Fluorescent Sample Labeling for DNA Microarray Analyses

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Summary

Three fluorophor-labeling methods for gene expression profiling on deoxyribonucleic acid (DNA) microarrays are described. All three techniques start from total ribonucleic acid (RNA) samples. Two procedures are based on first-strand complementary DNA synthesis by reverse transcription. Label is introduced either by direct incorporation of fluorescently labeled nucleotides or indirectly by incorporation of aminooallyldUTP and subsequent coupling of fluorescent dyes. The third method is based on an amplification of antisense RNA by in vitro transcription subsequent to first- and second-strand complementary DNA synthesis. While the first two methods are applied mainly in analyses on microarrays made from spotted polymerase chain reaction products or long oligonucleotides, the last procedure is mostly used for experiments on in situ synthesized oligonucleotide arrays.

Key Words: DNA microarray; oligonucleotide array; hybridization; fluorescent labeling.

1. Introduction

Deoxyribonucleic acid (DNA) microarrays of all formats, whether made by spotting prefabricated molecules or by in situ synthesis, have become a popular and versatile technique to analyse gene expression profiles on a genome-wide basis (1,2). All ribonucleic acids (total RNA) are extracted from the samples, labeled appropriately, and analyzed on arrays of gene-specific DNA fragments. A very common analysis mode is the labelling of two related samples with a pair of fluorescent dyes, Cy3 and Cy5 for example, followed by their simultaneous hybridization to a single microarray (3). Upon binding, the relative fluorescence intensities produced at the microarray spots are determined, instantly indicating in amount of each transcript (Fig. 1).

Although quite a number of labeling protocols exist, there are three procedures that are currently used most frequently. All three rely on oligo(dT)-priming so that there is no need to isolate mRNA before labeling. Direct labeling
Fluorescent Sample Labeling

only attached upon completion of complementary DNA (cDNA) synthesis by a chemical coupling of reactive NHS esters of the respective dye. With the latter method, usually better labeling efficiency and consistency is achieved because the nucleotides with the relatively small aminoallyl-group have a much better incorporation rate compared to those, which have the rather bulky fluorescence molecules attached.

A third method is based on the production of antisense RNA (aRNA) by in vitro transcription amplification (5). First-strand cDNA synthesis is performed using an oligo(dT)-primer that is linked to the promoter sequence of T7 RNA polymerase. After second-strand cDNA synthesis, aRNA is synthesized in rather large quantities via in vitro transcription. The labeling of the aRNA can be performed during the in vitro transcription by incorporation of fluorescence-labeled or biotinylated nucleotides. Alternatively, unlabeled aRNA can be produced and used as template in yet another round of reverse transcription as described previously. When biotinylated nucleotides are used, a reaction with streptavidine–phycoerythrine conjugate is necessary for staining the target after hybridization.

2. Materials

2.1. Reverse Transcription With Direct Incorporation of Fluorescently Labeled Nucleotides

2.1.1. Reagents

1. Oligo(dT)12-18 primer (0.5 μg/μL), Invitrogen (Karlsruhe, Germany).
2. SuperScript™II RNase H- reverse transcriptase (200 U/μL), 5X first-strand buffer, 0.1 M DTT.
3. Ribonuclease H (2 U/μL), Invitrogen.
4. RNaseOUT™ (40 U/μL), Invitrogen.
5. Deoxyribonucleotide-5′-triphosphates dATP, dCTP, dGTP, and dTTP (100 mM), MBI Fermentas (St. Leon-Roth, Germany).
6. Cy3-dCTP, Cy5-dCTP (1 mM), Amersham Biosciences (Freiburg, Germany).
7. QIAquick™ PCR purification kit, Qiagen (Hilden, Germany).

2.2. Aminoallyl Labeling

2.2.1. Reagents

1. Oligo(dT)12-18 Primer (0.5 μg/μL), Invitrogen.
2. SuperScript™II RNase H-reverse transcriptase (200 U/μL), 5X first-strand buffer, 0.1 M DTT, Invitrogen.
3. Deoxyribonucleotide-5′-triphosphates dATP, dCTP, dGTP, and dTTP (100 mM), MBI Fermentas.
4. 5-(3-Aminoallyl)-dUTP (aminoallyl-dUTP) Sigma-Aldrich (Deisenhofen, Germany).

during reverse transcription of the RNA is performed by incorporation of nucleotides, to which a fluorophor is bound. Alternatively, the dye is added in a two-step process: initially, aminoallyl-dUTP is incorporated, and the fluorophor is
Fluorescent Sample Labeling

2.3.2. Buffers

1. 10 mM dNTP-mix.
2. 7.5 mM Ammonium acetate.
4. 80% Ethyl alcohol.

3. Methods

3.1. Reverse Transcription With Direct Incorporation of Fluorescently Labeled Nucleotides

Starting from total RNA, a first-strand cDNA synthesis is performed, during which fluorescently labeled nucleotides are incorporated. Subsequently, the RNA template is hydrolyzed, and the single-stranded cDNA is purified. It can be used directly for hybridization onto microarrays made of spotted polymerase chain reaction (PCR) products or long oligonucleotides (see also Note 3).

