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Table 1. Procedure for Biotinylation of Antibodies

1. Dialyze antibody^a at 4°C against 50 mM sodium bicarbonate, 50 mM sodium chloride, pH 8.5, to remove free amines.
2. Prepare a fresh 10 mg/mL working stock of EZ-Link™ Sulfo-NHS-LC-biotin^b (Pierce Chemical, Rockford, IL, USA) in distilled water. The biotin stock should be made immediately before use.
3. Mix the biotin stock with the dialyzed antibody to give a final concentration of NHS-LC-biotin = 0.1 mg/mL. Incubate on ice for 45–60 min.
4. Add 1/20th volume of 1 M glycine, 1 M Tris-HCl, pH 6.8, to quench the reaction.
5. Dialyze biotinylated antibody extensively^c at 4°C against Tris-buffered saline containing 50 mM Tris-HCl, pH 7.4, 150 mM sodium chloride with 0.02% sodium azide. Store at 4°C.

^aUse antibody that has been either antigen affinity-purified or purified with protein-A. Use a microdialyzer for small volumes of antibody; typical volumes can range 20–500 µL. Antibody concentration can range approximately 0.1–1.0 mg/mL.

^bThe NHS-LC-biotin is very sensitive to water. Always make sure the stock bottle has equilibrated to room temperature before opening.

^cIt is critical to remove all traces of free biotin with several changes of dialysis buffer.

the background staining was not seen when duplicate blots were probed with the avidin-peroxidase conjugate (Figure 1B, lanes 5'–8') ExtrAvidin® (Sigma, St. Louis, MO, USA). Unlike the secondary antibody conjugate used in Figure 1A, the avidin-peroxidase conjugate used in Figure 1B did not recognize the electrophoresed immunoglobulins within the immunoprecipitate. Moreover, specific bands of ezrin were readily detected in immunoprecipitates using biotinylated ezrin antibody and the ExtrAvidin-peroxidase, regardless of whether samples were reduced or not (Figure 1B, lanes 6 and 8). Comparison of serial dilutions of total placental microvilli revealed that biotinylation of the affinity-purified ezrin antibody had little or no detectable effect on its sensitivity or specificity (compare Figure 1A, lanes 1–4, and Figure 1B, lanes 1–4). Moreover, we have found that the sensitivity and specificity of more than a half dozen other biotinylated rabbit polyclonal antibodies were retained on blots. Because a wide range of biotinylated antibodies from different species is available through commercial suppliers, we speculate that biotin-labeling will also be applicable to many monoclonal antibodies. However, the effect of biotinylation on antibody sensitivity or specificity must be determined empiri-

cally for each immunoreagent. Table 1 describes a procedure for biotinylation.

Although ezrin was detected in immunoprecipitates electrophoresed under reducing conditions using the conventional method for blotting, the considerable amount of background staining would preclude visualization of many other bands, especially in the region of the IgG-heavy chain (Figure 1A, asterisk). However, using the improved method for blotting, it is possible to detect polypeptides even when they co-migrate with the IgG-heavy chain (4). Thus, our improved method considerably expands the number of different-sized bands that can be visualized on immunoblots of immunoprecipitates. In addition, this method is simple, rapid, economical, and requires only small quantities of antibody.

REFERENCES

1. **Berryman, M., R. Gary, and A. Bretscher.** 1995. Ezrin oligomers are major components of placental microvilli: a proposal for their involvement in cortical morphogenesis. *J. Cell Biol.* 131:1231–1242.
2. **Bretscher, A.** 1989. Rapid phosphorylation and reorganization of ezrin and spectrin accompany morphological changes induced in A431 cells by epidermal growth factor. *J. Cell Biol.* 108:921–930.
3. **Laemmli, U.K.** 1970. Cleavage of structural

proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

4. **Reczek, D., M. Berryman, and A. Bretscher.** 1997. Identification of EBP50: a PDZ-containing phosphoprotein that associates with members of the ERM family. *J. Cell Biol.* 139:169–179.

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Received 24 April 2001; accepted 15 June 2001.

