Research Report

Utility and Accuracy of Template-Directed Dye-Terminator Incorporation with Fluorescence-Polarization Detection for Genotyping Single Nucleotide Polymorphisms

BioTechniques 32:1072-1078 (May 2002)

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ABSTRACT

There are little independent data available about how well single nucleotide polymorphism (SNP) genotyping technologies perform in the typical molecular genetics laboratory. We evaluated the utility and accuracy of a widely used technology, template-directed dye-terminator incorporation with fluorescence-polarization detection (FP-TDI), in a sample of 177 SNPs selected solely on the basis of map location. Genotypes were generated without optimization using standard protocols. Overall, 81% of the SNPs we studied generated readable genotypes by FP-TDI. Thirty-two SNPs were genotyped in duplicate by PCR-RFLP or fluorescent dye-terminator sequencing. Out of a total of 631 duplicate genotypes, no true discrepancies were detected. The true error rate has a 95% chance of lying between 0 and 6 out of 1000 genotypes. We also tested for deviations from Hardy-Weinberg Equilibrium in 33 SNPs genotyped in 50 unrelated individuals, and no significant deviations were detected. Our FP-TDI data were readily adaptable to automated genotype calling using our own method of cluster analysis, which assigns a probability score to each genotype call. We conclude that FP-TDI is both efficient and accurate. The method can easily fill the needs of SNP genotyping projects at the scale typically used for regional or candidate-gene association studies.

INTRODUCTION

Single nucleotide polymorphisms (SNPs) continue to grow in importance for studies of human genetic variation, evolutionary genetics, and complex genetic traits. However, there has not been a commensurate growth in the availability of accurate, high-throughput SNP genotyping technologies (10). Most currently available methods rely on detecting differences between alleles in molecular weight (3), enzymatic template activity (13), or single-base extension (1,2,5,14). Little is known about the utility and accuracy of these methods, aside from the data presented by the laboratory that first developed that method. Even less is known about how well any given method performs in the hands of the small-to-medium-sized laboratories that are the main consumers of SNP genotyping technologies.

Template-directed dye-terminator incorporation with fluorescence-polarization detection (FP-TDI) was first described in 1999 (2). The method relies on the ability of Thermo Sequenase™ (Amersham Biosciences, Piscataway, NJ, USA) to extend accurately an annealed probe by a single base that is complementary to the opposite strand. A fluorescent plate reader then detects differences in the polarization of light by incorporated versus unincorporated nucleotides previously labeled with a fluorescent dye. The method was efficient and accurate in the hands of its developers but has not, to our knowledge, been formally evaluated for utility and accuracy in other laboratories. We undertook such a study using an unselected sample of approximately 180 SNPs and more than 600 duplicate genotypes. We found that FP-TDI is both efficient and accurate, even in a small laboratory setting.

MATERIALS AND METHODS

SNP Selection

We studied a total of 177 SNPs, most of which were selected from public databases (http://snp.cshl.org; http://www.ncbi.nlm.nih.gov/SNP/) that mapped to chromosome 18q21-22, a bipolar disorder candidate region. A small portion of the SNPs (n = 7) were discovered in our own laboratory through the resequencing of sequence-tagged sites. SNPs were selected for the study solely on the basis of map location. All public SNP sequences were mapped back to the human draft sequence using Blastn; SNPs that mapped back to multiple chromosomes were dropped.

The sample sizes for each validity assay were as follows: success rate, 177 SNPs in 20 samples; cross-test accuracy, 32 SNPs in 20 samples; Hardy-Weinberg Equilibrium, 33 SNPs in 50 samples; and automatic genotype calling, 20 SNPs in 186 samples.

Oligonucleotide Primers and Probes

PCR primer sequences were selected using OLIGO® 6.0 (Molecular Biology Insights, Cascade, CO, USA). For each SNP, the best pair of primers fitting the following criteria was selected: minimum length of 21 bp, an an-

nealing temperature of 55°C–68°C, and a product length of 90–150 bp (9). The extension probe sequences were designed to encompass 25–32 bp 5′ to the nucleotide adjacent to the variable site using OLIGO 6.0. The shortest probe sequence free of 3′ dimer and hairpin structures and with an annealing temperature of 65°C–85°C was selected. Both forward (sense) and reverse (antisense) probes were tested for each SNP. SNPs that produced well-clustered genotypes with either probe were scored as successes, and those with a minor allele frequency estimated at greater than 5% were used in the subsequent experiments. Oligonucleotides were synthesized commercially (Invitrogen, Carlsbad, CA, USA) using standard protocols, without HPLC purification.

