Real-time PCR with molecular beacons provides a highly accurate assay for detection of Tay-Sachs alleles in single cells

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The results presented here provide the first single-cell genetic assay for Tay-Sachs disease based on real-time PCR. Individual lymphoblasts were lysed with an optimized lysis buffer and assayed using one pair of primers that amplifies both the wild type and 1278 + TATC Tay-Sachs alleles. The resulting amplicons were detected in real time with two molecular beacons each with a different colored fluorochrome. The kinetics of amplicon accumulation generate objective criteria by which to evaluate the validity of each reaction. The assay had an overall utility of 95%, based on the detection of at least one signal in 235 of the 248 attempted tests and an efficiency of 97%, as 7 of the 235 samples were excluded from further analysis for objective quantitative reasons. The accuracy of the assay was 99.1%, because 228 of 230 samples gave signals consistent with the genotype of the cells. Only two of the 135 heterozygous samples were allele drop-outs, a rate far lower than previously reported for single-cell Tay-Sachs assays using conventional methods of PCR. Copyright © 2002 John Wiley & Sons, Ltd.

KEY WORDS: real-time PCR; molecular beacons; Tay-Sachs disease; single cell PCR; preimplantation genetic diagnosis

INTRODUCTION

The challenge in pre-implantation genetic diagnosis (PGD), as well as other fields that employ single cells for genetic diagnosis, is to achieve accurate and rapid detection of specific alleles located on single chromosomes. The speed, reliability, and experimental convenience of such assays depend on the particular technology employed. It is therefore not surprising that procedures used to carry out single-cell analysis have been repeatedly upgraded as new molecular technologies for gene amplification and detection have been invented. New technologies also introduce more rigorous diagnostic criteria with which to judge the accuracy of single-cell genetic assays. Real-time polymerase chain reaction (PCR) is a quantitative method of sequence amplification that employs fluorescent probes, such as molecular beacons, to reveal the kinetics of amplicon accumulation in the course of the reaction.

A molecular beacon is a single-stranded oligonucleotide 25 to 35 bases-long in which the last 5 to 7 bases on the 3′ and 5′ ends are complementary. Thus, a molecular beacon forms a hairpin structure at ambient temperatures. Molecular beacons have a fluorophore attached to their 5′ end and a quencher attached to their 3′ end, but they do not fluoresce at room temperature because the hairpin conformation brings these two moieties into close proximity. However, if the molecular beacon is heated or allowed to hybridize to a target strand complementary to the sequence within the loop of the molecular beacon, the fluorophore and the quencher are separated and the molecule fluoresces. Molecular beacons with different loop sequences can be constructed with differently colored fluorophores in order to monitor amplification of sequences that differ by as little as one base (Tyagi *et al.*, 1998; Kostrikis *et al.*, 1998). Real-time PCR assays using molecular beacons take only two to three hours to complete and are carried out in a closed tube, thereby minimizing the likelihood of laboratory contamination.

We have previously described the use of real-time PCR with molecular beacons to detect moderately repeated, highly conserved sequences in the TSPY locus on the Y-chromosome and in the U2 locus on chromosome 17 (Pierce *et al.*, 2000). The cells employed for this study were lysed using a protocol optimized for maximum availability of the target templates for amplification (Pierce *et al.*, 2002). The use of repeated genes eliminated the possibility of allele dropout (ADO), a condition that occurs when one allele fails to amplify while the other allele amplifies successfully. As a result, we were able to correctly identify the gender of 99.7% of male and female lymphocytes, and 100% of blastomeres recovered from donated human embryos with fragmentation under 15%.

This communication extends the use of real-time PCR with molecular beacons to the detection of specific alleles of single-copy genes in single cells. To accomplish this goal we analyzed single lymphoblasts, homozygous or heterozygous for the normal, and/or mutated forms of the gene responsible for Tay-Sachs disease. The Tay-Sachs gene encodes the alpha subunit of the β-N-acetylhexosaminidase (HEXA), a lysozymal enzyme required for the breakdown of G_{M2} ganglioside. Individuals homozygous for certain mutated forms of this gene die of neural degeneration within three to four years of birth. Detection of the Tay-Sachs trait

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can be accomplished via an enzymatic assay starting with blood samples or via DNA assays from various sources, but DNA assays are more accurate (Robinson and Stirling, 2001; Kolodny, 2001). One allele in particular, composed of a four base-pair insertion at position 1278 + TATC, accounts for 82 to 90% of Tay-Sachs carriers within the Ashkenazi Jewish population (Bach *et al*., 2001). For many thorough recent reviews, see *Advances in Genetics, vol. 44,* edited by Desnick and Kaback (2001), which is entirely devoted to Tay-Sachs disease.