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Digital Image Analysis for Rapid Quantification of Total RNA and cDNA for SMART™-PCR

BioTechniques 31:746-752 (October 2001)

Many molecular biology techniques such as cDNA library construction, differential display, subtractive or northern hybridization, microarrays, and direct cDNA selection need a high quantity of initial mRNA or cDNA, typically several micrograms. In many instances when experimental material is very limited or unique, a researcher does not have enough starting RNA to implement these powerful approaches. Fortunately, the development of SMART™ (switching mechanism at 5' end of RNA transcript) technology

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(Clontech Laboratories, Palo Alto, CA, USA) and the availability of long-distance (LD) PCR allow one to overcome this problem. SMART technology produces ss-cDNA enriched for complete 5' untranslated regions (UTRs) of mRNAs. Full-length cDNA molecules contain incorporated sequences for two primers and can be amplified exponentially in the following LD-PCR. Truncated molecules contain sequence only for one primer and can only be amplified linearly. Consequently, after 15–25 cycles of LD-PCR, it is possible to receive several micrograms of full-length ds-cDNA, starting only with 50–1000 ng total RNA (1) and maintaining at the same time the original message profile (2,3). The most important step in this approach, which dramatically influences the final result, is the condition of the ds-cDNA amplification (number of PCR cycles and longevity of different PCR steps). To obtain the best results, PCR should be in the exponential phase of the reaction. Fewer thermal cycles are less likely to generate nonspecific PCR products and are therefore best for optimal cDNA quality. At the same time, it is necessary to produce enough cDNA for downstream applications. Overcycling produces nonspecific PCR products that will be cloned in subsequent steps and substantially contaminate the final cDNA library. Different samples in one experiment, depending on the quality and quantity of initial total RNA, may need different numbers of cycles for successful amplification. Therefore, it is necessary to estimate the quantity and quality of amplified cDNA several times during the reaction. A low quantity of PCR product does not allow multiple measurements of the reaction yield with a spectrophotometer. Estimation of product concentration can be accomplished by spotting aliquots of the reaction on an agarose gel or minigel alongside samples of DNA of known concentration (4). These methods produce a rather rough estimation of the product concentration and were developed for measurement of individual bands. The relative amount of the ds-cDNA product has been evaluated by performing, in parallel with the experimental samples, cDNA synthesis using control mRNA of known concentration (1). During

PCR amplification, aliquots of each sample are analyzed on an agarose/ethidium bromide gel until the concentrations of cDNA approach the required level. This technique depends on a researcher's experience in visual estimation of DNA concentrations and can frequently result in over- or underestimating the amount of product.

In the present report, we described a simple, rapid, and robust electrophoresis-based assay for simultaneous evaluation of the quantity and quality of ds-cDNA during PCR amplification. Aliquots of reactions are analyzed by agarose/ethidium bromide gel alongside known dilutions of a 1-kb DNA ladder, and appropriate software is then used for the evaluation of the digital image. A calibration curve is then constructed for the particular gel using dilutions of the DNA ladder, and exact concentrations of experimental samples are subsequently calculated using the composed curve. This method provided rapid and accurate measurements of cDNA concentrations that allowed us to efficiently perform cDNA library construction and subtractive hybridization.

For cDNA library construction, total RNA was extracted from different sam-

ples of WI-38 fibroblasts using the TRIzol[®] method according to manufacturer's instructions (Invitrogen, Burlington, ON, Canada). Total fibroblast RNA (500 ng) or 250 ng placenta poly(A) RNA were used for the first-strand cDNA synthesis using the SMART III oligonucleotide (5'-AAGCAGTGGTA TCAACGCAGATGGCCATTATGGC-CGGG-3') and CDSIII/3' PCR Primer (5'-ATTCTAGAGGCCGAGGCGGCC-GACAACATG-d(T)₃₀VN-3', where N = any nucleotide; V = A, G, or C) from the SMART cDNA Library Construction kit (Clontech Laboratories) and SUPERScript[™] II (Invitrogen) reverse transcriptase. Amplification of cDNA by LD-PCR was performed using the Advantage[®]-GC 2 cDNA PCR kit (Clontech Laboratories), CDSIII/3' PCR Primer, and 5' PCR primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'). PCR was performed in 100- μ L reactions using the GeneAmp[®] PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA) with the following program: 95°C for 25 s, and *n* cycles of 95°C for 10 s, 68°C for 6 min, where *n* equals the number of cycles determined experimentally. The relationship between the amount of starting

Figure 1. Implementation of digital image analysis for measurement of ds-cDNA concentrations. Lanes 1–5 and 9–13, 5, 10, 25, 50, and 100 ng of 1-kb DNA ladder; lanes 6–8, experimental samples for cDNA libraries construction; lanes 6 and 7, 500 ng total RNA from different sources of WI-38 fibroblasts were amplified for 21 and 25 cycles, respectively; lane 8, 250 ng placenta mRNA was amplified for 18 cycles; lanes 14–18, inserts in the placenta cDNA library were amplified by LD-PCR for 7, 10, 13, 16, or 19 cycles; a band specific for placenta mRNA is visible. Top, digital images of agarose/ethidium bromide gels. Center, analysis of images using the Scion Image software. Bottom, calibration curves for DNA ladders constructed using Excel and data exported from Scion Image.