3.1.1. First-Strand cDNA Synthesis

1. Dissolve 10–15 μg of total RNA in 15 μL of water, add 5 μL of oligo(dT)$_{12-18}$ primer and mix by pipetting. After an incubation at 70°C for 10 min, place the mixture on ice.
2. On ice, add 8.5 μL of first-strand buffer, 3.5 μL of DTT; 10 μL each of dATP, dGTP, and dTTP; 2 μL of Cy5-dCTP; respectively, 1 μL of RNaseOUT and 2 μL of SuperScript™III transcriptase; mix well.
3. Incubate the reaction for 1 h at 42°C, add another 2 μL of SuperScript™III transcriptase and incubate again at 42°C for at least 3 h (see also Note 4).
4. Incubate at 70°C for 10 min, add 1 μL of RNase H and incubate 20 min at 37°C. Continue immediately with cDNA purification or store overnight at −20°C.

3.1.2. Purification of Labeled cDNA

The purification of the labeled cDNA is done with the QIAquick® PCR purification kit according to the manufacturer’s instructions. All centrifugation steps are performed at room temperature at 16,000 g.

1. Add 225 μL of buffer PB to the sample and mix carefully by pipetting.
2. Apply the sample to the QIAquick column and centrifuge for 1 min.
3. After discarding the flow-through, add 750 μL of buffer PE to the column for washing and centrifuge for 1 min. Discard the flow-through and centrifuge again for 1 min to remove the buffer completely.
4. For elution, place the QIAquick column in a clean tube, add 50 μL of water, incubate 1 min at room temperature, and centrifuge for 1 min (see also Note 5). Repeat this elution step with another 50 μL of water.
3.2. Aminoallyl Labeling

This method is also based on reverse transcription of total RNA. However, the incorporation of fluorescence-labeled dCTP is replaced by the use of aminoallyl-dUTP. After reverse transcription, fluorescent dyes in form of monoreactive NHS-esters are coupled to the amino residues of the single stranded cDNA. After removal of unbound dye, the sample is ready for hybridization to spotted microarrays (see Note 3).

3.2.1. First-Strand cDNA Synthesis

1. Dissolve 10–15 μg of total RNA in 9.5 μL of water; add 5 μL of oligo(dT)12-18 primer and mix by pipetting. After incubation at 70°C for 10 min, place the mixture on ice.
2. On ice, add 6 μL of 5X first-strand buffer, 0.6 μL of 50X dNTP-mix, 3 μL of DTT, 3 μL of water, and 1.9 μL of SuperScript II transcriptase.
3. Incubate at 42°C for at least 3 h (see Note 4).
4. For hydrolysis of the RNA, add 10 μL of NaOH and 10 μL of ethylenediamine tetraacetic acid and incubate at 65°C for 10 min. Subsequently, neutralize with 25 μL of N-Hydroxyethylpiperazine-N'-2-ethanesulfonate.

3.2.2. Purification of First-Strand Synthesis Reaction

Purification of the first-strand synthesis product is performed with the QIAquick PCR purification kit according to the manufacturer's instructions. All centrifugation steps are conducted at room temperature at 16,000g.

1. Add 25 μL of water and 500 μL of buffer PB to the sample and mix carefully by pipetting.
2. Apply the sample to the QIAquick column and centrifuge for 1 min.
3. After discarding the flow-through, add 750 μL of buffer PE for washing and centrifuge for 1 min. Discard the flow-through and centrifuge again for 1 min to remove the buffer completely.
4. For elution, place the QIAquick column in a clean tube, add 30 μL of water, incubate 1 min at room temperature, and centrifuge for 1 min (see Note 5). Repeat this elution step with another 30 μL of water.

3.2.3. Coupling of Monofunctional NHS Esters of the Cy-Dyes

1. Dry the cDNA sample in vacuo.
2. Dissolve dried cDNA pellet in 9 μL of NaHCO3.
3. Add the dissolved cDNA to one aliquot of either Cy3- or Cy5-monofunctional dye and incubate at room temperature in the dark for 1 h.
4. For quenching unbound Cy-dye, add 4.5 μL of hydroxylamine and incubate 15 min at room temperature in the dark.
5. Add 70 μL of buffer PB and mix carefully by pipetting.
6. Apply the sample to the QIAquick column and centrifuge for 1 min.
7. After discarding the flow-through, add 750 μL of buffer PE for washing and centrifuge for 1 min. Discard the flow-through and centrifuge again for 1 min to remove the buffer completely.
8. For elution, place the QIAquick column in a clean tube, add 30 μL of buffer EB, incubate 1 min at room temperature and centrifuge for 1 min. Repeat this elution step with another 30 μL of buffer EB.

3.3. Antisense RNA Amplification by In Vitro Transcription

This labeling method was extensively tested for working on in situ synthesized oligonucleotide arrays using the Geniom technology of febit (see Note 6). In a first step, total RNA is reverse transcribed into double-stranded cDNA. After purification, the cDNA is used as template for an in vitro transcription with concomitant incorporation of biotinylated nucleotides (see Note 3). The fluorescence labeling of the target is performed after hybridization to an oligonucleotide array by an incubation with streptavidine–phycoerythrin conjugate.

