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QuantiLyse™: Reliable DNA Amplification from Single Cells

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BioTechniques 32:1106-1111 (May 2002)

ABSTRACT

Amplification of DNA sequences from single cells via PCR is increasingly used in basic research and clinical diagnostics but remains technically difficult. We have developed a cell lysis protocol that uses an optimized proteinase K solution, named QuantiLyse™, and permits reliable amplification from individual cells. This protocol was compared to other published methods by means of real-time PCR with molecular beacons. The results demonstrate that QuantiLyse treatment of single lymphocytes renders gene targets more available for amplification than other published proteinase K methods or lysis in water. QuantiLyse and an optimized alkaline lysis were equally effective in terms of target availability, although QuantiLyse offers greater flexibility, as it does not require neutralization and can comprise a higher percentage of the final PCR volume. Maximum gene target availability is also obtained following QuantiLyse treatment of samples containing up to 10 000 cells (the largest number tested). Thus, QuantiLyse maximizes the chances that targeted DNA sequences will be available for amplification during the first cycle of PCR, thereby reducing the variability among replicate reactions as well as the likelihood of amplification failure or allele drop-out. QuantiLyse will be useful in a range of investigations aimed at gene detection in small numbers of cells.

INTRODUCTION

PCR is sensitive enough to detect individual genes in single cells and is therefore useful in preimplantation genetic diagnosis, forensics, oncology, and other fields. However, accuracy, rapidity, and convenience are also critical for routine use of PCR-based assays in both scientific investigations and clinical diagnosis. Although the introduction of real-time PCR in the last few years has improved the quantitative rigor of such assays, their reliability at the single-cell level remains problematic. Such sensitivity requires that both sample preparation and PCR components be thoroughly optimized. Primer design, magnesium concentration, and cycle profile are all variables that affect the reproducibility of the reaction. Cell lysis is also critical because it determines whether or not all possible templates within the host cell genome are intact and sufficiently free of bound proteins to initiate amplification in the first thermal cycle.

Incomplete cell lysis can delay the start of sequence amplification or can prevent it entirely. In the case of heterozygous cells, suboptimal lysis conditions can prevent amplification of one of the two alleles. This phenomenon is called allele drop-out (ADO) and can result in clinical misdiagnosis. Such an outcome is particularly worrisome in the field of preimplantation genetic diagnosis, since the presence of potentially debilitating or lethal alleles has to be determined by analysis of one or two cells biopsied at an early embryonic stage. Individual clinics carrying out such analysis have adopted different protocols for cell lysis. These methods include freeze-thaw in water (1), heat denaturation in water followed by freeze-thaw (13), treatment in an alkaline buffer containing potassium hydroxide and DTT (2,5), and treatment with proteinase K and SDS (3,7). Proteinase K has also been used with nonionic detergents (8,9) or in the presence of magnesium chloride (13). Variations in sample preparation, including small variations in component concentrations or the precise technique used in preparing working solutions, could

account for the wide range of ADO rates reported in different studies (10,14,17). However, it is difficult to determine the best lysis methods from these reports, since virtually all studies also employed different amplification conditions. It is also not easy to compare different cell lysis protocols experimentally on the basis of their ADO rates because analysis of hundreds of single-cell samples is required to prove statistically even fairly large differences in ADO rates.

For all of these reasons, we decided to use real-time PCR to quantitatively compare different methods for lysing single cells. We based our analysis on real-time amplification of a repeated sequence in the TSPY genes that are present in about 30 copies on the human Y chromosome (11). Variations among replicate assays largely reflect differences in the number of TSPY sequences available to initiate amplification. The cell lysis protocol that releases those gene targets from chromatin most reliably is revealed by consistent generation of fluorescent signals in the fewest number of PCR cycles. This strategy has allowed us to develop an optimized lysis solution, QuantiLyse™, and to compare its performance to several other published methods for single cell lysis. QuantiLyse also proved useful for quantitative release of DNA in samples comprised of up to 10 000 cells. Commercial production of QuantiLyse by Hamilton Thorne Biosciences (Beverly, MA, USA) promises to make PCR analysis of small numbers of cells rapid and convenient.

