

Molecular Beacon–Based Temperature Control and Automated Analyses for Improved Resolution of Melting Temperature Analysis Using SYBR I Green Chemistry

CHRISTOFFER NELLÅKER,^{1*} ULF WÅLLGREN,² and HÅKAN KARLSSON¹

Background: Melting temperature analysis of products amplified with SYBR I Green chemistry is a cheap and effective method for identification of sequence differences. When used in conventional quantitative real-time PCR instruments (qPCR), this method is limited by temperature variations over the heating block and low numbers of fluorescence measurements during the dissociation step, which hamper the ability of most instruments to report accurate and precise melting temperatures.

Methods: We designed a molecular beacon–based temperature indicator probe (T_m-probe) to control for variations in temperatures over the heating block of the instrument. In addition, we wrote an automated curve-fit analysis algorithm of dissociation data to use multiple data points with a gaussian curve fit to extrapolate precise melting temperatures.

Results: Use of the T_m-probe in conjunction with the analysis algorithm and multiple dissociations improved SDs of melting temperatures over a 96-well plate from 0.19 to 0.06 °C

Conclusions: Melting temperature analyses with SYBR I Green chemistry on conventional qPCR instruments can be improved by the use of a T_m-probe in conjunction with curve-fit analysis of data. Resolution improvement up to 3-fold is possible and allows additional melting temperatures to be identified.

© 2007 American Association for Clinical Chemistry

Quantitative real-time PCR (qPCR) analyses are commonly used and are rapid methods for the analysis of nucleic acid levels within both clinical and basic sciences. This method uses fluorescent dyes to indicate levels of amplified PCR products as the reactions proceed (i.e., in real time). Sequence specificity of amplicon detection in qPCRs varies from the most specific Minor Groove–Binding-probes, to Taqman-probes (1), to the indiscriminate SYBR I Green detection system, which merely fluoresces with increased intensity when bound to double-stranded DNA. For economic reasons, however, SYBR I Green chemistry is one of the more widely used detection systems in qPCR. To check for erroneous products, a dissociation step must be run after the amplification has completed. During this step, the instrument gradually heats the amplified reactions and measures the decrease in fluorescence signal as the 2 strands of the products dissociate. The temperature at which the rate of signal decline is maximal (i.e., the peak of the negative derivative of the fluorescence measurements) is defined as the melting temperature (T_m) and is related to the base-pair composition of the product. Therefore in addition to detecting erroneous products, T_m analyses can indicate sequence variations between amplicons. T_m analyses have been recently employed for strain identification in clinical and veterinary virology (2, 3), typing of bacterial strains (4), identification of expression patterns of highly homologous genomic elements (5), and genotyping of HLA variants (6). Furthermore, this approach has previously been used to detect translocations in cancers (7) and to scan for single-nucleotide polymorphisms (8).

¹ Department of Neuroscience, Karolinska Institutet, Stockholm, Sweden.

² FLIR Systems AB, Danderyd, Sweden.

* Address correspondence to this author at: Retzius väg 8, 171 77 Stockholm, Sweden. Fax: +468325325; e-mail christoffer.nellaker@ki.se.

Received June 19, 2006; accepted October 19, 2006.

Previously published online at DOI: 10.1373/clinchem.2006.075184

³ Nonstandard abbreviations: qPCR, quantitative real-time PCR; T_m, melting temperature; FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine; BHQ, Black Hole Quencher-2; FRET, fluorescence resonance energy transfer; GcT_m, Gaussian curve fit analysis of T_m.