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Table 1. Estimation of cDNA Concentrations by Digital Image Analysis and Spectrophotometry

N	Number of LD-PCR Cycles	Concentration of DNA (ng/ μ L) Estimated by Digital Image Analysis ^a	Concentration of DNA (ng/ μ L) Measured by Spectrophotometer
1	7	8.9 $\pm$ 0.2	— ^b
2	10	38.2 $\pm$ 3.8	30
3	13	55.8 $\pm$ 6.1	54
4	16	75.3 $\pm$ 7.0	83
5	19	106.7 $\pm$ 8.2	100

Inserts in the placenta cDNA library were amplified by LD-PCR for 7–19 cycles and purified over GFX columns before spectrophotometric measurements and electrophoresis.

^aMean $\pm$ SD for three independent gels

^bLower than the sensitivity of spectrophotometer

RNA and the optimal number of thermal cycles may vary significantly between samples of different origin and different models of thermal cycler. The ds-cDNA appears as a 0.1–10 kb smear on the gel, with some bright bands corresponding

to the abundant mRNAs specific for a particular cell type.

After 15 cycles and after each additional three cycles, 5- μ L aliquots of reactions were analyzed on an agarose gel. For quantitation purposes, 5–100

ng of 1-kb ladder were loaded per lane on the same gel. This ladder has a size distribution very close to those of mammalian cDNAs and consists of 10 bands ranging from 75 to 517 bp and 13 bands from 1 to 12 kb. For a relative measurement using absorbance, it is desirable to have the same molecular weight distribution in both reference and experimental samples. After a short electrophoretic run (10 min at 20 V/cm), we received the digital image of the gel using the Gel Documentation System (UVP, San Gabriel, CA, USA). Any CCD or digital camera producing images in TIFF or BMP format can be used. It is important not to overrun the gel because experimental samples can become invisible over time. The image of the gel containing amplified cDNAs and dilutions of the ladder is represented in Figure 1A. The freeware program Scion Image (www.scioncorp.com) was used for background subtraction and quantitation of integrated optical density in cDNA smears and 1-kb ladder dilutions. Rectangular selection for measurement comprised the entire range of the 1-kb ladder. Primers occasionally can be seen after the RT-PCR but run lower than the first band in the ladder. Any freeware or commercial software intended for the analysis of one-dimensional electrophoretic gels can be used at this step. Data from such software can then be easily copied to the clipboard and processed by Microsoft® Excel® or another spreadsheet program. Using the series of 1-kb ladder dilutions, we can use Excel to create a calibration curve for the gel under investigation. It should be mentioned that by its nature the calibration curve is specific for a particular gel, run conditions (e.g., voltage, time, and agarose concentration), and conditions during photography (e.g., exposure time, distance to camera, and illumination). For the image in Figure 1, the 1-kb ladder concentrations are linear over more than one order of magnitude ranging from 5 to 100 ng. For the measurement of cDNA concentrations exceeding this range, additional dilutions of the ladder can be added, and exposure time should be adjusted. With the help of the calibration curve, the quantity of cDNA in each experimental sample can be calculated.

Simple macros for Excel can addi-

Figure 2. Estimation of total RNA concentrations using digital image analysis. Total RNA was extracted from HeLa cells using TRIzol reagent according to the manufacturer's recommendations (Invitrogen). Top, digital image of ethidium bromide-stained 1.1% native agarose gel. Lanes 1–7, dilutions of total RNA (1500, 1000, 500, 250, 100, 50, and 25 ng); lanes 8–16, experimental RNA samples, 3 μ L per lane; lanes 8 and 16, control cells; lanes 9–15, HeLa cells 15, 30, 60, 120, 180 min, 24 and 48 h after UV irradiation, 50 J/m²; lane M, GeneRuler™ 100-bp DNA ladder (MBI Fermentas, Hanover, MD, USA). Bottom left, lane profile plot generated by Scion Image for the gel. Rectangular selection for measurement is shown on the gel image. Bottom right, calibration curve for the gel, quantity of total RNA in lanes 1–7 are plotted against measured absorbance.

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tionally accelerate plot drawing and calculations. In the course of cDNA library construction, we measured cDNA concentrations during LD-PCR, after *Sfi*I digestion, and before ligation to vector. It allowed us to generate high-quality cDNA libraries, more than 2×10^6 independent clones, 90%–99% of recombinant clones, with less effort.