PCR

The 10-µL reaction contained 20 ng genomic DNA, 0.05 mM dNTPs (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 0.025 U Platinum *Taq* DNA polymerase, 1× Platinum *Taq* buffer (Invitrogen), and 0.125 µM each primer (synthesized by Invitrogen). PCR and all subsequent reactions were done in black, skirted 96-well plates utilizing a PTC-100™ thermal cycler (MJ Research, Waltham, MA, USA). Most reactions were carried out under a silicon mat (Dot Scientific, Burton, MI, USA); some reactions were carried out under 10 µL mineral oil to address evaporation problems. PCR amplification was carried out over 14 step-down cycles (68°C–53°C) and 20 amplification cycles, with a final extension at 68°C for 7 min (9). A small amount of PCR product was electrophoresed on a 2% agarose gel and visualized with ethidium bromide to confirm amplification. Shrimp alkaline phosphatase (Roche Applied Science, Indianapolis, IN, USA) and exonuclease I (Amersham Biosciences) were used to remove unincorporated primers and dNTPs. The shrimp alkaline phosphatase along with exonuclease I (1 U each) in 1× shrimp alkaline phosphatase buffer were added in a 10-µL volume to the PCR product and incubated at 37°C for 45 min. The enzymes were then heat-inactivated at 95°C for 15 min.

FP-TDI

We used the most recently published protocol (9), without modification or further optimization. The FP-TDI mixture consisted of 0.0125 µM TAMRA ddCTP, Rox ddGTP, R6G ddUTP, BODIPY® ddATP (Perkin Elmer Life Sciences, Gaithersburg, MD, USA) with 0.04 U Thermo Sequenase, 1 µM probe in an FP-TDI buffer consisting of 50 mM Tris-HCl, pH 9.0, 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, and 8% glycerol. Ten microliters of the mixture were added to the completed PCR/shrimp alkaline phosphatase and incubated at 93°C for 2 min, and then 50 cycles of 93°C for 10 s and 50°C for 30 s (PTC-100; MJ Research).

Following the extension reaction, 0.22 µg ssDNA binding protein were added to some assays in a volume of 10 µL FP-TDI buffer and incubated for 1 h at 37°C. After incubation, 75 µL 33% ethyl alcohol in 5.3% glycerol, 33.3 mM Tris-HCl, 33.3 mM KCl, and 3.3 mM $MgCl_2$ were added as a reading buffer. The plates were read on an Ana-