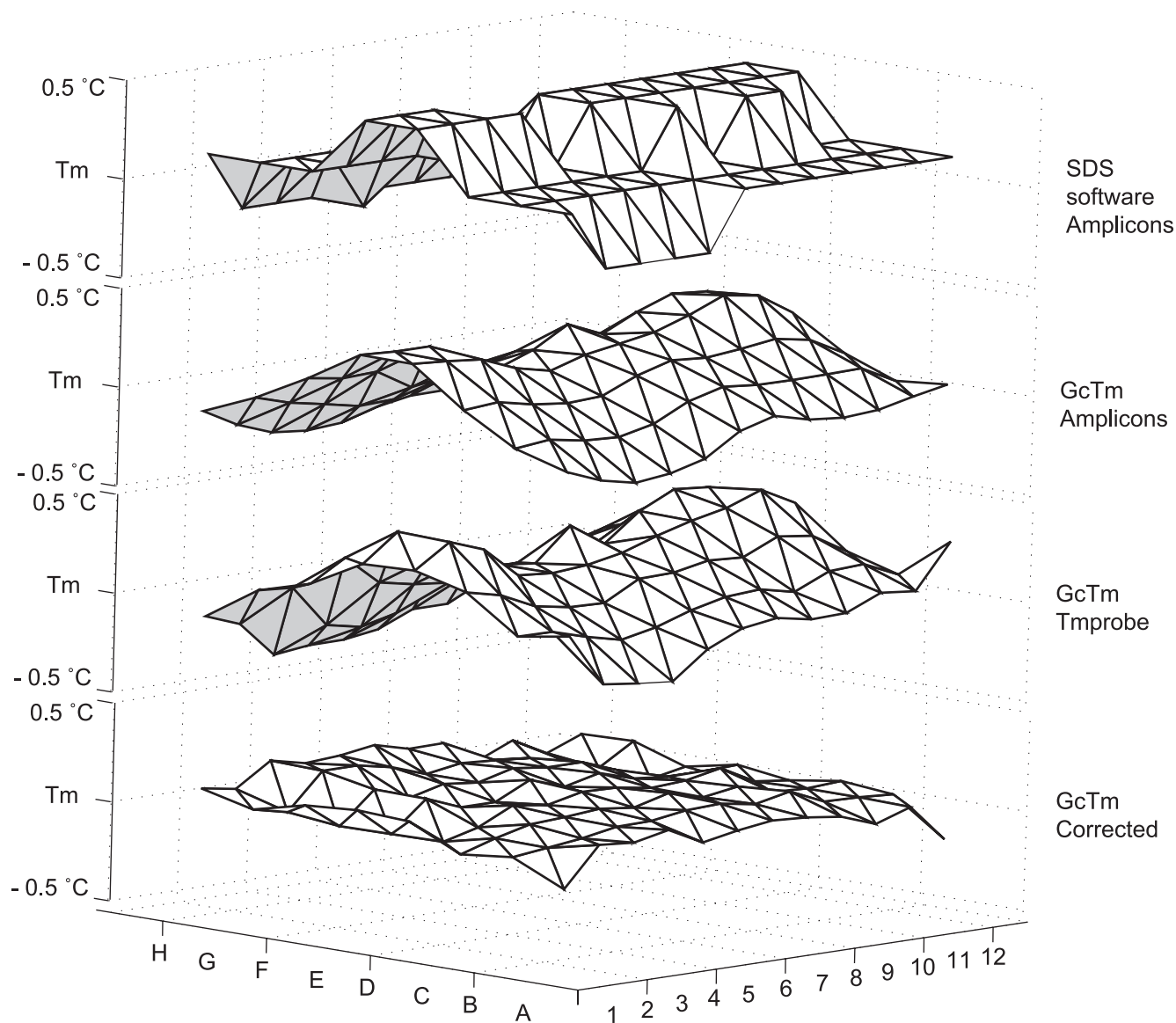


Fig. 3. Distribution of reported temperatures over a 96-well plate in an ABI Prism 7000 for 1 template sequence.

Top, one example of the Tms reported by the SDS software. *Upper middle*, indicates the amplicon Tms reported by SYBR and calculated by the Tm-analysis program. *Lower middle*, Tm-probe Tms as calculated by GcTm Tm-analysis program. *Bottom*, the normalized Tms of the amplicons, (i.e., calculated Tms corrected for temperature variations with the Tm-probe data). The lower 3 plots represent data averaged for 3 dissociation curves.

cies of the assay in the presence or absence of the Tm-probe were calculated as previously described (1).

TM PROBE DESIGN

To allow detection in an ABI Prism 7000 simultaneously as SYBR I Green fluorescence, a molecular beacon (12, 13) was designed to have a stem structure with a Tm higher than those observed for the target transcript amplicons (85 °C), for which the SYBR signal is minimal [Web page for tm analysis and generation of Fig. 2 folding <http://www.bioinfo.rpi.edu/applications/mfold/dna/form1.cgi> (14–16)]. During denaturation, the fluorescence of molecular beacons increases rather than decreases upon melting, allowing the derivative curve of dissociation data to be easily

distinguished from that of any SYBR products. To obtain absorption and emission wavelengths appropriate for the instrument, we used a wavelength-shifting molecular beacon design (17). The molecular beacon was triple-labeled with 6-carboxyfluorescein (FAM) in the 5' end, 6-carboxytetramethylrhodamine (TAMRA) attached to the 6th thymidine from FAM, and Black Hole Quencher (BHQ)-2 in the 3' end (Fig. 1). In the hybridized configuration the FAM in the Tm-probe absorbs the 485-nm excitation provided by the instrument. The high-energy state FAM undergoes fluorescence resonance energy transfer (FRET) to TAMRA, which in turn donates its energy through FRET to the BHQ-2. At temperatures >85 °C the molecular beacon undergoes a conformational change, and the TAMRA will no longer

transfer any energy to BHQ-2 because it is no longer in close enough proximity and will thus fluoresce at 580 nm. The Tm probe was purchased from MedProbes (Eurogentec).