There are several previous reports of single-cell PCR assays for Tay-Sachs disease (Snabes *et al*., 1994; Gibbons *et al*., 1995; Sermon *et al*., 1995; Liu *et al*., 2000). The most recent of these publications (Liu *et al*., 2000) describes amplification efficiency and ADO frequency for the 1278 + TATC Tay-Sachs gene allele of 85.3% and 4.8%, respectively. The results presented here provide the first single-cell genetic assay for Tay-Sachs disease based on real-time PCR. We demonstrate that the kinetics of amplicon accumulation provide novel objective criteria by which to evaluate the validity of each reaction, providing additional information to lower potential misdiagnosis.

MATERIALS AND METHODS

The following human lymphoblast cell lines were obtained from Coriell Cell Repositories (Camden, NJ, USA): homozygous for TSD 1278 + TATC allele (cell line #GM11852), heterozygous for TSD 1278 + TATC allele (cell line #GM03575), homozygous for the normal sequence of the HEXA gene (cell line #GM01531). Lymphoblasts were prepared and manipulated as described elsewhere (Pierce *et al*., 2000). Single lymphoblasts were transferred directly to MicroAmp optical PCR tubes (Applied Biosystems, Foster City, CA, USA) containing 10 μl QuantiLyse (Hamilton Thorne Biosciences, Beverly, MA, USA), an optimized lysis solution containing proteinase K (Pierce *et al*., 2002). Samples were incubated at 50 °C for 30 minutes, then 95 °C for 10 minutes in an ABI 7700 (Applied Biosystems).

Molecular beacons were designed according to previously described methods (Tyagi and Kramer, 1996; Pierce *et al*., 2000) and were purchased from Research Genetics (Huntsville, AL, USA). The molecular beacon sequence for the normal allele was 5′ FAM—CGGCGTCACCGTATATCCTATGGCCCTGAGACGCCG—Dabcyl 3′ and for the 4-base pair insertion was 5′ TET—CGGCGTCACCGTATATCTATCCTATGGCCCTGAGACGCCG—Dabcyl 3′. Primer sequences for the amplification of a 78-base pair (bp) region that included the 4 bp; insertion had been designed and previously tested using higher quantities of target DNA (Tyagi and Kramer, unpublished results). The upper primer sequence was 5′ CCTTCTCTCTGCCCCCTGGT 3′ and the lower primer sequence was 5′ AGGGGTTCCACTACGTAGAA 3′. Specific amplification of the 78 bp amplicon from single lymphoblasts was confirmed using SYBR® Green (Molecular Probes, Eugene, OR, USA) detection during amplification and product denaturation.

Fifteen microliters of a concentrated PCR reagent mixture was added to each tube containing a lysed cell (or no-cell control) to yield a final sample volume of 25 μl with final concentrations of 1X PCR buffer (Invitrogen, Carlsbad, CA, USA), 3.0 mM $MgCl_2$, 0.25 mM each dNTP, 500 nM each primer, 0.6 μM each molecular beacon, and 1.25 units Platinum Taq DNA polymerase (Invitrogen). Amplification and fluorescence detection were carried out in an ABI 7700. Thermal cycling consisted of an initial 3 min denaturation at 95 °C followed by 10 cycles of 95 °C for 10 s, 65 °C for 30 s, and 72 °C for 15 s, followed by 50 cycles of 95 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s, with fluorescence acquisition during the 55 °C step. Contamination control procedures for reagent preparation and sample handling described previously (Pierce *et al*., 2000) were strictly followed.

Reactions initiated with a single cell take more than 30 cycles to accumulate enough amplicons to be detected with a molecular beacon. The first cycle at which the fluorescent signal rises above the background is known as the C_T value. The fluorescent reading after 60 cycles, F_{60} value, was also utilized for sample evaluation. Amplicon accumulation had plateaued in most reactions by this cycle.

For the purposes of two-dimensional plots of C_T and F_{60} values, the raw data were subjected to the Extreme Studentized Deviate (ESD) test to identify outliers at the 95% confidence level. These samples were removed from each data set and the remaining samples were used to calculate the means and standard deviations of each set. The boxes shown in Figure 2 are three standard deviations from the mean-C_T and mean F_{60} value for each data set.

