Real-Time PCR Assay for Quantitative Mismatch Detection

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ABSTRACT

We describe here a quantitative real-time PCR assay for the detection of single-base-pair differences that does not require fluorescently labeled gene-specific probes or complicated primer combinations. Following PCR or RT-PCR of a gene segment that may contain allele-specific differences, 100 pg amplified product are used for a real-time PCR with allele-specific primers and SYBR® Green. The use of HEPES buffer at a pH of 6.95 together with AmpliTaq® DNA polymerase results in a threshold difference between the correct template and the mismatched template of as many as 20 cycles, depending on the mismatch. Correct matches can be detected in an excess of mismatched template at least at the 0.01 level for the six primer-template matches versus mismatches tested: GC vs. A·C, AT vs. G·T, GC vs. C·C, GC vs. G·G, AT vs. C·T, and GC vs. G·A. Because the initial amplification is separate from real-time detection, conditions can be independently optimized for each step, making the assay particularly suitable for the detection of allele-specific expression in single cells.

INTRODUCTION

Mismatch detection is important not only for mutation detection but also for studies of imprinting or allelic exclusion. Previous work has shown that amplification of mismatches by primers flanking the allelic difference, followed by assay of the resulting amplified product, allows quantitative measurement of allelic ratios in small amounts of material (1), including single cells (2). Real-time PCR assays described thus far differ from this approach in that real-time PCR is performed directly on genomic DNA or total RNA and generally involves gene-specific fluorescently labeled primers or probes (3–5), although the fluorescent dye SYBR® Green (Molecular Probes, Eugene, OR, USA) has also been employed (6,7). In a recent study, SYBR Green was used to detect allelic variation present at the 0.1%–1% level, but single-base-pair mismatches were not studied, and the range in threshold cycles between correct and mismatched templates was low (8).

The method we describe here differs from the above assays in that the amplification step remains distinct from detection. This allows the use of two sets of conditions: the first optimized for

amplification of small amounts of material and the second optimized for quantitative real-time detection of mismatches. In addition, because the template for the assay is a specific amplified product rather than genomic DNA or RNA, SYBR Green can be used with greater confidence that amplification of the target of interest is being measured. Other SYBR Green-based allele-specific assays have been reported (9), but the technique we describe allows more accurate quantification because the buffer we utilize results in greater allele-specific differences in threshold cycles, most likely because of its low pH. Under the conditions we describe, allele-specific PCR is quantitative at least at the 0.01 level for all mismatches tested. Although the calculated cost per assay is comparable to that of other methods in which *Taq* DNA polymerase is used, such as the LightTyper SNP Analysis System™ (Roche Applied Science, Mannheim, Germany), this assay is more sensitive and does not require specialized instrumentation, tubes, or kits, so the actual cost may be considerably less.

MATERIALS AND METHODS

Templates and Primers

Templates were synthesized from genomic DNA of mouse strains C57BL/6 or AT29 (10): the first template was a 572-bp PCR product synthesized from the *Pgk1* promoter (GenBank® accession no. X15339) with primers 5′-AGCATGCGCTTTAGCAGCCCC-3′ and 5′-GAGGGCTTGAGGGCAGCAGTA-3′. The second was a 398-bp product synthesized from the *Xist* promoter (GenBank accession no. U29341) with primers 5′-TTTTGCATAGACAGGTGTGTGACCTAATGT-3′ and 5′-CTTGGTGGTAGGGGAACTAAAAAGTTC-3′. Details of template synthesis are available upon request. Templates were gel-purified before use and then stored at -20°C. A diagnostic agarose gel was used to estimate the concentration of each amplified product. Table 1 shows the primers used for match/mismatch detection.

Conditions of the Allele-Specific Assay

For *Pgk1*, each reaction contained in a final volume of 25 µL: 20 mM HEPES buffer, pH 6.95, 50 mM KCl, 10 mM $(NH_4)_2S0_4$, 1.5 mM $MgCl_2$, 5% DMSO (Roche Applied Science), 100 µg/mL BSA (New England Biolabs, Beverly, MA, USA), 40 µM each dNTP, 0.1 µM each primer, 1 U AmpliTaq® DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 0.005% SYBR Green (diluted from a

0.1% working solution in HEPES buffer, pH 6.95), and 100 pg template in varying allelic ratios as indicated. For *Xist*, reaction conditions were the same, except that for G·A detection, the reaction mixture contained 2.5 mM $MgCl_2$, and for T·C detection, 3.0 mM $MgCl_2$ and 2% DMSO were used.

Templates, primers, and dNTPs were distributed to Eppendorf® tubes on ice; then, an equal volume of a master mixture containing the remaining reagents was added. Samples were distributed to Hard-Shell™ thin-walled skirted microplates (MJ Research, Waltham, MA, USA) at room temperature and covered with Microseal™ "B" Adhesive Film (MJ Research).