TM ANALYSIS

We used the MATLAB™ (The MathWorks) version 7.0.1.24704 with The Optimization Toolbox to write an automated analysis algorithm for data from Sequence Detection Software version 1.2.3 used in conjunction with an ABI Prism 7000. The program, gaussian curve fit analysis of TM (GcTm), was designed to determine Tms of amplicons by fitting gaussian curves to derivative data from dissociation analyses (Fig. 2). The peak of the negative derivative data is automatically selected by taking the values differing from the mean derivative over all temperatures by at least 1.2 SDs. We also designed the program to use the Tm of the Tm-probe to normalize temperatures of amplified products reported from the instrument in each well. The Tm normalization calculation took the Tms, determined by GcTm, of the amplicon minus that of the corresponding Tm-probe plus the average of all the Tm-probes used in that experiment. The program is available for download at <http://www.neuro.ki.se/kristensson/tmanalysis.html>.

Results

To evaluate the Tm analysis methods we used a plasmid template in 25- μ L reactions in all wells of a 96-well plate. The Tms reported by the SDS software over the 96-well plate show a range of 0.6 °C in reported temperatures (SD 0.19 °C). Each Tm was reported as 1 of 3 discreet temperatures (Fig. 3). Analysis with the GcTm algorithm of the dissociation data gave Tms with higher resolutions than those reported by the SDS software, based on a gaussian curve fitted to multiple data points (on average 5.25, Fig. 3), but the systematic variations in reported temperatures remained (range 0.66 °C). To control for the temperature variations over the heating block, a molecular beacon-based Tm-probe was designed (Fig. 1). Gaussian curves fitted to derivative dissociation curve data for the SYBR and TAMRA detectors are shown in Fig. 2. The negative derivative dissociation data for the Tm-probe with the TAMRA detector displays an artifactual peak with changes in SYBR fluorescence, because in our assay SYBR has a broad emission spectrum and fluoresces an order of magnitude more than TAMRA. In the interest of cost-effectiveness, we used the lowest concentration of Tm-probe giving reliable Tms with low variation. The Tms for the Tm-probe calculated with GcTm showed variations over the heating block similar to those of the amplicons (Fig. 3). Correcting the Tms of the amplicons by subtracting those of the Tm-probe (and adding the average Tm of all the Tm-probes) gave a profile over the 96-well plate with no apparent systematic variations with regard to well position (Fig. 3). Variations in reported Tms had an SD of 0.18 °C for the SYBR alone and an SD of 0.12 °C when corrected with the Tm-probe (Fig. 4). The

Tm-probe showed a higher variation in Tms within wells than the amplicons. In addition, although the TAMRA fluorescence displayed spatial variation similar to that of SYBR I Green, the TAMRA fluorescence fluctuated more. Therefore the Tm-probe showed high accuracy but lower precision. To reduce variations, we set the ABI Prism 7000 to run 2 additional dissociation curve analyses immediately after the primary run. These dissociations were analyzed individually with GcTm and the resulting Tms averaged. The mean of 3 dissociations resulted in an SD of 0.17 °C for the SYBR alone and an SD of 0.06 °C when corrected with the Tm-probe (Fig. 4). Additional dissociations beyond the 3 described did not further decrease the SDs of the corrected Tms. To illustrate the improved precision and accuracy, we analyzed 4 different plasmids with variations in the target sequences (Fig. 5A). The addition of the Tm-probe allowed the identification of 3 distinct groups of Tms, indicating at least 3 unique sequences that were indistinguishable without correction of amplicon Tms (Fig. 5B). Uncorrected and corrected Tms for each of the 4 plasmid templates are shown in Fig. 5C to demonstrate that sequence variations do not necessarily give Tm differences. Although the Tm-probe described in the present work had no effect on reaction efficiency (data not shown), the fluorescence emitted by FAM, which is not transferred by FRET to TAMRA, increased the background and thereby reduced the signal-to-noise ratio from 9 to 4.5.