RESULTS

In order to exploit real-time PCR for detection of Tay-Sachs disease, we used a single pair of primers and two allele-discriminating molecular beacons for simultaneous amplification and detection of both the normal and the 1278 + TATC-mutant sequences in each tube. Out of a total of 248 such assays containing a single lymphoblast, 235 generated at least one molecular beacon signal, for an overall assay utility of 94.8%. The results described below are based on these 235 assays. Figures 1A and 1B display the fluorescent signals generated by individual lymphoblasts homozygous for either the normal or the 1278 + TATC-allele, respectively. All 48 cells homozygous for the normal allele had a strong signal from the FAM-labeled molecular beacon targeted to the normal allele sequence, but no signal from the TET-labeled molecular beacon complementary to the mutant allele sequence (Figure 1A). Conversely, all 45 cells homozygous for the 1278 + TATC-insertion had a strong TET signal but no FAM signal (Figure 1B). Taken together these results confirm that the two molecular beacons are allele discriminating under the conditions of the assay.

When the same analysis was carried out on lymphoblasts heterozygous for the 1278 + TATC allele,
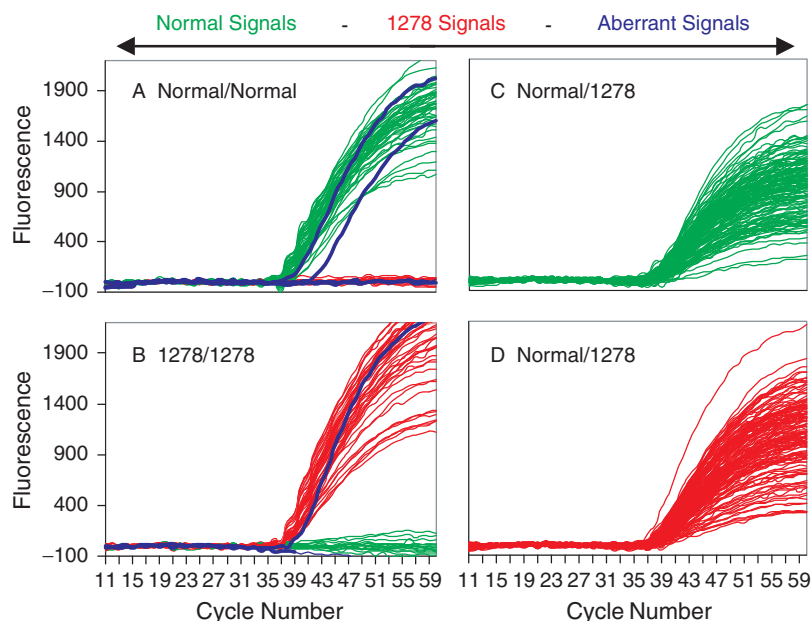
Figure 1—Real-time PCR plots of fluorescent signals generated from individual lymphoblasts. All reactions were simultaneously assayed for the normal allele using an FAM-labeled molecular beacon (in green) and for the 1278 + TATC mutant allele using a TET-labeled molecular beacon (in red) and were placed in boxes A to D based on whether they exhibited one or two signals, regardless of the known genotypes of the cells from which they were derived. (A) homozygous for the normal allele, plus heterozygotes positive for the normal allele only; (B) homozygous for the 1278 + TATC allele, plus heterozygotes positive for the 1278 + TATC allele only. Note, three heterozygous cells yielded only one signal and therefore were included with the homozygous cells (blue lines in A,B). (C&D) heterozygous cells that gave two signals, (C) results for the normal allele and (D) results for the 1278 + TATC allele

97.9% (139 of 142) of the cells generated both the normal and the mutant signals (Figure 1C&1D). Most signals had very similar C_T values and displayed typical amplification kinetics. Two reactions, however, displayed a signal for the normal allele without a signal for the mutant allele and are therefore represented in Figure 1A as blue lines. One additional sample had a signal for the mutant allele without a signal from the normal allele (Figure 1B, blue line). In the absence of further information, these three samples would be regarded as homozygous samples and might, under clinical conditions, result in a misdiagnosis. However, as discussed below, real-time PCR provides quantitative information with which to refine this conclusion.

The data in Figure 1 were next analyzed to establish the mean and standard deviations of C_T and F_{60} values for each set of samples (see Materials and Methods). Table 1 shows that the mean-C_T value for each allele

present in a heterozygous cell having both signals, is approximately one cycle greater than the mean-C_T value for the same allele present in homozygous cells. Similarly, the mean-F_{60} value for each set of samples derived from heterozygous cells is approximately one half of the corresponding mean-F_{60} values for the homozygous cells. These results are consistent with reactions initiated with homozygous cells that contain two copies of a particular allele, as compared to heterozygous cells that contain only one copy of each allele.

