Benchmarks

MD, USA) were used. In the case of comparing DNA of different origins (e.g., cloned cDNAs, isolated genomic DNA or λ-cloned genomic DNA), preliminary PCRs were performed initially with flanking 5’ and 3’ primers (Figure 1B, hatched arrows) to obtain DNA templates of similar size for the analytical PCRs. We used 2 μL of a 1/1000 dilution of the preliminary PCRs as template. All PCRs contained 1× PCR Buffer (Life Technologies), 2 mM MgCl₂, 200 μM dNTP, 0.5 μM of each primer and 1 U Taq DNA polymerase per 50 μL volume. To minimize pipetting variations, master mixtures were prepared containing all ingredients except primers and templates. Thirty cycles were performed as follows: 1 min at 94°C, 1 min at the respective annealing temperature and 1 min at 72°C. Ten microliters of each 50 μL PCR were loaded onto a standard TBE 1.2% agarose gel. The concentration of Taq DNA polymerase was found to be the most crucial parameter for a successful discriminating reaction.

Interestingly, the frame-shift discrimination works over a broad annealing temperature range, although to variable extent (Figure 2A). However, the appropriate annealing temperature has to be tested in accordance with the calculated melting temperature of the respective primers before the ARMS application. The Taq DNA polymerase concentration that gave the most consistent success in discriminating between 5 T and 6 T residues in a variety of DNA templates, including cDNA and genomic clones, was 1.0 U/50 μL reaction (Figure 2B). The use of higher concentrations of Taq DNA polymerase diminished the discriminating capacity of the ARMS technique (Figure 2B, lanes 10–13). Using optimized conditions, sequences of cDNA containing 5 T residues (V.1) were distinguished from cDNA and genomic clones containing 6 T residues (Figure 2C; λ3.2, IV.8 and II.2.4). The PCR results in Figure 2C have been confirmed by DNA sequencing.

Clones obtained from site-directed mutagenesis experiments of cDNA V.1 (5T) using the MORPH™ Mutagenesis Kit (5 Prime→3 Prime, Boulder, CO, USA) and primer GFA12-T6 (5’-CG-ACTCTGCTTTTTATATCGAGT-CCG-3’) were analyzed under the same conditions (Figure 2D). Analytical PCR was performed with both primer sets (Figure 2D). Clone VI.1 was found positive for 6 T residues, which was confirmed by DNA sequence analysis. Clones VI.2–VI.6 contained 5 T residues.

The method presented here distinguishes wild-type and mutant DNA sequences within homopolymeric nucleotide tracts by means of their different performances as PCR templates. Mutations in homopolymeric DNA tracts can be detected using the ARMS technique; however, diagnostic (analytical) primers must be designed so that the mutated nucleotide to be detected does not lie at the 3’ end of the primer, as in the standard method, but approximately 2–4 bases towards the 5’ end. However, analytical primers have to be designed very carefully in respect to the properties of the homopolymeric tract; short [3–4 nucleotides (nt)] and/or G/C homopolymeric tracts require longer extensions (3–4 nt) beyond the homopolymeric tract, while longer (>4 nt) and/or A/T homopolymeric tracts require short (2–3 nt) 3’ extensions of the primers. Having 2–4 non-repetitive bases at the 3’ end of the primer worked consistently in the experiments reported here. We have also successfully applied this method for discriminating poly(G) tracts of different lengths (data not shown). The method presented expands the possible applications of the ARMS. However, the discrimination of both the wild-type and the mutated templates is only quantitative in most cases. Therefore, PCRs using both primer sets must be performed in parallel. High template concentrations may enable some amplification from the “wrong” primer pair.

REFERENCES


Received 16 February 1999; accepted 6 July 1999.

Hanns-Rüdiger Graack and Horst Kress
Institut für Genetik
Freie Universität Berlin
Berlin, Germany

Empirical Midpoint Dissociation Temperature (Tₘ) Determination for Oligonucleotide Probes Using a PCR Thermal Cycler

BioTechniques 27:666-670 (October 1999)

Investigators in applied microbiology and molecular microbial ecology use oligonucleotide probes to quantify nucleic acids from specific microbial
populations in environmental samples against a background of many unknown populations (1,3,5,7). Such applications require that probes discriminate against unknown, nontarget nucleic acids that may have only a one-base mismatch from the intended target. Therefore, an essential feature of quantitative probing of environmental samples is accurate determination of the melting characteristics of the probe-target duplex. For this purpose, it is not sufficient to calculate melting temperatures based on free-energy parameters, which frequently misestimates melting temperatures by 2°C–10°C (2). Instead, empirical determination of the melting temperature is required (8).