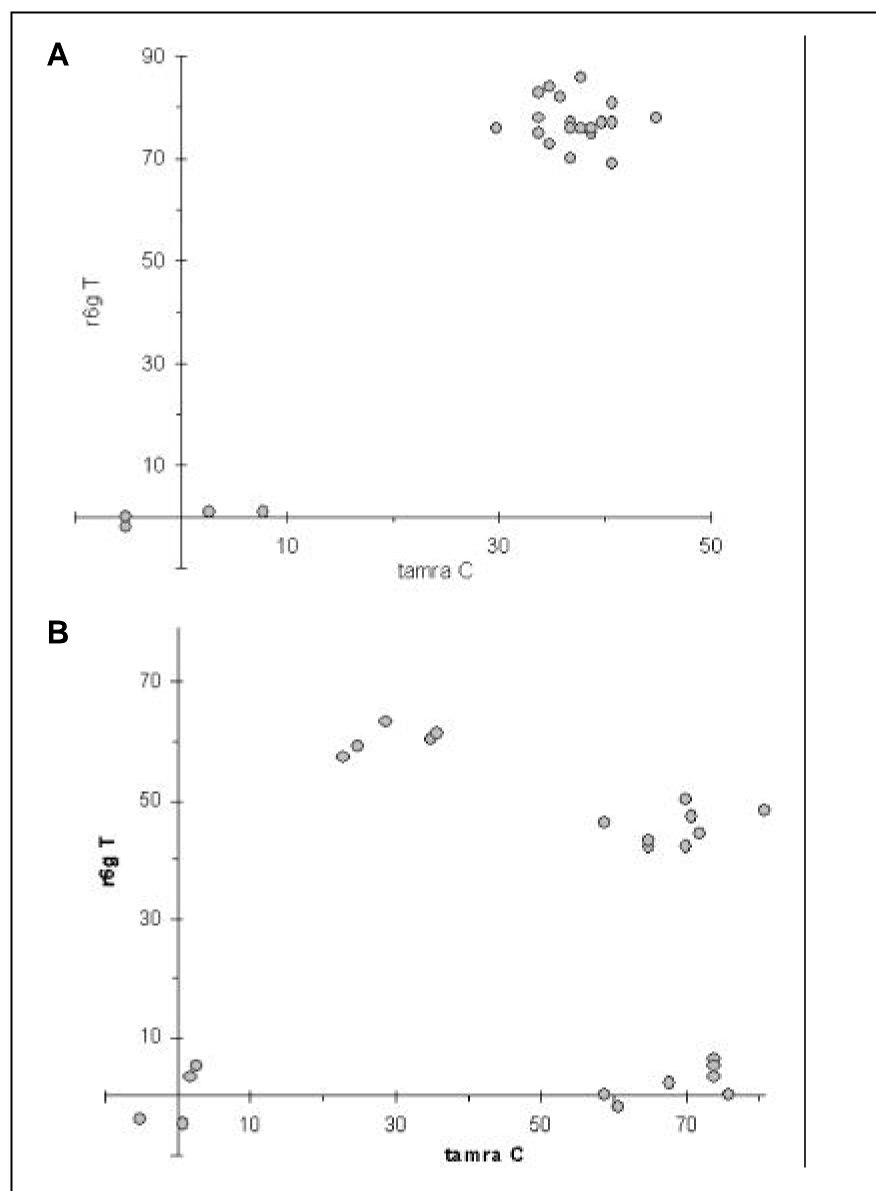

Figure 1. Representative FP-TDI results for a monomorphic (top) and polymorphic (bottom) SNP genotyped on our sample of 20 unrelated subjects and four water controls. Results are plotted in mP units above the average polarization of the controls.

lyst AD™ (Molecular Devices, Sunnyvale, CA, USA) for all dyes present in the FP-TDI mixture.

PCR-RFLP

DNA that flanked selected SNPs was amplified using the same primer pairs designed for the FP-TDI. The PCR products were digested with restriction endonucleases selected on the basis of a change in the restriction site that resulted from allelic variation at the SNP of interest. The resulting fragments were visualized by electrophoresis on agarose gel (1% NuSieve® plus 1% SeaKem® agarose; BMA, Rockland, ME, USA) stained with ethidium bromide.

Sequencing

Double-stranded PCR fragments were amplified using the same primer pairs designed for FP-TDI. These PCR fragments were purified on a MultiScreen®-PCR 96-well filtration system (Millipore, Bedford, MA, USA) and then sequenced directly using the BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). All sequencing reactions were processed with MultiScreen-HV N45 (Millipore) containing Sephadex® G-50 (Sigma, St. Louis, MO, USA) to remove the unincorporated dye and then run on an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems). The resulting data were aligned using Sequencher™ 4.0 (Gene Codes, Ann Arbor, MI, USA).

Genotype Calling

All genotypes were called manually by an experienced reader blind to the results of the other methods. Automated genotype calling was performed in two steps. (*i*) Each data point was assigned a genotype by k-means cluster analysis. This statistical method initially partitions the data into any number of clusters the user specifies, continuing iteratively until subsequent partitions fail to decrease the variance within clusters. We specified three or four clusters (based on an initial inspection of the scatterplot) with a random initial partition, 10 repetitions, and a maximum of 50 iterations at a precision of 1×10^{-5}. In this case, each cluster corresponds to one of the genotypes present in the sample: heterozygote, major homozygote, minor homozygote, or negative control/PCR failure. We employed k-means cluster analysis because it is an approach that is widely used when the expected number of clusters is known in advance. (*ii*) Each genotype call was assigned a score, representing the probability that the data point belongs to that genotype cluster. This probability was estimated, conditional on the distance from the geographic center of the cluster (centroid). That distance was calculated as the Mahalanobis Distance, a more accurate measure than Euclidean Distance when—as in the case of FP data—the x and y values are correlated (6). Equality of the variance/covariance matrices was assumed, since this is conservative. Analyses that did not assume variance/covariance equality did not perform as well as those that did (results not shown), particularly when the data did not cluster well. The cluster and distance analysis was implemented with Microsoft® Excel®, using the software package XLStat 5.0 (http://www.xlstat.com/).