MATERIALS AND METHODS

Preparation and Handling of Lymphocytes

Mononuclear leukocytes (primarily lymphocytes) were isolated and manipulated as previously described (11). Single cells were picked up with a finely drawn glass pipet and transferred directly into lysis solution in 0.2-mL MicroAmp™ optical PCR tubes (Applied Biosystems, Foster City, CA, USA). Samples were processed using the different lysis protocols as described below.

To prepare samples containing larger numbers of cells, a hemacytometer was used to determine cell concentration, and serial dilutions were made using calcium- and magnesium-free PBS (Sigma, St. Louis, MO, USA) using low-adhesion pipet tips and tubes (USA Scientific, Ocala, FL, USA). PBS (5 µL) containing the desired number of cells was added to each optical PCR tube containing 20 µL QuantiLyse. Samples were incubated as described below. Additional samples received purified human genomic DNA (Sigma). PBS (5 µL) containing 60 pg (equivalent to 10 genomes) or 60 ng (equivalent to 10 000 genomes) was added to each optical PCR tube containing heat-inactivated QuantiLyse, and the samples were processed for PCR as described below.

Protease-Based Lysis

QuantiLyse was used for a protease-based lysis protocol. The mixture contains proteinase K and other components at concentrations that have been optimized by similar testing procedures. Samples of single lymphocytes in 10 µL QuantiLyse were transferred from ice to a preheated thermal cycler block (ABI PRISM® sequence detector; Applied Biosystems) and incubated at 50°C for 30 min and then 95°C for 10 min.

The proteinase K/SDS protocol described by Thornhill et al. (14) was used as an alternative protease-based lysis. Single lymphocytes were transferred into 5 µL freshly prepared working solution of 17 µM SDS (Sigma) and 125 µg/mL proteinase K (Roche Applied Science, Indianapolis, IN, USA). Samples were incubated at 37°C for 1 h and then 98°C for 15 min.

Freeze-Thaw in Water

The method of Chong et al. (1) was used with only slight modification. Lymphocytes were transferred to 10 µL water (18 MΩ, molecular biology grade, Sigma). Samples were initially maintained on ice, frozen to -20°C, and then heated to 37°C. Freezing and thawing were repeated for a total of three cycles.

Heat Denaturation/Freeze-Thaw in Water

A modification of the method of Schaaff et al. (13) was used. Lymphocytes were transferred to 10 µL water. Samples were initially maintained on ice, placed in the thermal cycler, heated to 95°C for 10 min, cooled, and immediately frozen on dry ice. Samples were thawed at room temperature. Freezing and thawing were repeated for a total of three cycles.

Alkaline Lysis

The procedure of Cui et al. (2) as modified by Gitlin et al. (5) was followed. Lymphocytes were transferred directly into 5 µL published KOH solution (200 mM KOH/50 mM DTT), or KOH solution with 5 mM DTT, or KOH solution with no DTT. The samples were heated at 65°C for 10 min. Five microliters of neutralizing solution (900 mM Tris-HCl, pH 8.3, 300 mM KCl, 200 mM HCl) were then added. In some experiments, KCl was omitted from the neutralizing solution to reduce the final concentration of potassium in the PCR to 20 mM.

Real-Time PCR

Primers, molecular beacons, and reagent conditions used for amplification and detection of TSPY in a volume of 25 µL were identical to those previously described (11). In the tests of alkaline lysis versus QuantiLyse, all samples were brought to a final volume of 50 µL with the same final reagent concentrations plus potassium ion concentrations at either 20 or 50 mM. Samples containing multiple cells were also brought to a final volume of 50 µL for PCR.

Amplification and fluorescence detection was carried out using an ABI PRISM7700 sequence detector. The cycling profile included an initial denaturation at 95°C for 3 min, followed by 45 cycles of 95°C for 10 s, 58°C for 45 s, and 72°C for 10 s, with fluorescence readings taken during the 58°C step. For reactions containing KCl, the PCR extension step was increased to 30 s. The threshold was set at 10 standard deviations above baseline fluorescence readings.

Contamination Control

Strict measures were taken to minimize the possible contamination of samples, as described previously (11). Possible contamination of reagents was monitored by the inclusion of negative controls (inactivated lysis buffer plus PCR mixture)

in each experiment. A total of three positive reactions were observed in 70 control samples of all lysis conditions, including zero positive reactions in 32 controls of QuantiLyse. The C_t values of those three controls ranged from 39.0 to 40.5, considerably above the C_t values generated by the multiple copies of TSPY in single lysed cells, and suggest contamination by a single TSPY molecule.