The preferred parameter for characterizing melting dynamics of oligonucleotides (<50 bp) is the midpoint dissociation temperature (T_d), the temperature at which 50% of the probe initially hybridized with its target sequence, which is bound to a support membrane, dissociates from the target within a specified time period and in a wash buffer of specified composition (8). An approach used in molecular microbial ecology (e.g., References 4 and 6) is to place dot/slot blots with hybridized probe into scintillation vials containing 2–3 mL of wash buffer. The vials are then incubated in a water bath, which must be adjusted to successively higher temperatures to complete the assay. The T_d is determined based on the amount of probe that dissociates from the blot at each temperature.

A complete dissociation curve consists of 10–15 different temperatures; at 10 min each, the incubations alone require 2–3 h. If a single water bath is used, the total time to complete the assay may be increased substantially by downtime required to adjust the temperature of the heating apparatus between incubations. This procedure may require 5–6 h. Some laboratories reduce or eliminate downtime by using 3–4 water baths, so that vials preheat while the blots incubate at a lower temperature in a different bath. This approach increases equipment needs and is tedious if inexpensive analog thermostats are used, requiring the temperature to be monitored until it equilibrates at the desired set point. Under this scenario, we could complete up to 3 dissociation curves in approximately 4 h. To reduce downtime and eliminate the tedium of monitoring multiple water baths, as well as to improve temperature precision and stability, we adapted the protocol of Raskin et al. (6) to be carried out in a polymerase chain reaction (PCR) thermal cycler.

Oligonucleotide probe S-D-Bact-0338-a-A-18 (5'GCTGCTCCCCGTAGGAGT-3') targets a segment of the 16S rRNA of virtually all members of the domain Bacteria and is widely used in quantitative hybridization studies in laboratory and environmental samples (1). We used this probe targeted against Escherichia coli 16S rRNA extracted from a pure culture to compare T_d curves generated in a water bath to curves generated in a PCR cycler. Total RNA was isolated from approximately 10^9 late-log-phase E. coli cells using the RNeasy® Kit (Qiagen, Valencia, CA, USA). Dot blots were prepared with 100 ng of E. coli 16S rRNA per dot. The dot blots were prepared as described previously (4) using a Minifold® II Microsample Filtration Manifold (Schleicher & Schuell, Keene, NH, USA) with 4-mm diameter dots. Commercially synthesized oligonucleotide (DNagency, Malvern, PA, USA) was end-labeled enzymatically with 32P as described previously (6). The labeled oligonucleotide was hybridized to the dot blots overnight at 30°C in 5 mL of hybridization buffer (0.9 M NaCl, 50 mM sodium phosphate, pH 7.0, 5 mM EDTA, 10× Denhardt solution, 0.5% sodium dodecyl sulfate (SDS)), finishing with two 30-min rinses in 15 mL of wash buffer (1% SDS–1× SSC [0.15 M NaCl, 0.015 M sodium citrate, pH 7.0]) at 40°C. Each dot was cut into approximately 6-mm squares for insertion into 6-mL scintillation vials or 0.5-mL microcentrifuge tubes.

For comparison, a pair of probe-labeled dot blots were assayed using a water bath, and a replicate pair were assayed using a PCR cycler. A Model 254 Circulating Water Bath with a digital thermostat (GCA, Precision Scientific Group, Chicago, IL, USA) was used for the water bath protocol. Twenty 6-mL scintillation vials containing 2 mL of wash buffer were placed in the water bath. An additional vial held a thermometer for monitoring the temperature of the wash buffer. Duplicate dot blots were placed in separate, preheated vials and incubated at 30°C for 10 min. The blots were then removed from the vials and sealed in plastic wrap to prevent drying. The temperature of the water bath was increased to 35°C, and the blots were placed in a new pair of vials and incubated for 10 min. This cycle was repeated, increasing the temperature by 5°C each time, until the final incubation at 75°C was completed for a total of 10 temperatures. Equilibration of the buffer temperature between incubations required 10–20 min with downtime increasing with temperature. After the final incubation, the blots were placed into separate vials containing 2 mL of fresh wash buffer.