Table 1. Duplicate Genotypes Generated on 30 Unique SNPs by FP-TDI, PCR-RFLP, and Fluorescent Dye-Terminator Sequencing

Duplicate Method	No. of SNPs	No. of Genotypes	Discrepant Genotypes
PCR-RFLP	27	500	0
Sequencing	7	131	2
Total	34[a]	631	2[b]

[a]Two SNPs, one of which produced two genotypes that were discrepant between FP-TDI and sequence, were subsequently and blindly assayed by PCR-RFLP.
[b]PCR-RFLP genotypes of the two discrepant samples agreed with the original FP-TDI results.

Statistics

The 95% confidence interval of the discrepancy rate was estimated using the approximate normal method for proportions in the program Confidence Interval Analysis (7). Since neutral polymorphisms are expected to be at Hardy-Weinberg Equilibrium in most populations, significant deviations can suggest biased genotype calls (8). Deviation from Hardy-Weinberg Equilibrium was tested using the program HWSIM (http://krunch.med.yale.edu/hwsim/), which estimates Chi square values by a Monte Carlo permutation procedure when expected cell frequencies are less than 5.

RESULTS

Success Rates

A complete assessment of the success rate of a SNP genotyping method should consider both the proportion of SNPs that generate readable data and the proportion of samples that can be successfully amplified under the PCR constraints of the method. FP-TDI performed well on both counts. Overall, 143 of the 177 genotyped SNPs (80.8%) produced data of sufficient quality to allow the grouping of most of the genotypes into heterozygous and homozygous clusters (Figure 1). Among these SNPs, 80 of 2860 reactions (2.8%) failed to amplify on the first attempt. Out of 170 SNPs recovered from the dbSNP and The SNP Consortium, 134 (79%) were found to be polymorphic. Overall, there was no significant difference in success rates between different types of nucleotide substitutions.

Accuracy

To assess accuracy, 631 duplicate genotypes were generated by FP-TDI, PCR-RFLP, or sequencing for 30 different SNPs (Table 1). No discrepant calls were made between FP-TDI and 500 duplicate PCR-RFLP genotypes. Among 131 genotypes generated by sequencing, two samples were called heterozygous that FP-TDI had identified

as homozygous. Subsequent PCR-RFLP genotyping of these same samples produced genotypes that agreed with the FP-TDI results (i.e., the samples were found to be homozygotes, indicating that the sequencing results and not the FP-TDI results were likely in error). The true discrepancy rate was thus 0/631 among the SNPs and samples we studied. The 95% confidence interval of this proportion ranges from 0.00% to 0.58%, or a maximum of less than six errors in 1000 genotypes.

We assessed the agreement between observed and expected (Hardy-Weinberg Equilibrium) proportions of heterozygotes and homozygotes for 33 SNPs genotyped in unrelated subjects. No significant deviations were observed at the $P \leq 0.05$ level.