Real-time PCR was carried out in a DNA Engine Opticon™ (MJ Research). Conditions of real-time PCR were as follows. For *Pgk1* (A·C and G·T mismatches): 94°C for 2 min, 60°C for 2 min, and 72°C for 1 min, followed by 35 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 1 min, and 82°C for 2 s, plate read (i.e., fluorescence detection). For *Pgk1* (C·C and G·G mismatches): 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, followed by 35 cycles of 94°C for 15 s, 64°C for 30 s, 72°C for 1 min, 82°C for 2 s, plate read. For *Xist*: 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 35 cycles of 94°C for 15 s, 60°C for 1 min, 72°C for 1 min, and 82°C for 2 s, plate read. Melting curve analysis was performed at the end of each reaction.

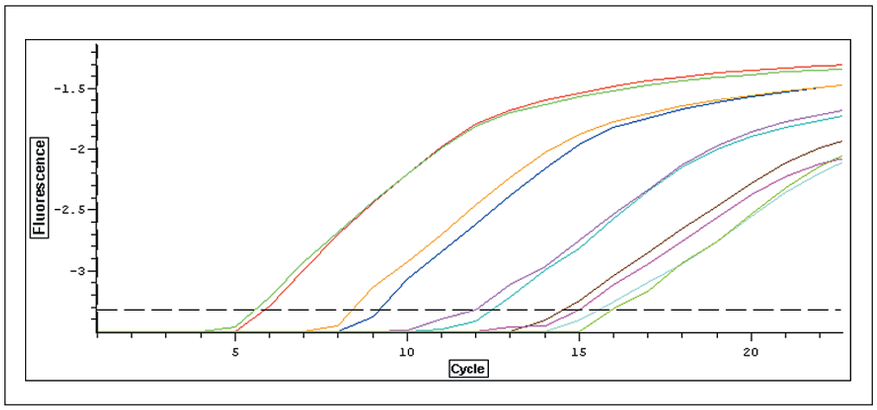

Figure 1. Illustration of assay. The real-time PCR assay was performed with a mixture of the C57BL/6 and AT29 *Pgk1* templates and the primer set for the detection of an AT base pair vs. a G·T mismatch (Table 1). The fraction of the matched (AT29) template was, from left to right, in duplicate, 1, 0.1, 0.01, 0.001, and 0. The dashed line shows the threshold, which was manually selected after baseline subtraction. x-axis, cycle number; y-axis, log fluorescence of SYBR Green.

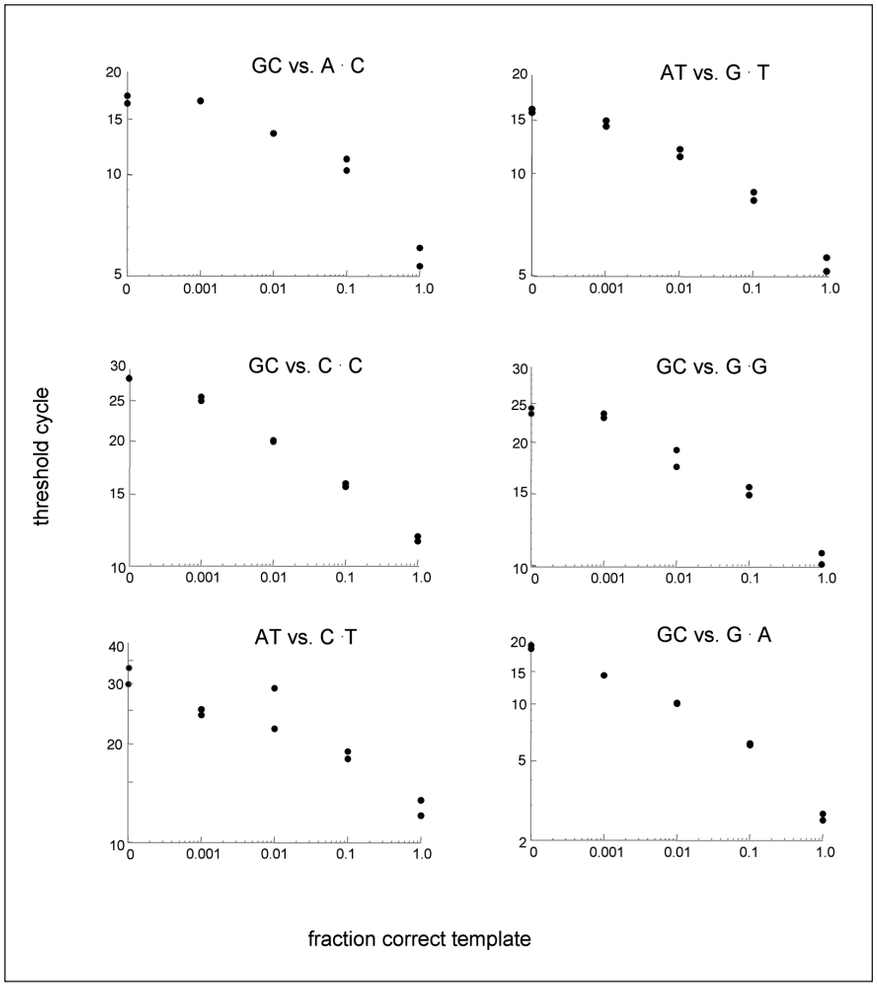

Figure 2. Sensitivity of the assay for detection of transitions (top two panels) and transversions (bottom four panels). Correct and mismatched templates were mixed in the ratios indicated on the x-axis and analyzed by the real-time PCR assay. Table 1 shows the templates and primers used. The match and mismatch probed by each template/primer set are shown at the top of each panel, with the mismatched pair denoted by a dot. All experiments were carried out at least in duplicate, with the threshold cycle determined after selection of the threshold, as illustrated in Figure 1.

