNase protection (Ribonuclease Protection: Mapping RNA with Ribonuclease and Radiolabeled RNA Probes), or quantitative PCR (Quantitative PCR). In situ mRNA hybridization can be used to localize the transcript to a diseased or developing tissue.

REFERENCES


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**Caution**

Dithiothreitol (DTT)

Dithiothreitol (DTT) is a strong reducing agent that emits a foul odor. It may be harmful by inhalation, ingestion, or skin absorption. When working with the solid form or highly concentrated stocks, wear appropriate gloves and safety glasses and use in a chemical fume hood.

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**Caution**

Formamide

Formamide is teratogenic. The vapor is irritating to the eyes, skin, mucous membranes, and upper respiratory tract. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses. Always use in a chemical fume hood when working with concentrated solutions of formamide. Keep working solutions covered as much as possible.
Caution

Radioactive substances

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

Recipe

Amplification Buffer, 10X

- 500 mM KCl
- 100 mM Tris-Cl (pH 8.3 at room temperature)
- 15 mM MgCl₂

Autoclave the 10x buffer for 10 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. Divide the sterile buffer into aliquots and store them at -20°C.

Recipe

DD-PCR reverse transcriptase buffer

- 250 mM Tris-Cl (pH 8.3)
- 375 mM KCl
- 15 mM MgCl₂

Autoclave the 5x buffer for 10 minutes at 15 psi (1.05 kg/cm²) on liquid cycle. Divide the sterile buffer into aliquots. Store the aliquots at -20°C.

Recipe

Dithiothreitol (DTT) (1 M)

- DTT (DL-dithiothreitol, anhydrous m.w. = 154.25)

Dissolve 1.5 g of DTT in 8 mL of H₂O. Adjust the total volume to 10 mL, dispense into 1-mL aliquots, and store them in the dark (wrapped in aluminum foil) at -20°C (indefinitely). Do not autoclave DTT or solutions containing it.
**Recipe**

**Formamide loading buffer**

- 80% (w/v) deionized formamide
- 1 mg/ml xylene cyanol FF
- 1 mg/ml bromophenol blue
- 10 mM EDTA (pH 8.0)

Purchase a distilled deionized preparation of formamide and store in small aliquots under nitrogen at -20°C.

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**Recipe**

**dNTP solution**

Dissolve each dNTP (deoxyribonucleoside triphosphates) in H₂O at an approximate concentration of 100 mM. Use 0.05 M Tris base and a micropipette to adjust the pH of each of the solutions to 7.0 (use pH paper to check the pH). Dilute an aliquot of the neutralized dNTP appropriately, and read the optical density at the wavelengths given in the table below. Calculate the actual concentration of each dNTP. Dilute the solutions with H₂O to a final concentration of 50 mM dNTP. Store each separately at -70°C in small aliquots. For polymerase chain reactions (PCRs), adjust the dNTP solution to pH 8.0 with 2 N NaOH. Commercially available solutions of PCR-grade dNTPs require no adjustment.

<table>
<thead>
<tr>
<th>Base</th>
<th>wavelength (nm)</th>
<th>Extinction Coefficient (E) (M⁻¹cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>259</td>
<td>1.54 x 10⁴</td>
</tr>
<tr>
<td>G</td>
<td>253</td>
<td>1.37 x 10⁴</td>
</tr>
<tr>
<td>C</td>
<td>271</td>
<td>9.10 x 10³</td>
</tr>
<tr>
<td>T</td>
<td>267</td>
<td>9.60 x 10³</td>
</tr>
</tbody>
</table>

For a cuvette with a path length of 1 cm, absorbance = EM. 100 mM stock solutions of each dNTP are commercially available (Pharmacia).