3.3.1. First-Strand Synthesis

1. 15 μg of total RNA is dissolved in 10 μL of water and placed on ice.
2. Add 1 μL of T7-T(24)-primer, mix well and incubate at 70°C for 10 min by shaking gently (500 rpm; see also Note 7).
3. During this incubation, mix in a second tube the reverse transcription mix, made of 4 μL of 5X first-strand buffer, 2 μL of DTT, 1 μL of dNTP-mix, and 0.5 μL of RNaseOUT. Preheat this mix to 50°C.
4. Place the primer mix also at 50°C, incubate briefly and add the reverse transcription mix.
5. Incubate at 50°C for 2 min. Then add 1.5 μL of SuperScript II transcriptase.
6. Incubate at 50°C for 1 h, then place immediately on ice and proceed directly to second-strand synthesis.

3.3.2. Second-Strand Synthesis

1. Prepare the second-strand synthesis mix on ice: mix 30 μL of 5X second-strand buffer, 4 μL of DNA polymerase I, 3 μL of dNTP-mix, 1 μL of DNA ligase, 1 μL of RNase H, and 91 μL of water.
2. Add the second-strand synthesis mix to the first-strand synthesis reaction and incubate at 16°C for 2 h.
3. Add 2 μL of T4 DNA polymerase and incubate at 16°C for 5 min.
4. Place the reaction on ice and proceed immediately to cDNA purification.

3.3.3. cDNA Purification
1. Add 1 volume rotiphenol to the second-strand synthesis reaction, mix by vortexing at maximum speed for 1 min and centrifuge at 16,000g for 5 min.
2. Transfer the aqueous phase carefully to a new tube and place the sample on ice.
3. Repeat the two steps with 1 vol chloroform/isoamyl alcohol.
4. For DNA precipitation, add 0.5 vol 7.5 M ammonium acetate and 2.5 vol 100% ethyl alcohol and centrifuge at 4°C at 16,000g for 20 min.
5. After removing the supernatant, wash the pellet twice with 375 μL of 80% ethyl alcohol. In between, centrifuge at 4°C at 16,000g for 7 min.
6. Dry the cDNA pellet in vacuo and dissolve it in 1.5 μL of water. The cDNA can either be used directly for in vitro transcription or stored overnight at -20°C.

3.3.4. In Vitro Transcription
1. Prepare on ice the nucleotide mix, made up of 2 μL of ATP, 2 μL of GTP, 1.5 μL of CTP, 1.5 μL of UTP, 3.75 μL of Biotin-11-CTP, 3.75 μL of Biotin-16-UTP, and 2 μL of 10X reaction buffer.
2. Add the nucleotide mix and 1.5 μL of 0.1 M Tris base in a total volume of 15 μL of the kit’s enzyme mix to the purified cDNA.
3. Seal the tube with parafilm and incubate the reaction at 37°C for 6 h shaking gently.
4. For hydrolysis of the DNA, add 1 μL of DNase I and incubate at 37°C for 15 min.
5. Place the tube on ice and proceed immediately to aRNA purification.

3.3.5. aRNA Purification
For purification of the aRNA, the RNeasy Mini Kit (Qiagen) is used as recommended by the manufacturer. All centrifugation steps are performed at room temperature at 16,000g.
1. Adjust the volume to 100 μL by adding 20 μL of water to the aRNA. Then, add 350 μL of buffer RLT and mix by vortexing.
2. Add 250 μL of ethyl alcohol and mix carefully by pipetting.
3. Transfer the sample gently to an RNeasy column and centrifuge for 1 min.
4. Place the RNeasy column into a fresh 2-mL collection tube and add 500 μL of RPE buffer. Centrifuge for 1 min and discard flow-through. Repeat this washing step a second time.
5. For elution, place the RNeasy column into a fresh tube. Add 40 μL of water, incubate at room temperature for 4 min and centrifuge the column for 1 min. Repeat this elution step with another 40 μL of water.

Fluorescent Sample Labeling

4. Notes
1. When working with RNA, all buffers must be prepared with DEPC-treated water and be autoclaved.
2. When aliquoting the Cy-dyes, be sure to hide them from light. The DMSO used for aliquoting needs to be completely water-free and stored over molecular sieve.
3. Before hybridization, target is dried in vacuo and dissolved in an appropriate volume of hybridization buffer.
4. For a higher yield, the incubation of the reverse transcription reaction must be at least for 3 h; we recommend incubation times up to 12 h.
5. The elution from the QIAquick columns is very pH sensitive. Therefore, take care that the water used really has at least pH 7. This results in a much higher yield. If the water has a pH below 7.0, adjust the pH with 1 N NaOH. Using buffer EB as recommended by the manufacturer is not possible, because this buffer contains Tris, whose amino groups would react with the NHS-esters of the Cy-dyes.
6. Using biotinylated aRNA for hybridisation to spotted cDNA microarrays is not recommended. The subsequent reaction with streptavidin-phycoerythrin conjugate would produce a high background.
7. All incubation steps during this protocol are performed by gentle shaking at 500 rpm.

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References