Using a replicate pair of dot blots, a similar procedure was followed using a PCR thermal cycler (Delta Cycler II™; Ercomp, San Diego, CA, USA) equipped with a block accommodating 60 0.5-mL microcentrifuge tubes. Twenty tubes were filled with wash buffer (0.5 mL). The Delta Cycler II uses an external temperature probe inserted into a reference microcentrifuge tube, which was also filled with wash buffer. Separate PCR cycler programs were used for each incubation temperature, and the programs were initiated and terminated manually. Each program would hold the heating block at a specified temperature for 30 min, allowing the dissociation of probe from multiple blots to be monitored at a single temperature. This was repeated for each of the 10 temperatures, resulting in 10 dissociation curves in approximately 3 h.

Figure 1. Dissociation curves from the water bath and PCR cycler incubations. Each symbol represents the mean dissociation of probe from duplicate dot blots. Error bars represent ±1 standard deviation. When not visible, error bars are contained within the symbols. The dashed lines show extrapolation from the midpoint of the y-axis to the corresponding T_d on the x-axis.
lowing excess time for equilibration of wash buffer to the set temperature and manual timing of the 10-min incubations. The blots were incubated at 10 temperatures ranging from 30°–75°C as above. Downtime between incubations was <2 min. Following the incubations, the blots were placed into separate microcentrifuge tubes containing 0.5 mL of fresh wash buffer. The contents of each microcentrifuge tube were poured into a 20-mL scintillation vial, and the tube itself was inserted into the vial.

For both sets of samples, 3 mL of scintillation cocktail (Ultima Gold™; Packard Instrument, Meriden, CT, USA) were added to each scintillation vial, and the radioactivity counts per minute (cpm) of each sample was measured using a Model LS 1801 Scintillation Counter (Beckman Instruments, Fullerton, CA, USA). For a given blot, the activities from all incubations plus the washed blot were summed to determine the total probe activity. The individual activity for each temperature and the washed blot was then divided by the total activity and multiplied by 100 to determine the percent of probe that was dissociated from the blot at each temperature and the percent of probe that remained bound to the blot after washing. These activities were plotted against the corresponding incubation temperatures to produce a dissociation curve.

Extrapolation from the midpoint of the y-axis (50% bound probe) to the corresponding temperature on the x-axis shows that both curves produced the same estimated T_d of 53°C (Figure 1). This agrees well with the previously published T_d value of 54°C for probe S-D-Bact-0338-a-A-18 (8). We have also found agreement between the water bath and PCR cycler protocols for several other oligonucleotides (not shown). Because oligonucleotide melting dynamics are concentration dependent (8), we were concerned initially that the smaller volume of wash buffer used in the PCR cycler protocol would produce higher estimated T_d's than the water bath protocol. This concern was alleviated, however, as the two protocols produced virtually identical results.

Generating T_d curves in a PCR cycler reduced the time required to produce the plots in Figure 1, from approximately 5 h (using a single water bath) to approximately 2 h, yet the results were comparable. In a 60-well PCR cycler, T_d values for up to 6 different probes could be determined simultaneously in <3 h, thus effectively increasing the data/time ratio, relative to using a single water bath, about tenfold. Other benefits included tighter control over temperature precision and fluctuation resulting from the superior design of PCR cyclers over most water baths, a smaller work area over which radioactive samples were distributed, no risk of spilling radioactive liquid into a water bath and reduced consumption of supplies. This modified protocol will be particularly useful for those laboratories that have a PCR cycler but are not equipped with several water baths that can be used simultaneously for T_d determinations. Laboratories currently using multiple water baths to reduce downtime may find this protocol more convenient, less tedious and potentially more reproducible.

REFERENCES


We thank M.F. Polz for technical assistance and suggestions to improve the manuscript. We thank M.A.J. Hullar for useful discussions about the protocol. This work was funded by a National Science Foundation grant (DEB-9708092). Jay Gulledge is a DOE-Life Sciences Research Foundation Postdoctoral Fellow. Address Correspondence to Colleen M. Cavanaugh, Harvard University, The Biological Laboratories, 16 Divinity Ave., Cambridge, MA 02138, USA. Internet: cavanaugh@fas.harvard.edu

Received 15 April 1999; accepted 15 June 1999.

Jay Gulledge and Colleen M. Cavanaugh
Harvard University Cambridge, MA, USA