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Concentration dependency of nonequilibrium thermal dissociation curves in complex target samples

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ABSTRACT

The nonequilibrium thermal dissociation (NTD) methodology has been proposed to provide a superior discrimination between specific and nonspecific hybridizations than the commonly used array techniques involving hybridization followed by a single stringent wash. Multiple studies have used this method on gel-pad, planar, and nylon membrane arrays to identify specific microbial targets in complex target mixtures. A recent physicochemical study revealed several problems, particularly when the method was used to examine complex target samples. In the present study, we investigated the effect of target concentration on NTD of complex target samples obtained from an anaerobic bioreactor. Our purpose was to experimentally demonstrate that variation in the concentrations of both specific and nonspecific targets determines the course of dissociation, which was not evaluated in initial microbiological studies. We also present an approach for analyzing the dissociation curves that is less error prone compared to those used in the previous studies. Our results show that: (i) a specific target in a mixture, at a certain concentration, may have a higher dissociation temperature/time than that of the same pure target, and (ii) the concentration dependence of the dissociation precludes usage of reference curves for identifying a target. Contrary to the previous studies, an explicit calibration is required, which makes the NTD approach impractical for high throughput analysis.
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1. Introduction

For microbial identification, nonequilibrium thermal dissociation (NTD) has been suggested to offer better discrimination between target (specific) and nontarget (nonspecific) nucleic acids than measuring signal at an appropriate wash stringency (DeLosReyes et al., 1997, 1998; ElFantroussi et al., 2003; Eyers et al., 2006; Hansen et al., 1999; Kelly et al., 2005; Koizumi et al., 2002; Li et al., 2004; Liu et al., 2001; Loy et al., 2002; McMahon et al., 1998; Mobarry et al., 1996; Siripong et al., 2006; Urakawa et al., 2002, 2003; Zheng et al., 1996). The NTD approach was first developed for membrane arrays (DeLosReyes et al., 1997; DeLosReyes et al., 1998; Hansen et al., 1999; Koizumi et al., 2002; McMahon et al., 1998; Mobarry et al., 1996; Raskin et al., 1994a,b; Zheng et al., 1996), and later adapted for gel-pad (ElFantroussi et al., 2003; Eyers et al., 2006; Kelly et al., 2005; Liu et al., 2001; Siripong et al., 2006; Urakawa et al., 2002, 2003) and planar (Li et al., 2004) arrays. The rationale for this approach is that, while probe signal intensities may vary, dissociation behavior is supposedly dependent upon whether or not the binding resembles that of a perfectly matching duplex. The NTD approach is performed on an array by increasing the temperature of a buffer solution and recording the signal, which is interpreted in the form of a dissociation curve (Liu et al., 2001; Pozhitkov et al., 2005). A conceptual example of the NTD is presented in Fig. 1. The approach relies on two assumptions: nonspecific duplexes dissociate faster than specific ones, and a dissociation curve is unique to a probe–target duplex (*i.e.*, a target in a mixture can be identified by curve matching).

At face value, the NTD approach seems quite appealing, since it is promising to alleviate the difficulties associated with the interpretation of microarray signals obtained at one stringent wash condition. Specifically, these problems arise from the fact that probes naturally have different binding energies (Pozhitkov et al., 2006), and targets occur at different concentrations in a complex target sample. In a mixed target sample, for example, it is not possible to determine *a priori* if the signal intensity of a probe is due to differences in the binding energies of hybridized targets or to differences in their concentrations. On the other hand, comparison of NTD curves, if proven valid, would provide an attractive alternative for ensuring the specificity of a probe to a target. For example, comparison of a curve of a reference pure target to that of a sample could reveal whether or not the reference target was present in an environmental sample, which has been alluded to in previous studies (*e.g.*, ElFantroussi et al., 2003; Raskin et al., 1994a,b). At that time, little was known about how a target, or a mixture of targets, dissociates from probes in solution, or from probes immobilized on the surface of a

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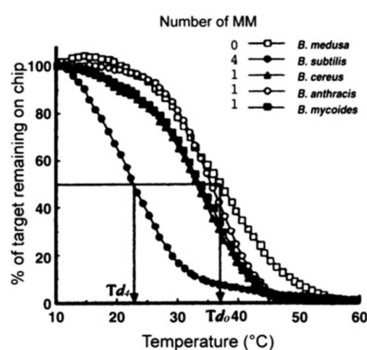

Fig. 1. Normalized signal intensity of targets to a probe specific for *Bacillus medusa* based on gel-pad arrays. Shown is the number of mismatches and symbol. Adapted from Liu et al. (2001).

microarray. Only recently has extensive practical and theoretical studies of the hybridization and dissociation of duplexes on a microarray been conducted, *i.e.*, hybridization thermodynamics (Held et al., 2006; Mei et al., 2003; Naef and Magnasco, 2003; Pozhitkov et al., 2006; Wu and Irizarry, 2004; Zhang et al., 2003).