Statistical Analysis of C_t Values

Ten samples that showed no amplification were regarded as cell transfer failures and were not included in the analysis. Each group of samples was evaluated for the presence of outliers using the extreme studentized deviate (ESD) statistic. Five samples in these experiments had C_t values several cycles greater than the mean for their groups and were identified as statistical outliers. These samples are not included in the table or in the data analysis of the sample groups, since such results are likely to reflect technical problems with cell transfer, lysis, or amplification rather than differences due to the lysis solution itself. The remaining C_t values were evaluated using the *F* test for the equality of variances. Mean C_t values were compared using the Student's *t* test for independent samples.

RESULTS

Direct comparison of several methods of cell lysis was accomplished by amplification of a TSYP repeated-gene sequence in single cells and real-time detection of the accumulating PCR product with a molecular beacon. Molecular beacons are hairpin-shaped oligonucleotides whose loops hybridize to complementary target sequences with allelic specificity. As a product accumulates, it hybridizes to its corresponding molecular beacon during the annealing phase of the PCR cycle. The beacon molecules only fluoresce when they are in the open, hybridized conformation and thereby give a direct measure of the product concentration (15). The PCR cycle at which the fluorescent signal first exceeds a detection threshold is called the C_t value. The C_t value of a reaction reflects both the number of DNA sequences that initiate the reaction and the efficiency with which the reaction proceeds. However, because all samples in a single experiment were amplified under the same PCR conditions and each lymphocyte contains the same number of TSPY gene copies (about 30), the variations observed reflect the extent to which the TSPY sequences were rendered accessible by a particular method of cell lysis; the smaller the C_t value is, the greater the number of available target sequences. Thus, the most efficient and reliable method of cell lysis generates the lowest mean C_t value.

Experiment 1: QuantiLyse versus Lysis in Water

Figure 1 illustrates the increase in molecular beacon fluorescence during PCR in three sets of replicate samples containing single lymphocytes, one set prepared with QuantiLyse, a second set prepared by freezing/thawing in water, and a third set lysed in water and heated to 95°C before freeze/thaw. The graph shows that QuantiLyse-treated samples have an earlier and more reproducible fluorescence increase than either set of water-treated samples. The mean C_t value of the QuantiLyse-treated samples was 34.39 cycles, significantly lower ($P < 0.001$) than that obtained by freeze-thaw in water, 39.41 cycles, or in water heated to 95°C before freeze-thaw, 35.00 cycles (Table 1). The large difference between the two methods of water lysis suggests that early heating may prevent the loss of targets by denaturing endogenous nucleases. Although the difference in mean C_t values between the QuantiLyse and heat-treated samples is small (0.6 cycle), it is significant ($P < 0.01$) and indicates that about one-third of the targets are not available during the initial amplification, assuming each cycle reflects an approximate 2-fold difference in target number.

Experiment 2: QuantiLyse versus Proteinase K/SDS

QuantiLyse was compared directly with a proteinase K/SDS protocol recently used for analyzing ADO rates following single-cell lysis (14) and similar to other protocols that have been used for single-cell lysis (3,7). The mean C_t values, 33.78 and 34.62, for those two treatments were significantly different (Table 1) and underscore the improvements in the proteinase K-based QuantiLyse relative to the proteinase K/SDS protocol typically used for single cells.

Experiment 3: QuantiLyse versus Proteinase K with Nonionic Detergents

As noted in the Introduction, some published lysis protocols utilize nonionic detergents such as Triton® X-100 (9) or Nonidet™ P-40 (8). Comparisons of samples treated with QuantiLyse or proteinase K solutions containing either Triton X-100 or Nonidet P-40 are provided in Table 1. The mean C_t values for Triton X-100, 34.99, and Nonidet P-40, 35.25, were both significantly higher ($P < 0.001$) than the mean C_t value attained for QuantiLyse, 34.10, in the same experiment. Thus, these nonionic detergents with proteinase K are not as effective as QuantiLyse.

