electrophoretic system for peptides. In conclusion, laborious work and time required for electrophoresis can be shortened using the single gel system for peptides. This gel system could be a very useful method for peptide analysis in conjunction with mass spectrometry methods.

REFERENCES

1. Swank, R. T., and Munkres, K. D. (1971) Molecular weight analysis of oligopeptides by electrophoresis in polyacrylamide gel with sodium dodecyl sulfate. *Anal. Biochem.* **39,** 462–477.
2. Kyte, J., and Rodriguez, H. (1983) A discontinuous electrophoretic system for separating peptides on polyacrylamide gels. *Anal. Biochem.* **133,** 515–522.
3. Hashimoto, F., Horigome, T., Kanbayashi, M., Yoshida, K., and Sugano, H. (1983) An improved method for separation of low-molecular-weight polypeptides by electrophoresis in sodium dodecyl sulfate–polyacrylamide gel. *Anal. Biochem.* **129,** 192–199.
4. Anderson, B. L., Berry, R. W., and Telser, A. (1983) A sodium dodecyl sulfate–polyacrylamide gel electrophoresis system that separates peptides and proteins in the molecular weight range of 2500 to 90,000. *Anal. Biochem.* **132,** 365–375.
5. West, M. H. P., Wu, R. S., and Bonner, W. M. (1984) Polyacrylamide gel electrophoresis of small peptides. *Electrophoresis* **5,** 133–138.
6. Fling, S. P., and Gregerson, D. S. (1986) Peptide and protein molecular weight determination by electrophoresis using a high-molarity Tris buffer system without urea. *Anal. Biochem.* **155,** 83–88.
7. Bothe, D., Simons, M., and von Döhren, H. (1985) A sodium dodecyl sulfate–gradient gel electrophoresis system that separates polypeptides in the molecular weight range of 1500 to 100,000. *Anal. Biochem.* **151,** 49–54.
8. Okajima, T., Tanabe, T., and Yasuda, T. (1993) Nonurea sodium dodecyl sulfate–polyacrylamide gel electrophoresis with high-molarity buffers for the separation of proteins and peptides. *Anal. Biochem.* **211,** 293–300.
9. Schägger, H., and von Jagow, G. (1987) Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166,** 368–379.
10. Ploug, M., Jensen, A. L., and Barkholt, V. (1989) Determination of amino acid compositions and NH_2-terminal sequences of peptides electroblotted onto PVDF membranes from tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis: Application to peptide mapping of human complement component C3. *Anal. Biochem.* **181,** 33–39.
11. Ahn, T., Yim, S-K., Choi, H-I., and Yun, C-H. (2001) Polyacrylamide gel electrophoresis without a stacking gel: Use of amino acids as electrolytes. *Anal. Biochem.* **291,** 300–303.
12. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.
13. Marshall, T. (1984) Sodium dodecyl sulfate polyacrylamide gel electrophoresis of serum after protein denaturation in the presence or absence of 2-mercaptoethanol. *Clin. Chem.* **30,** 475–479.

Recovery of Cyanine-Dye Nucleotide Triphosphates

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Using DNA microarrays or DNA chips, global gene expression changes of diverse physiologic and pathologic states, single nucleotide polymorphisms, or mutations can be followed (1–3). In recent years, this analysis is based on hybridization of fluorescent-labeled probes prepared from mRNA, total RNA, or DNA obtained from diverse biological samples to microarrays having complementary sequences as targets on their surfaces (3, 4). For each hybridization usually a mixture of two fluorescent labeled probes is applied, where one labeled probe is obtained from a control (untreated or unaffected) and the other is from a treated or affected sample. This direct comparative hybridization method allows a quantitative comparison of the relative abundance of individual sequences.

In most cases cyanine dyes (Cy3 and Cy5) linked to deoxyuridine-triphosphate or deoxycytidine-triphosphate are used for preparation of labeled nucleic acid probes. For labeling of RNA templates these dye-labeled nucleotide triphosphates (NTPs)[2] are incorporated during reverse transcription (RT) reaction. For DNA templates, the same triphosphates and usually Klenow polymerase are used for the synthesis of labeled DNA (5). In labeling reactions dye labeled NTPs are in large excess: the incorporated monomers are approximately 2–5% of the unincorporated, unused ones (estimated from the absorbance of the unincorporated dye before and after the labeling reaction). Owing to the high cost of the dye-labeled monomers, we intended to develop a fast and efficient purification protocol for the recovery of unincorporated fluorescent labeled NTPs, which can be performed without any high-performance chromatography instruments and

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[2] Abbreviations used: NTP, nucleotide triphosphates; RT, reverse transcription; DEPC, diethylene pyrocarbonate; TEAC, tetraethylammonium chloride; aRNA, antisense RNA.

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the amount is not restricted for large-scale purification.