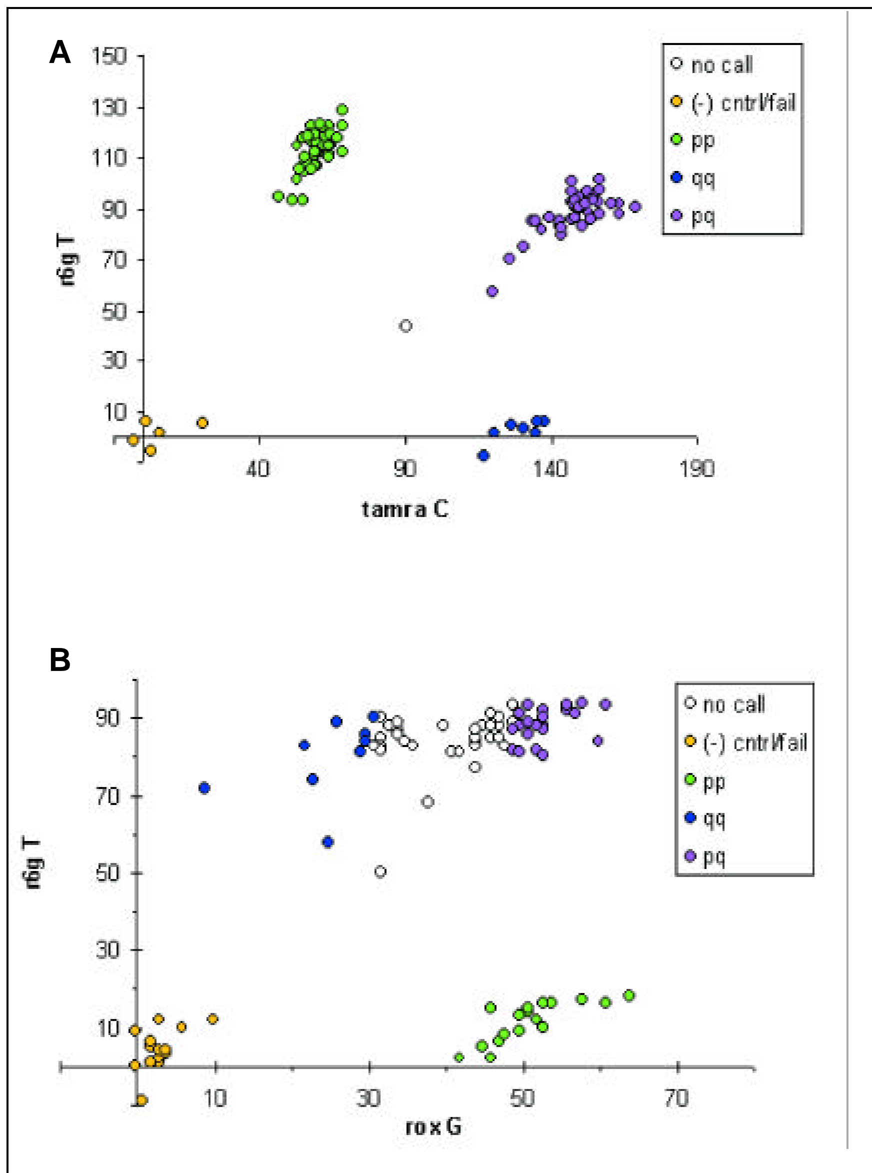

Figure 2. Example of automated genotype calling for a typical SNP (top) and one with poor clustering (bottom). Reducing the probability threshold to 95% for the poorly clustered SNP led to 26 additional genotype calls, all of which agreed with the expert reader, suggesting that more data could be recovered for some SNPs without sacrificing accuracy if the probability threshold is reduced. However, in practice, very few SNPs cluster poorly. Abbreviations: no call indicates data points that could not be assigned to any cluster at greater than 99.9% probability (see Materials and Methods); (-) cntrl/fail includes water controls and PCR failures; pp indicates the major allele homozygote; qq, the minor allele homozygote; and pq, heterozygotes. Results are plotted in mP units above the average polarization of the controls.

Automatic Genotype Calling

An automated method for calling genotypes and assigning a statistical confidence to each call is an essential component of any high-throughput method. Automatic genotype calls were generated for 20 SNPs genotyped on each of 186 unrelated subjects. Each genotype was called both automatically and manually by an expert reader blind to the automatic genotype calls.

Overall, 91.4% of all genotypes could be called automatically at greater than 99.9% probability (Figure 2). Of these 3509 genotype calls, 3500 (99.7%) agreed with the expert reader. The nine discrepant genotype calls were not considered by the expert reader to be assignable to a cluster. Two of these calls were clearly in error (scored as minor allele homozygotes by the algorithm), while seven were considered reasonable upon subsequent inspection.