Experiment 4: The Effect of Magnesium on Lysis Using Proteinase K

Magnesium has been added to lysis buffers by some investigators (13), perhaps to increase the half-life of proteinase K activity and because it is a required component of the subse-

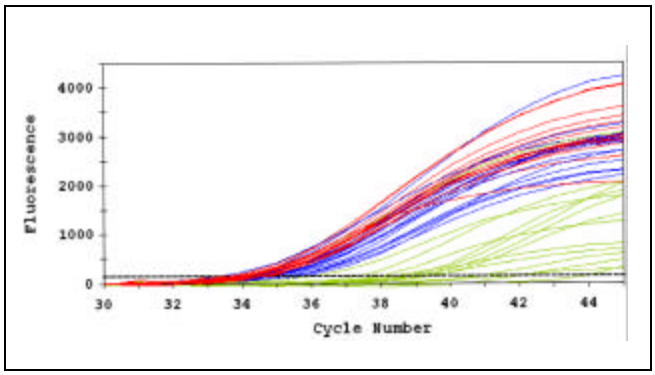

Figure 1. Molecular beacon detection of TSPY amplification during real-time PCR. Each sample contains a single male lymphocyte prepared with QuantiLyse (red), heat denaturation in water (blue), or freeze-thaw in water (green). Detection threshold for determining C_t values is indicated by dashed line.

Table 1. Comparisons of TSPY Amplification from Single Lymphocytes Following Different Lysis Methods

	Treatment A			versus Treatment B			
Experiment[a]	Lysis Protocol	Number of Samples	Mean C_t[b]	Lysis Protocol	Number of Samples	Mean C_t[b]	*P*-value
1	QuantiLyse	15	34.39 ± 0.34	freeze-thaw in water	16	39.41 ± 2.58	$P < 0.001$
				Denature in water	14	35.00 ± 0.66	$P < 0.01$
2	QuantiLyse	14[c]	33.78 ± 0.20	PK/SDS	14[c]	34.62 ± 0.78	$P < 0.01$
3	QuantiLyse	15[c]	34.10 ± 0.26	PK/TX-100	16	34.99 ± 0.45	$P < 0.001$
				PK/NP-40	16	35.25 ± 0.69	$P < 0.001$
4	QuantiLyse	16	33.01 ± 0.81	QuantiLyse + Mg^{++}	16	34.15 ± 1.16	$P < 0.01$
5	KOH + 50 mM DTT	16	36.16 ± 0.59	KOH + 50 mM DTT	15[c]	35.61 ± 0.69	$P < 0.05$
				KOH + no DTT	15	33.76 ± 0.56	$P < 0.01$
6[d]	QuantiLyse	14[c]	33.29 ± 0.53	KOH + no DTT	29	33.27 ± 0.78	NS
7[e]	QuantiLyse	31	33.86 ± 0.43	KOH + no DTT	32	33.87 ± 0.52	NS

[a]Some PCR parameters, including molecular beacon lots, vary between experiments. Therefore, C_t values can only be compared within each experiment.

[b]Mean ± SD. Lower mean C_t value indicates a higher number of gene targets available for amplification.

[c]One statistical outlier is not included. See Materials and Methods.

[d]PCR conditions include final potassium ion concentration of 50 mM.

[e]PCR conditions include final potassium ion concentration of 20 mM.

PK, proteinase K; TX-100, Triton X-100; NP-40, Nonidet P-40; NS, not significant.

quent PCR. Therefore, we compared the efficacy of QuantiLyse with and without the addition of 1.5 mM $MgCl_2$. The presence of magnesium increased the mean C_t value significantly ($P < 0.01$), from 33.01 to 34.15 (Table 1). Higher concentrations of magnesium caused even greater increases in mean C_t values, although this effect could be partially overcome by the use of higher incubation temperatures (data not shown). In addition to reducing proteinase K activity directly, magnesium may act indirectly by stabilizing chromatin structure and thereby preventing proteinase K from completely releasing the target DNA.

Experiment 5: Alkaline Lysis and the Effect of DTT

Cell lysis in KOH plus a reducing agent is another well-established method for preparing genomic DNA. Initial tests of the alkaline lysis technique yielded mean C_t values that averaged 2–3 cycles higher than those obtained with QuantiLyse (data not shown). This large difference suggested that residual DTT reduced the efficiency of the PCR, independently of whether alkaline lysis was or was not effective. To test this possibility, comparisons were made of alkaline lysis containing 50 mM DTT (standard protocol), 5 mM DTT, or no DTT. Lowering the DTT concentration resulted in a significant reduction in the mean C_t value from 36.16 to 35.60 (Table 1). Eliminating DTT from the lysis solution further reduced the mean C_t value to 33.76, a highly significant difference from each of the other groups ($P < 0.001$).