To confirm the accuracy of the measurements in our assay, we compared data obtained by digital image analysis of gels and by spectrophotometer measurements. Using LD-PCR, we amplified the pool of DNA from constructed placenta cDNA library after 7, 10, 13, 16, or 19 cycles, purified each on a GFX column (Amersham Pharmacia Biotech, Baie d'Urfe, QC, Canada), and eluted products with 50 μ L water. The quantities of the resultant PCR products were estimated after electrophoresis (Figure 1B). Using the series of 1-kb ladder dilutions, we created a calibration curve for this gel and calculated the concentrations of PCR samples. In parallel, we quantified the concentrations of PCR products by spectrophotometry on a Hewlett Packard 8451A spectrophotometer. The comparison of spectrophotometer measurements and digital image analysis showed a very good concordance (Table 1).

Remarkably, we could use only 5 μ L from 50 μ L purified PCR product for digital analysis, but it was necessary to use at least 20 μ L of the same product for spectrophotometric measurement. The use of new intercalating dyes such as GelStar[®] (BioWhittaker, Walkersville, MD, USA) or SYBR[®] Green (Sigma, St. Louis, MO, USA) for staining of nucleic acids in gels can additionally increase the sensitivity of the entire assay. Digital image analysis not only requires less material than a spectrophotometer but also can be faster, especially when multiple samples are analyzed. This procedure takes only 20 min, including the 10-min gel run.

In addition, the substitution of the DNA ladder for dilutions of total RNA with known concentrations allowed us to use the proposed digital image analysis technique for estimation of the quantity and quality of total RNA samples (Figure 2). Note that multiple experimental samples can be analyzed simultaneously in contrast to spec-

trophotometric measurements. Data generated by digital imaging for total RNA demonstrated good correlation with spectrophotometer measurements (data not shown).

Digital image analysis is becoming a more powerful instrument for life research. We demonstrate that this technique can be successfully implemented for the measurement of ds-cDNA and total RNA concentrations. In comparison with traditional spectrophotometric measurements, digital image analysis has higher sensitivity, demands less material, and is a timesaving tool.

REFERENCES

1. **Chenchik, A., Y.Y. Zhu, L. Diatchenko, R. Li, J. Hill, and P.D. Siebert.** 1998. Generation and use of high-quality cDNA from small amounts of total RNA by SMART[™] PCR, p. 305-319. *In* Gene Cloning and Analysis by RT-PCR. Eaton Publishing, Natick, MA.
2. **Ende, W.O., K.E. Steinmann, L.A. Boardman, S.N. Thibodeau, and R. Schlegel.** 1999. Representative cDNA libraries and their utility in gene expression profiling. *BioTechniques* 26:542-548.
3. **Herrler, M.** 2000. Use of SMART-generated cDNA for differential gene expression studies. *J. Mol. Med.* 78:B23.
4. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.

This work was supported by a grant from the Defense Advance Research Project Agency of the Department of Defense, U.S.A. to Eugenia Wang. Address correspondence to Dr. Eugenia Wang, Department of Biochemistry and Molecular Biology, School of Medicine, University of Louisville, Louisville, KY 40292, USA. e-mail: eugenia.wang@louisville.edu

Received 19 March 2001; accepted 15 June 2001.

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Protocol for Small-Scale RNA Isolation and Transcriptional Profiling of Developing Arabidopsis Seeds

BioTechniques 31:752-758 (October 2001)

Seeds provide much of the economic value in agricultural products, and numerous research efforts are aimed toward understanding the regulation of the storage compound accumulation in crop seeds. With its fully sequenced genome, *Arabidopsis thaliana* (thale cress) has now become the dominant model system in dicot plant science, including seed biology. Unfortunately, Arabidopsis seeds are very small and not always amenable to study. For example, the application of microarray technology to large-scale gene expression profiling in Arabidopsis seeds can be limited because of the relatively large amount of RNA needed to prepare probes. Another potentially restrictive factor is that seeds are difficult material for RNA isolation because of high amounts of secondary metabolites and polysaccharides. In our experience, commercial kits and widespread plant RNA extraction methods are unsatisfactory in preparing microarray probes using Arabidopsis or other seeds. Here, we describe a rigorous procedure that enabled us to isolate high-quality RNA and perform successful microarray experiments using small amounts of developing Arabidopsis seeds.

A. thaliana (ecotype Col-2) seeds were dissected from siliques between 5 and 13 days after flowering, immediately frozen with dry ice, and stored at -80°C. The fresh weight of developing Arabidopsis seeds is in the range of 10–30 μ g each, so 3000–10 000 seeds (80–250 siliques) were harvested for each extraction. The extraction buffer presented here is a combination of buffers described in Schultz et al. (10) and Chung et al. (4). Thiourea was used as a chaotropic reagent because RNA extractions from plant material using guanidine buffer often fail (2). The addition of three different detergents (7) also improved both yield and quality of