DISCUSSION

The chief strength of this study is that it demonstrates the accuracy and utility of a widely used SNP genotyping method in a small laboratory setting. We studied an unbiased set of SNPs, mostly from public databases, that are typical of SNPs that would be employed in regional or candidate-gene association studies. All comparisons were made blind to the results of the other methods. While our assays were performed in a 96-well format, the technology should easily scale to 384-well plates. Thus, it should be possible to generate nearly 7700 genotypes per week with one technician, one fluorescent plate reader, and one four-block thermal cycler.

This study is limited mainly by the relatively small sample of SNPs we studied, all of which map to the same broad region on chromosome 18q21-22. However, our sample of more than 600 duplicate genotypes allowed us to estimate an error rate of 0% with a narrow 95% confidence interval that does not exceed 0.58%. Because the error rate is so low, even a trebling of the number of duplicate genotypes would not have substantially changed the estimate. All of the SNPs we studied mapped to a region that happens to have a GC content of 37%, somewhat below the human genome average of 41% (11). Since GC content affects both the ease of PCR amplification and the likelihood of hairpin formation within the FP-TDI probes, the results in regions of high GC content may differ from those we observed.

At this rate of throughput, automated genotype calling is essential. Here we present a method based on cluster and distance analysis that works efficiently with widely available software and agreed with our expert reader on

99.7% of all genotypes. Most of the genotype calls that did not agree were corrected by additional runs of the cluster analysis, which suggested that they were due to an unfortunate initial partition, an inherent problem with the k-means method (12). This problem could be addressed by selecting exemplary data points from each cluster to drive the initial partition. Independently, Ranade et al. (16) applied a similar method of cluster analysis to data generated by the fluorescent resonance energy transfer (TaqMan®) method, also with good results. We go one step further, estimating the probability that each data point belongs to a particular genotype cluster. Commercial software is available for calling FP-TDI genotypes, but the clustering algorithm is proprietary and no statistical certainty is applied to individual genotype calls. A Microsoft Excel template that implements our method is available at http://biplab.bsd.uchicago.edu.

FP-TDI performed well compared to other methods of comparable throughput. Published studies report success rates of 81% for Invader (13), 89% for dynamic allele-specific hybridization (DASH) (15), and 87% for TAG-SBE (4), which are similar to the rate of 81% we determined for FP-TDI. We could locate no published reports of success rates for denaturing HPLC, TaqMan, or Pyrosequencing™ (Pyrosequencing, Westborough, MA, USA). Our accuracy rate of at least 99.42% is at the high end of the published accuracy rates for other methods, which range from 99.95% for TaqMan (16) to 95.2% for TAG-SBE (4).

While FP-TDI performs well, it is no panacea. Well-clustered genotypes are typically generated by either the sense or the antisense probe—but often not both. We could discern no way to predict which probe would work best, so we designed, synthesized, and tested both the sense and antisense probes for each SNP. This, combined with the reagent costs for the PCR clean up and single-base extension reaction ($0.67/well or $0.38/well when a two-dye mixture is used) and the opaque plates required by FP-TDI ($2.50/plate), suggests that the cost might become prohibitive for projects requiring millions of genotypes. Yet, the ease of assay development, comparatively low initial costs, and excellent performance of the automated genotype calling method make FP-TDI an ideal technology for small-to-medium-sized laboratories, particularly those that genotype a relatively large number of different SNPs in relatively few samples.

In an ideal world, laboratories would have a choice among a variety of simple, accurate, and inexpensive SNP genotyping technologies. However, for the foreseeable future, the choice will be limited by the efficiency, accuracy, and expense of available methods. Our results indicate that FP-TDI is both efficient and accurate and can easily fill the needs of SNP genotyping projects at the scale typically used for regional or candidate-gene association studies.

ACKNOWLEDGMENTS

Supported by grants from the National Institutes of Health and the Edward F. Mallinckrodt, Jr. Foundation. We thank Tony Hsu and Pui-Yan Kwok for technical advice and for allowing one of the authors (N.A.) to visit their laboratory. We thank Edwin Cook and Dave Gonen for advice and technical assistance with the fluorescent plate reader.

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Received 26 October 2001; accepted 2 January 2002.

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