Experiments 6 and 7: QuantiLyse versus Alkaline Lysis without DTT

To compare the QuantiLyse and alkaline lysis protocols accurately, adjustments had to be made in the PCR condi-

tions. Since the published alkaline lysis protocol employs 50 mM potassium for subsequent PCR, KCl was added to each QuantiLyse-treated sample following digestion so that any differences in mean C_t values would reflect differences in target availability and not amplification efficiency due to the potassium concentrations. However, previous testing with TSPY had shown that increasing KCl to 50 mM produced changes in amplification efficiency that lowered the mean C_t value but increased the variance among replicate samples (data not shown). Therefore, to insure that possible differences in lysis would not be masked by high levels of potassium, QuantiLyse treatment and alkaline lysis without DTT were also compared by omitting KCl from the neutralization buffer and adjusting the final KCl concentration in all reactions to 20 mM. The results show that the two methods of cell lysis exhibited equivalent mean C_t values under either PCR condition (Table 1, experiments 6 and 7). Nevertheless, a greater range of PCR salt concentrations and volumes are possible following QuantiLyse treatment, offering flexibility and convenience not possible with the alkaline lysis protocol (see Discussion).

QuantiLyse Treatment of Samples Containing 10–10 000 Cells

Figure 2 illustrates the quantitative results obtained from QuantiLyse-treated samples containing up to 10 000 lymphocytes. Each 10-fold change in cell number results in a shift of 3.8 cycles in the mean C_t value. The overall difference of 11.3 cycles between 10 000 and 10 starting cells was similar to the difference of 11.4 cycles obtained from reactions initiated with 10 000 and 10 genomes of purified genomic DNA. These results indicate that the efficiency of QuantiLyse does not decrease with increasing cell concentrations. The higher variance among samples with approximately 10 cells as compared to samples with higher numbers of cells was likely due to variations in the exact number of cells pipetted into the PCR tube from a dilute suspension of cells. We conclude that 20 µL QuantiLyse is equally effective in releasing genomic DNA from 10 000 cells as from single cells.

DISCUSSION

Successful PCR from cellular DNA requires the removal of nuclear proteins under conditions that minimize the damage to target genes. Real-time PCR has allowed us to quantitatively demonstrate that QuantiLyse is an excellent reagent for accomplishing this goal. Highly reproducible results were obtained when this method of cell lysis was tested via amplification of the multi-copy TSPY gene from single cells, as well as with samples containing up to 10 000 cells. Similar consistency has been observed for U2, another multi-copy gene (11). Other lysis methods, including freeze-thaw in water or other variations of proteinase K treatment, resulted in relatively delayed PCR amplification, suggesting that genomic DNA was either not fully released from residual proteins or was partially damaged. Either possibility could result in misdiagnosis in assays requiring quantitative, reliable DNA amplification starting from single cells or small numbers of cells.

Historically, the alkaline lysis protocol with DTT was first designed for genetic analysis of highly condensed sperm nuclei that contain protamines, small proteins cross-linked by numerous cysteine residues (2). Real-time PCR now demonstrates that inclusion of DTT is not required for complete lysis of somatic cells such as lymphocytes and, in fact, decreases amplification efficiency. Alkaline lysis in the absence of DTT results in efficient DNA release and subsequent PCR but, nevertheless, has some limitations compared to QuantiLyse. First, additional components are required to neutralize the solution following the lysis incubation. The addition of these components, typically added before the PCR reagents, increases the chances of sample contamination, particularly when single human cells are being assayed. Second, the alkaline buffer must be diluted 10-fold before PCR, necessitating lysis in a very small volume and/or increasing the volume of PCR. Third, even after dilution, potassium and sodium salts remain at high concentrations that may not be desirable for some PCR applications. In contrast, QuantiLyse contains relatively low concentrations of these salts and commonly comprises 40%–50% of the final PCR volume and can be used at higher percentages, if necessary.

The tests described here were carried out using human lymphocytes, which are an easily obtained, relatively homogeneous type of quiescent cells. QuantiLyse has also been used successfully for the lysis of blastomeres from human embryos (11). Their larger cytoplasmic volume does not appear to influence the efficacy of QuantiLyse. QuantiLyse is also effective for samples of up to 10 000 lymphocytes (the highest number tested).