Materials and Methods

Purification of dye-labeled nucleotide triphosphates. Purification includes two steps: a reverse-phase column purification and a size fractionation using a membrane filter unit.

The flowthrough from PCR purification step containing the labeled NTPs in a high-salt binding buffer was collected and stored at −20°C. Before purification, the mixture was diluted with 1 vol of water, and the pH was adjusted with acetic acid to pH 6.0. Thirty milliliters of flowthrough mixture containing approximately 70 nmol dye-labeled triphosphate was applied onto an equilibrated Oligo-Pak oligonucleotide purification column (Glen Research, Sterling, VA) with a syringe. Equilibration was performed with 5 ml of acetonitrile and then with 5 ml of 1 M TEAC. After sample application the column was washed with 2 ml of 3% (w/v) aq. NH_3 and then with 8 ml of DEPC-treated water. Labeled triphosphate was eluted with 0.5 ml of 20% aq. acetonitrile, lyophilized, reconstituted in 100 μl of deionized water, applied onto Microcon YM-3 membrane filter unit (Millipore, Bedford, MA), and centrifuged at 10,000*g* for 20 min. The flowthrough was collected and the concentrated sample was diluted with 500 μl of water and applied onto a new filter unit. After centrifugation with the same parameters the two flowthrough solutions were combined, lyophilized, dissolved in 60 μl of DEPC-treated water, and used in labeling reactions or stored at −20°C.

HPLC analysis. HPLC chromatography was performed on a HP1100 instrument with the following conditions: Lichrospher RP select B column, 60 Å, 250 × 4 mm (Merck, Budapest, Hungary); detection at 550 nm in the case of Cy3-labeled nucleoside triphosphates or at 600 nm in the case of Cy5-labeled nucleoside triphosphates; flow rate, 1.0 ml/min; eluent A, 0.1 M aq. TEAC (pH 7.0); eluent B, 0.1 M aq. TEAC (pH 7.0):acetonitrile 1:4; gradient, 15–45% B in A in 30 min.

Construction of microarrays. Human control clones were obtained from a mixed human library, cloned in pBluescript SK II (−) plasmid (New England Biolabs, Hertfordshire, England) with traditional techniques. Amplified cDNA inserts were arrayed on amino-silanized slides (Sigma, Germany) by using a MicroGrid total array system (BioRobotics, Cambridge, UK) spotter with 16 pins with a 4 × 4 format. A total of 3200 PCR amplified cDNA clones were spotted in duplicate onto 20 slides. The diameter of each spot was approximately 250 μm.

Generation of microarray probes, array hybridization, and posthybridization processes. Commercially available human heart total RNA (Ambion Inc., Austin, TX) was amplified by an antisense RNA amplification method according to Berry *et al.* (6) and labeled by Cy5- or Cy3-dCTP (NEN, Boston, MA) using standard protocols (1, 3). The probe mix containing fluorescently labeled amplified human heart cDNA was denaturated by heating for 5 min at 80°C in formamide hybridization buffer. Twenty microliters of the mix was placed on the array under a 24 × 32-mm coverslip. Slides were incubated at 42°C for 18 h in a humid hybridization box and then were washed by submersion and agitation for 10 min in 1× SSC with 0.1% SDS, for 10 min in 0.1× SSC with 0.1% SDS, and for 10 min in 0.05× SSC at RT; rinsed briefly in deionized water; and dried.

Scanning and data analysis. Each array was scanned by using a ScanArray Lite (GSI Lumonics, Billerica, MA) confocal fluorescent scanner with 10 μm resolution. Image analysis was performed by ScanAlyze2 software (7). For background corrections those data were calculated as negatives where the average intensity of the spot was smaller than two times of the average background of the same area.

Results and Discussion

Fluorescent-labeled cDNA for microarray analysis is obtained by RT of an RNA template in the presence of dye-labeled nucleoside triphosphates. According to the labeling protocols the RNA part of the RNA/labeled-cDNA heteroduplex is hydrolized with alkali immediately after the RT. This hydrolysis would degrade the NTPs; thus for the recovery before the hydrolysis, an additional DNA purification is performed using any commercially available PCR or DNA fragment isolation kit in order to separate the high-molecular-weight nucleic acids from the unincorporated nucleotides and short oligonucleotides. The purified heteroduplexes are degraded afterward and can be used for hybridization in microarray experiments. The flowthrough from the first purification step contains the unincorporated fluorescent-labeled NTPs and can be applied for the recovery. All PCR purification kits working with high-salt binding buffer are suitable for the purification. After dilution of the flowthrough, the pH was adjusted to slightly acidic and applied onto an equilibrated Oligo-Pak oligonucleotide purification column with a syringe. This column was originally developed for the purification of tritylated oligonucleotides and contains reverse-phase resin suitable for the binding of molecules having hydrophobic groups. Up to 70 nmol fluorescently labeled NTP could be bound onto this column and purified. For a better yield washing steps were performed with volume smaller than suggested for the tritylated oligonucleotide purification and treatment of the column with trifluoroacetic acid solution was omitted. With this purification only the