Discussion

According to the manufacturer's specifications of the ABI Prism 7000 instrument, the temperature uniformity over a 96-well block is ± 0.5 °C, a feature that limits the obtainable resolution when Tms of amplicons are examined. Using the Tm of a plasmid template as a reporter, we

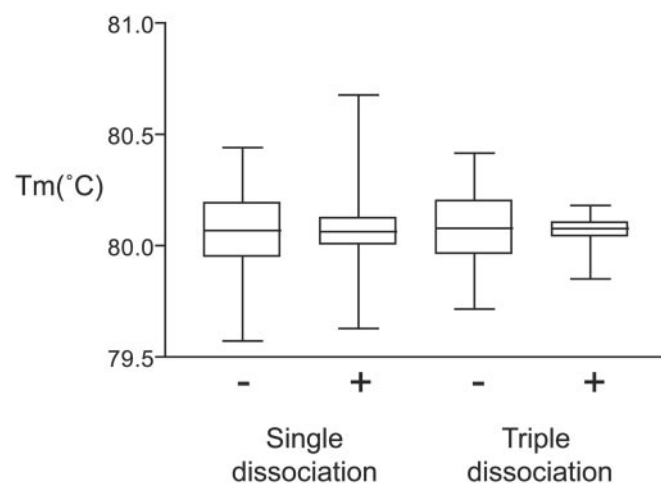
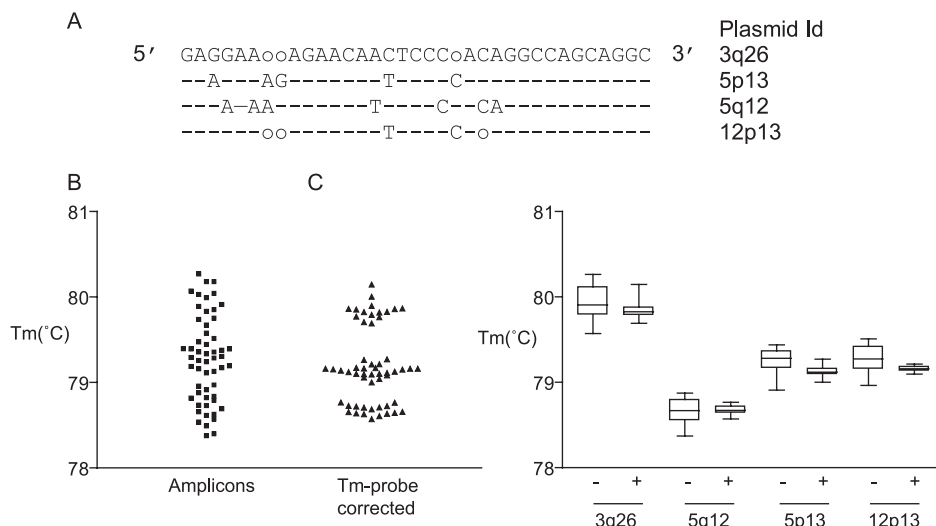


Fig. 4. Reported Tms for 1 template in a 96-well plate.

- represents amplicon Tms for the SYBR dye alone calculated with the Tm-analysis program. + indicates amplicon Tms corrected with the Tm-probe. *Single dissociation* indicates data from a single dissociation curve, analyzed with GcTm. *Triple dissociation* indicates that the data is the mean of 3 repeated dissociation analyses on the same 96-well plate and analyzed with GcTm.

Fig. 5. Demonstration of improved resolution in detection of 4 different amplification targets.

(A), target sequences of plasmid templates. *Dashes* indicate nucleotide identical to the alignment sequence. *Open circles* indicate gaps in sequences introduced to optimize the alignment. (B), T_m variations of amplicons from the 4 different templates detected with and without Tm-probe temperature correction. (C), the same data divided into the respective template sequences used, - indicate GcTm calculated Tms, + indicate GcTm-calculated Tms corrected with Tm-probe.



found that higher T_m s were reported consistently in the middle of the 96-well reaction plate and that T_m s were reported as discreet temperatures (Fig. 3). We wrote an analytical algorithm to extrapolate accurate T_m s from multiple data points. Using this algorithm in conjunction with a molecular beacon-based Tm-probe, we improved resolution 3-fold.

Some caveats should be considered with the use of the proposed T_m analyses. The reason for the observed variations in Tm-probe melting temperatures between wells is not known. However, the tests performed have shown a greater variation in wells flanked by empty wells or in wells at the corners of the plate, possibly a sign of evaporation effects and salt-concentration changes affecting the Tm-probe to a larger extent than the amplicons.