To more thoroughly investigate the NTD approach, we previously conducted an extensive experimental study using a large dataset of curves recorded on gel-pad microarrays (Pozhitkov et al., 2005). To our surprise, we discovered major problems associated with how the signal intensities from an array were processed by an image acquisition and processing system, as well as multiple problems associated with complicating physicochemical factors, such as the diffusivity of the target in the gel pad and the surrounding solution. These multiple overlapping problems have a substantial impact on the interpretation of signal in gel-pad studies. Beyond the technological problems, we then asked ourselves if the NTD approach had ever been theoretically and experimentally validated. Using a different (and less complicated) platform and signal processing algorithm (avoiding all the problems previously identified; Pozhitkov et al., 2005; Pozhitkov and Noble, 2007), we began unraveling nonequilibrium dissociation curves of perfectly matching duplexes from a physicochemical perspective (Pozhitkov et al., 2007). We found that, contrary to popular belief, nonspecific duplexes do not always dissociate faster than specific ones. Furthermore, interpretation of NTD curves was found to be complicated by the fact that the intensity of an array spot is a composite of all specific and nonspecific targets bound to the same probe (Zhang et al., 2005).

This study explicitly addresses the issue of identification of targets in complex target mixtures, which should be of concern to microbiologists that use, or have used, the NTD approach to identify microbes. Our study is unique compared to all previous NTD studies because: (i) it provides a method for comparing NTD curves that is a significant improvement from previous studies (*i.e.*, it is an objective measurement based on physical theory), (ii) we show that when a specific target is present at 1%, 5% or 10% of the total amount of DNA, the dissociation curves tend to shift to lower temperatures as the concentration of the specific target decreases, (iii) dissociation of the pure target is situated between 5% and 10% mixtures, and (iv), comparison of the 100% specific target to 0% (*i.e.*, mix of all targets but the specific one) revealed that a greater number of nonspecific curves actually dissociate at higher temperatures than the specific ones.

2. Materials and methods

2.1. Microarray and raw data

The 16S ribosomal RNA gene from *Burkholderia xenovorans* strain LB400 (accession number U86373) was used to design 220 perfect-match probes. The probes were synthesized *in situ* (in quadruplicate) on Xeotron (Invitrogen) microfluidic arrays. Hybridization protocols, solutions, and the way the dissociation curves were recorded have been previously described (Pozhitkov et al., 2007; Wick et al., 2006). After scanning, the microarray was washed for 2.2 min at 22 °C and scanned again. Washing and scanning cycles were repeated up to 70 °C. The data generated can be downloaded at http://staff.washington.edu/pozhit/default.htm. To minimize the effects of background noise, probes that had initial signal intensities of <200 a.u. before dissociation were excluded from all analyses. In addition, in some experiments at one temperature, there was a sudden drop of signal intensity followed by a continuation of the normal dissociation course. This drop was an apparent outlier and the corresponding temperature was excluded from the analysis for every probe on the microarray.

2.2. Targets

A fragment of the 16S rRNA genes (1466 bp) was amplified from a pure culture of the *B. xenovorans* strain LB400. A mixture of unknown microbial targets (that did not contain *B. xenovorans*) was obtained by amplifying rRNA genes from an anaerobic bioreactor. Amplicons tested on all batches were generated from the same pools of gDNA. Sample mixtures included 100, 10, 5, 1 and 0% pure culture in anaerobic bioreactor, and were mixed based on the mass of amplicons (*e.g.*, 2.5 ng of pure culture amplicons and 247.5 ng of bioreactor amplicons for 1% mixture). The targets were labeled with Cy3 as previously described (Wick et al., 2006).

2.3. Comparison of NTD curves

Concentration of a single bound nucleic acid target, s1, within the microarray spot after the first washing step at temperature T_1 for a fixed time period Δt is given by a combination of the first order kinetics and Arrhenius equations:

$$C_{T_1}^{s1} = C_0^1 \cdot \exp\left(-A^{s1} \cdot \exp\left(-\frac{E_a^{s1}}{RT_1}\right) \cdot \Delta t\right) \quad (1)$$

where C_0^{s1} is the concentration of the nucleic acid s1 before the washing, E_a^{s1} is the activation energy, A^{s1} is the preexponential coefficient, and R, the universal gas constant. The same equation can be used to calculate the concentration following the next washing step except that C_0^{s1} was changed to $C_{T_1}^{s1}$, and T_1 in the exponent was changed to T_2. All subsequent washing steps can be described analogously. Thus, the concentration of the nucleic acid following the nth washing step can be expressed as follows:

$$C_{T_n}^{s1} = C_0^{s1} \cdot \exp\left(-A^{s1} \cdot \Delta t \cdot \sum_{i \le n} \exp\left(-\frac{E_a^{s1}}{RT_i}\right)\right). \quad (2)$$

In order to use the equation above for comparing one dissociation curve to another, one has to log transform this equation. Since the signal intensity is proportional to the surface concentration, C can be substituted with I (signal intensity):

$$\log\left(I_{T_n}^{s1}\right) = \log\left(I_0^{s1}\right) - A^{s1} \cdot \Delta t \cdot \sum_{i \le n} \exp\left(-\frac{E_a^{s1}}{RT_i}\right). \quad (3)$$

Considering another target, s2, whose dissociation profile is being compared to s1, the same formalism holds true. If we assume that $E_a^{s1} \approx E_a^{s2}$, which is reasonable in accordance with Ikuta et al. (1987) and our previous study (Pozhitkov et al., 2007), we can express log values of s2 profile via log values of s1 profile:

$$\log\left(I_{T_n}^{s2}\right) = \frac{A^{s2}}{A^{s1}} \log\left(I_{T_n}^{s1}\right) - \frac{A^{s2}}{A^{s1}} \log\left(I_0^{s1}\right) + \log\left(I_0^{s2}\right). \quad (4)$$

Hence, the profile s2 is expressed via s1 as a straight line with the slope related to the difference in dissociation kinetics (the last two

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