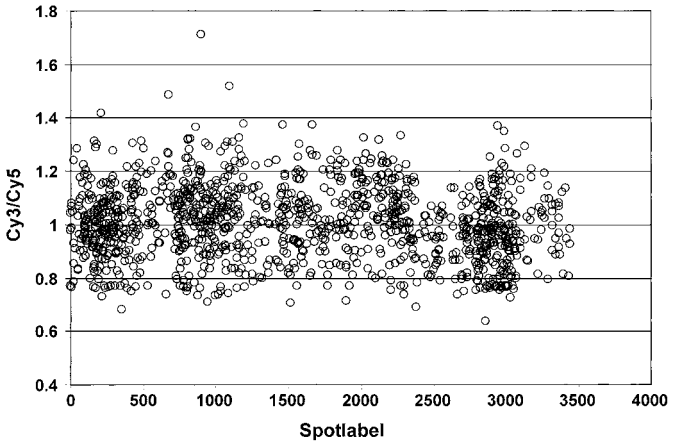

FIG. 1. Normalized, average signal distribution of 6400 spots (3200 data points) on the human cDNA microarray. Ratios were calculated from intensities of the Cy3 and Cy5 channels and normalized according to the mean intensities of the separate channels. Human heart aRNA was labeled with the commercially available Cy3-dCTP and with recovered Cy5-dCTP.

fluorescent labeled NTPs and labeled, short nucleic acids were recovered, and the unlabeled NTPs and nucleic acids were discarded. The reverse-phase column-purified labeled NTPs were further purified with size fractionation using membrane filters in order to separate the NTPs from the higher molecular weight, labeled nucleic acids. The purification protocol took approximately 4 h and provided an estimate yield of 60% according to the starting material. The purity of the recovered NTPs was confirmed by HPLC (data not shown). On HPLC chromatograms no impurities could be observed.

The recovered fluorescently labeled Cy5-dCTP and Cy3-dCTP were used for microarray analysis. Human heart amplified, antisense RNA (aRNA) was used as a template for synthesis of labeled cDNA during RT with standard protocols. As a control experiment the same template was labeled with commercially available Cy5-dCTP or Cy3-dCTP. cDNA labeled with recovered Cy5-dCTP was mixed with commercial Cy3-dCTP-labeled cDNA and hybridized onto a human microarray, and normalized, average signal distribution of 6400 spots (3200 data points) was determined. The same experiment was carried out with recovered Cy3-dCTP and commercial Cy5-dCTP-labeled cDNA. After data analysis all the low-confidence data derived from spots with high local background caused by dust particles or other impurity of the slide or from those exhibiting very low intensities were eliminated. Figure 1 demonstrates the tight distribution of data points deriving from the normalized ratio of intensities from commercially available Cy3-dCTP labeled cDNA and intensities from recovered Cy5-dCTP labeled cDNA. The color flip experiment gave the same tight distribution (data not shown). This means that purified NTPs did not distort the expression results and confirms that recovery can be performed without any risk in microarray experiments. Using recovered dye-labeled NTPs the expenses of microarray experiments can be decreased, especially when large number of samples are intended to be labeled and analyzed.

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REFERENCES

1. Schena, M., Shalon, D., Heller, R., Chai, A., Brown, P. O., and Davis, R. W. (1997) Parallel human genome analysis: microarray-based expression monitoring of 1000 genes. *Proc. Natl. Acad. Sci. USA* **93,** 10614–10619.
2. Gerry, N. P., Witowski, N. E., Day, J., Hammer, R. P., Barany, G., and Barany, F. (1999) Universal DNA microarray method for multiplex detection of low abundance point mutations. *J. Mol. Biol.* **292,** 251.
3. Eisen, M. B., and Brown, P. O. (1999) DNA arrays for analysis of gene expression. *Methods Enzymol.* **303,** 179.
4. Lockhart, D. J., Dong, H., Byrne, M. C., Follettie, M. T., *et al.* (1996) Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nature Biotechnol.* **14,** 1675.
5. Clontech Laboratories Inc. (2001) RNA Chip User Manual, No. PT3420–1, Clontech Laboratories Inc., Palo Alto, CA.
6. Barry, C., and Brown, P. O. (1999) Modified Eberwine ("Antisense") RNA Amplification Protocol, http://www.microarrays.org/pdfs/ModifiedEberwine.pdf.
7. ScanAlyze2 software (2000) http://www.microarrays.org/software.html.

pH-Stat Titration Allows the Continuous Determination of Ribonuclease A Activity toward Cytidine 2′,3′-Cyclic Monophosphate at High Substrate Concentrations

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The continuous spectroscopic determination of the hydrolysis of cytidine 2′,3′-cyclic monophosphate

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