Figure 2 provides a two-dimensional analysis of all samples having a normal allele signal only, (Figure 2A), a mutant allele signal only (Figure 2B), or both signals (Figure 2C, normal allele, Figure 2D, mutant allele), regardless of the actual genotypes of the cells that generated these signals. Each two-dimensional plot compares the threshold cycle values (C_T) and fluorescence values after 60 cycles (F_{60}) in each sample and each set of samples in Figure 2 is surrounded by a box indicating the boundaries defined by three standard deviations of the mean-C_T value and the mean-F_{60} for that particular set of reactions. Assuming a normal distribution, 99% of the samples in a set are expected to fall within the corresponding box. Values outside each box most probably reflect aberrant amplification kinetics due to problems with either the synthesis or the detection of that amplicon.

On the basis of the two-dimensional analysis of the data in Figure 1A, one of the two heterozygotes with aberrant signals can be ruled as atypical PCR, because its C_T value lies outside the box designating three standard deviations of the mean (Figure 2A, open triangles).

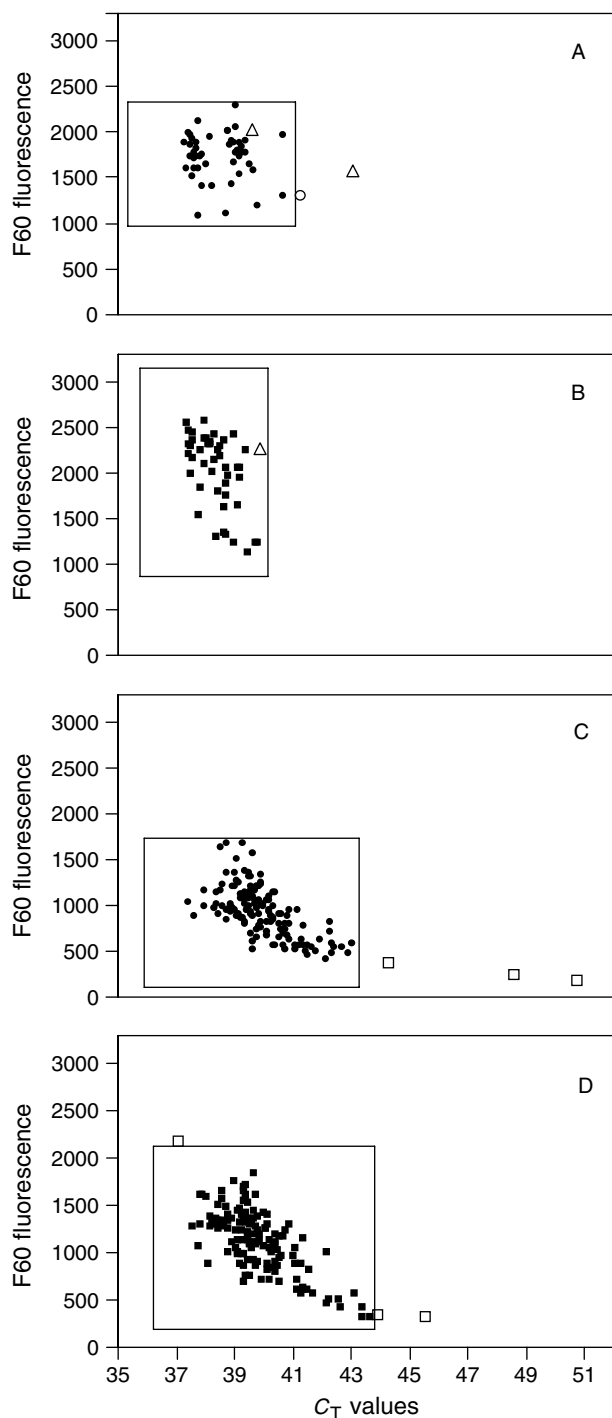| Cell Type	Mean C_T (SD)	Mean F_{60} (SD)
Lymphoblast with normal allele signal only	38.5 (0.90)	1728 (256)
Lymphoblast with mutant allele signal only	38.4 (0.68)	2015 (405)
Lymphoblast with both signals		
Normal allele signal	40.0 (1.17)	922 (276)
1278 allele signal	39.8 (1.29)	1117 (341)

SD; Standard Deviation.