Furthermore, for T_m analyses of multiple sequences in the same reactions, SYBR I Green chemistry may not be optimal and other dyes (such as LCGreen) have been suggested to be more appropriate (18). Although the use of LCGreen can improve the determination of amplicon T_m s, it will not eliminate the need for 3 dissociation analyses to be performed, because the primary source of variation stems from the Tm-probe measurements rather than those of the amplicons. SYBR I Green chemistry is adequate for comparison of T_m s of amplicons between wells. It should be noted that differing sequences can share the same T_m , as is the case with the plasmids corresponding to the HERV-W *gag* on chromosomes 5p13 and 12p13.

In conclusion, we report methodological improvements for T_m analyses on an ABI Prism 7000 instrument. By use of the addition of a Tm-probe in combination with a curve-fit method (GcTm) and repeated measurements to determine T_m s of amplicons, we have improved T_m precision from ± 0.36 °C to ± 0.12 °C (with a 95% confidence interval).

This study was generously supported by the Stanley Medical Research Institute and the Swedish Research Council (project no. K2006-21X-20047-01-3). The authors declare that they have no competing interests. We thank Cecilia Eckervig from MedProbes whose expertise aided the design of the Tm-probe. We also thank Dr. Yuanrong Yao in our laboratory for providing the plasmids used as templates.

References

1. Yao Y, Nellaker C, Karlsson H. Evaluation of minor groove binding probe and Taqman probe PCR assays: influence of mismatches and template complexity on quantification. *Mol Cell Probes* 2006; 20:311–6.
2. Waku-Kouomou D, Alla A, Blanquier B, Jeantet D, Caidi H, Rguig A, et al. Genotyping measles virus by real-time amplification refractory mutation system PCR represents a rapid approach for measles outbreak investigations. *J Clin Microbiol* 2006;44:487–94.
3. Pham HM, Konnai S, Usui T, Chang KS, Murata S, Mase M, et al. Rapid detection and differentiation of Newcastle disease virus by real-time PCR with melting-curve analysis. *Arch Virol* 2005;150: 2429–38.
4. Harasawa R, Mizusawa H, Fujii M, Yamamoto J, Mukai H, Uemori T, et al. Rapid detection and differentiation of the major mycoplasma contaminants in cell cultures using real-time PCR with SYBR Green I and melting curve analysis. *Microbiol Immunol* 2005;49:859–63.
5. Nellaker C, Yao Y, Jones-Brando L, Mallet F, Yolken RH, Karlsson H. Transactivation of elements in the human endogenous retrovirus W family by viral infection. *Retrovirology* 2006;3:44.
6. Graziano C, Giorgi M, Malentacchi C, Mattiuz PL, Porfirio B. Sequence diversity within the HA-1 gene as detected by melting temperature assay without oligonucleotide probes. *BMC Med Genet* 2005;6:36.
7. Bohling SD, Wittwer CT, King TC, Elenitoba-Johnson KS. Fluorescence melting curve analysis for the detection of the *bcl-1/JH* translocation in mantle cell lymphoma. *Lab Invest* 1999;79:337–45.
8. Germer S, Higuchi R. Single-tube genotyping without oligonucleotide probes. *Genome Res* 1999;9:72–8.

9. Herrmann MG, Durtschi JD, Bromley LK, Wittwer CT, Voelkerding KV. Amplicon DNA melting analysis for mutation scanning and genotyping: cross-platform comparison of instruments and dyes. *Clin Chem* 2006;52:494–503.
10. Dodge A, Turcatti G, Lawrence I, de Rooij NF, Verpoorte E. A microfluidic platform using molecular beacon-based temperature calibration for thermal dehybridization of surface-bound DNA. *Anal Chem* 2004;76:1778–87.
11. Kutyavin IV, Afonina IA, Mills A, Gorn VV, Lukhtanov EA, Belousov ES, et al. 3'-minor groove binder-DNA probes increase sequence specificity at PCR extension temperatures. *Nucleic Acids Res* 2000;28:655–61.
12. Bonnet G, Tyagi S, Libchaber A, Kramer FR. Thermodynamic basis of the enhanced specificity of structured DNA probes. *Proc Natl Acad Sci U S A* 1999;96:6171–6.
13. Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 1996;14:303–8.
14. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res* 2003;31:3406–15.
15. SantaLucia J, Jr. A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci U S A* 1998;95:1460–5.
16. Peyret N. Prediction of nucleic acid hybridization: parameters and algorithms [PhD dissertation]. Detroit, MI: Wayne State University, 2000.
17. Tyagi S, Marras SA, Kramer FR. Wavelength-shifting molecular beacons. *Nat Biotechnol* 2000;18:1191–6.
18. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003;49:853–60.