Our goal is to achieve the most reliable, experimentally convenient protocol for PCR analysis of clinically important single copy genes and their alleles in single cells. The quantitative nature of real-time PCR allowed us to evaluate and minimize the variability due to sample preparation, initially through the use of the multi-copy TSPY gene. Such detailed comparisons using single copy genes are impractical, since each test would require hundreds of individual cells to uncover small differences in amplification results, including ADO rates. However, the ability of QuantiLyse to maximize the availability of target genes implies a potential for lower rates

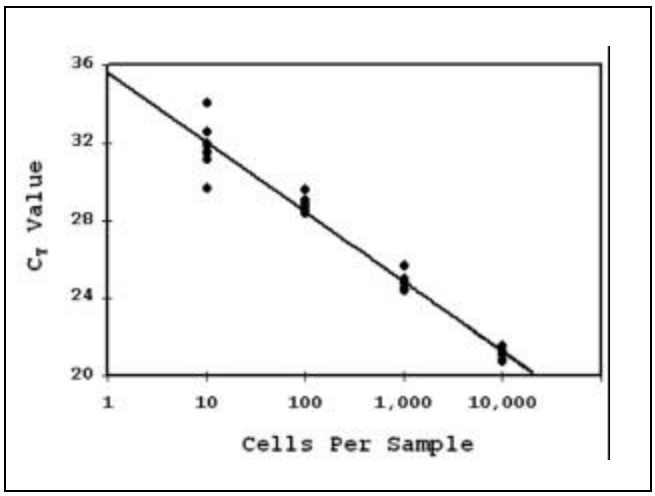

Figure 2. Standard curve generated from C_t values obtained for TSPY amplification in samples containing 10–10 000 cells treated in QuantiLyse. As anticipated, a linear decrease in C_t values is observed with a logarithmic increase in cell number. Eight samples were tested at each cell concentration. The mean values for 10 and 10 000 cells correspond approximately to samples containing an equivalent amount of purified genomic DNA.

of PCR failure and ADO. If the higher C_t values reflect solely the inability to amplify a corresponding number of targets, lysis with water (with heat denaturation) or with standard proteinase K/SDS protocols would result in ADO rates at least 30% higher than with QuantiLyse or alkaline lysis. A more likely explanation of the C_t differences is a combination of absence of amplification from some targets and delayed amplification from others. In the case of single copy genes, delayed amplification of one allele from a heterozygote would lead to preferential amplification and possibly to ADO, depending on differences in the initiation of amplification and the sensitivity of the detection method. Indeed, the use of the more sensitive fluorescent PCR methods has led to a decrease in ADO rates (10,16), at least in part due to the detection of extreme preferential amplification (4).

Our result showing reduced target availability following a standard proteinase K/SDS protocol is consistent with the higher ADO rates observed by Thornhill et al. (14) using that protocol compared with alkaline lysis. In contrast to the published proteinase K/SDS protocol, QuantiLyse treatment should provide ADO rates comparable to or lower than those obtained using alkaline lysis or other methods. We recently tested the amplification and molecular beacon detection of the Tay-Sachs and cystic fibrosis genes, following single-cell lysis in QuantiLyse and observed ADO in 3 of 142 (2.1%) samples of lymphoblasts heterozygous for the major Tay-Sachs mutation and in 6 of 172 (3.5%) lymphoblasts heterozygous for the cystic fibrosis ΔF508 mutation (Reference 12 and unpublished data). These rates are below the 5%–20% rates commonly reported for these genes (10,16) and comparable to the 2.7% (5/185) combined ADO rate recently reported for six different mutant alleles of the cystic fibrosis gene, following alkaline lysis and fluorescent PCR of single lymphoblasts (6).

We anticipate that in the future real-time PCR for the amplification and detection of DNA molecules released from single cells will be a fully optimized technique. The goal, of course, in both the clinical and research setting is to have confidence in the ability to detect and quantify target sequences that are present in a biological sample. The commercial production of QuantiLyse by Hamilton Thorne Biosciences will guarantee that the lysis step is rendered routine. This will make it easier to detect and circumvent other sources of variability.

ACKNOWLEDGMENTS

We thank Sanjay Tyagi and Fred Kramer for their assistance with molecular beacon technology. This research was supported by a grant from Hamilton Thorne Biosciences.

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