Figure 2—Two-dimensional analyses of the data in Figure 1. (A) reactions with FAM signals only; (B) reactions with TET signals only; (C) FAM signals from reactions with both signals; (D) TET signals from reactions with both signals. The rectangles in each panel display the boundaries for three standard deviations from the mean-C_T and the mean-F_{60} of each data set (see text for details)

The remaining aberrant heterozygote cannot be distinguished from homozygous normal cells because its signal lies within the box. Similarly, the single aberrant heterozygote in Figure 1B cannot be distinguished from homozygous 1278 + TATC cells (Figure 2B, open triangle). Finally, analysis of 60 additional no-cell control

reactions for the possible presence of both the normal and the mutant gene sequences generated one signal for the normal allele, suggesting the presence of a contaminating wild type HEXA gene sequence in one control tube. However, the two-dimensional analysis placed the signal from this sample outside of the box in Figure 2A (open circle), suggesting that it may also have arisen via a PCR error.

In light of the quantitative analyses shown in Figure 2, all lymphoblasts (100%) homozygous for either the normal or the mutant lymphoblasts were correctly diagnosed. In addition, 139 of 142 heterozygous cell samples had two signals and were also correctly identified as heterozygotes. However, a total of six of these 139 samples displayed one of their two signals outside of the box. Three lymphoblasts had a normal allele signal outside of the box (Figure 2C, open squares) while 3 other lymphoblasts displayed a mutant allele signal outside of the box (Figure 2D, open squares). On the basis of these objective quantitative criteria none of these six samples would be used for diagnosis in the clinical setting. These discards, as well as the one discarded ADO sample would reduce the efficiency of the assay to 97% (228/235). The overall accuracy of the assay can be regarded as the percentage of correctly diagnosed samples, among all samples with diagnosable signals. The diagnostic accuracy of this lymphoblast series is 99.1% (226/228). The rate of contamination was 1.67% (1/60), although two-dimensional analysis eliminated the positive sample from further consideration. One of the three cases of aberrant heterozygotes was eliminated from further consideration based on quantitative criteria. Among the diagnosable samples, the ADO rate was 2/135 (1.5%).

DISCUSSION

Single-cell PCR technology can be objectively judged on the basis of its utility, its efficiency, its accuracy, and its rate of ADO. The assay described here had an overall utility of 95%, based on detection of at least one signal in 235 of the 248 cell attempted tests. The 5% of samples that failed to generate any signal, is similar to our previous findings (Pierce *et al.*, 2000 and unpublished observations) and most probably arise because the chosen cell is either apoptotic, or is never actually transferred to the tube. Assay utility is the number of samples having any signal as a percentage of total assays attempted. In the present study, the utility was 95%, which is an improvement over previously reported tests for Tay-Sachs alleles at the single-cell level (85.3%) (Liu *et al.*, 2000) and is similar to the 97% utility reported by Gitlin *et al.* (1996) using cell lysis with potassium hydroxide and dithiothreitol. The efficiency of the assay described here is 97%, because 7 of the 235 samples with at least one signal were excluded from further analysis because one of those signals fell outside the limits set by three standard deviations from the mean-C_T or mean-F_{60} values. The accuracy of the real-time assay was 99.1% because 2 of the 230

samples with diagnosable signals were heterozygotes that appeared to be homozygotes due to ADO.

The total ADO rate for this real-time PCR assay was 1.5% because of 2 out of 135 heterozygous samples generated only one signal with amplification kinetics indistinguishable from homozygotes. This rate is far lower than the rate of 4.8% previously reported using conventional methods of PCR for detection of Tay-Sachs disease in single cells (Liu *et al.*, 2000), as well as the 6% rate reported for nested PCR following cell lysis with potassium hydroxide and dithiothreitol (Gitlin *et al.*, 1996). One of the aberrant heterozygote samples generated only a normal signal while the other generated only a mutant $1278 + \text{TATC}$ allele signal. Had these results been obtained with individual blastomeres recovered from human embryos from carrier parents, one of these assays could have resulted in the birth of a normal baby having a heterozygous normal rather than a homozygous normal genome. The embryo corresponding to the ADO assay that was positive for the $1278 + \text{TATC}$ allele would never have been transferred *in utero*. The very low rate of ADO achieved in the present study was a direct result of using previously optimized conditions for single-cell lysis (Pierce *et al.*, 2000; Pierce *et al.*, 2002), as well as optimized PCR primers and allele discriminating molecular beacons. The ADO rate was further reduced by taking advantage of the quantitative kinetic data produced by the real-time PCR method. The fluorescent signal in one case of apparent ADO could be clearly distinguished from the signal typical of a homozygous lymphoblast. Such a conclusion could not have been reached using conventional methods of PCR that depend on end-product analysis rather than kinetic analysis of the reaction and are semiquantitative at best.

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