RESULTS AND DISCUSSION

The Real-Time PCR Assay

Table 1 shows the primers and templates used for the real-time PCR assay. Each allele-specific primer, indicated by an asterisk, was used together with the reverse primer shown to assay for the corresponding match in an excess of mismatched template. For each primer set tested, templates amplified from strains AT29 or C57BL/6 were compared, as well as mixtures of the two templates, mixed in the ratios 0.1, 0.01, and 0.001 correct/mismatched template. Real-time PCR was performed with the fluorescent dye SYBR Green added to each reaction to mea-

Table 1. Templates and Primers for Real-Time PCR Assay

Gene	Primers Used (5′→3′)	Size of Product	% GC	Match/ Mismatch Detected	Reference
Pgk1	TTCTACTCCTCCCCTAGTCAGGAAGTTC* TTCTACTCCTCCCCTAGTCAGGAAGTTT* AGGCCTACCCGCTTCCATTGCTCAG	138	60	GC vs. A·C AT vs. G·T	16
Pgk1	GCTTTTGAAGCGTGCAGAATGCCGGGCCTCC* GCTTTTGAAGCGTGCAGAATGCCGGGCCTCG* TTCTACTCCTCCCCTAGTCAGGAAGTT	290	65	GC vs. C·C GC vs. G·G	16
Xist	GAGGAGTGGCCACAAAGATTGCAAT* GAGGAGTGGCCACAAAGATTGCAAG* TTTTGCATAGACAGGTGTGTGACCTAATGT	241	33	AT vs. C·T GC vs. G·A	17

The allele-specific forward primers are indicated by an asterisk. For each set, the first and second primers correspond to the C57BL/6 and AT29 alleles, respectively; the third primer is the reverse primer used with either forward primer.

sure amplification and determine the threshold cycle. Figure 1 illustrates results for the AT match versus G·T mismatch, where the dependence of threshold cycle on the match:mismatch ratio can be clearly seen. The spread between the threshold cycle obtained with the correct versus mismatched allele is at least 10 cycles, which is sufficient for quantitative detection with a small number of duplicate samples. Comparable results were obtained for the other five primer sets assayed; as shown in Figure 2, matches in all cases can be detected at least at the 0.01 level. The assay is also accurate enough that 2- to 3-fold changes in allele-specific expression are measurable (Figure 3).

Optimization of Conditions

The results shown in Figure 2 were obtained under the optimal conditions described in the Materials and Methods section. Among the components varied in the course of optimization were as follows.

Buffers. It was necessary to use HEPES buffer, pH 6.95, instead of the standard Tris-HCl, pH 8.3, for mismatch discrimination at the level shown in Figure 2. This result is consistent with previous results showing that misincorporation by *Taq* DNA polymerase is reduced at lower pH (11). In view of a recent study showing that SYBR Green is alkali labile and that its breakdown products inhibit PCR, the use of HEPES buffer may also ameliorate the inhibitory effect on PCR widely observed with SYBR Green.

Allele-specific primers. The primers shown in Table 1 varied in length from 25 to 32 bases and showed the greatest specificity at annealing temperatures in the range of 60°C–64°C. This was a surprising result as the T_m of the primers is far above this range, and much work on allele-specific PCR has been based on the assumption that maximum specificity is obtained by the use of much shorter primers at or near their T_m (12). It seems that at pH 6.95 the requirement of *Taq* DNA polymerase for perfect complementarity at the 3′ base is less related to the overall stability of the primer-template duplex than at pH 8.3.

Template concentration. Variation of the concentration of template showed that 4 pg/µL (100 pg/reaction) was optimal for mismatch detection. This concentration is similar stoichiometrically to that found to be optimal for direct detection of alleles in genomic DNA (8).

Product size and GC content. The assay worked well with real-time PCR products ranging in size from 138 to 290 bp, and over a wide range of GC content (Table 1), although the establishment of optimal conditions appeared more straightforward with the shortest product.

$MgCl_2$ concentration. For some allele-specific primer sets, the optimal $MgCl_2$ was unchanged by the addition of SYBR Green, while, for others, the use of SYBR Green changed the optimal $MgCl_2$ concentration by 0.5–1 mM, as previously reported (13). Thus, the $MgCl_2$ concentration should be determined empirically for each primer set.

BSA and DMSO. BSA and DMSO were essential components of the reaction, although the optimal level of DMSO varied from 2% to 5% and thus should also be determined empirically for each primer set.

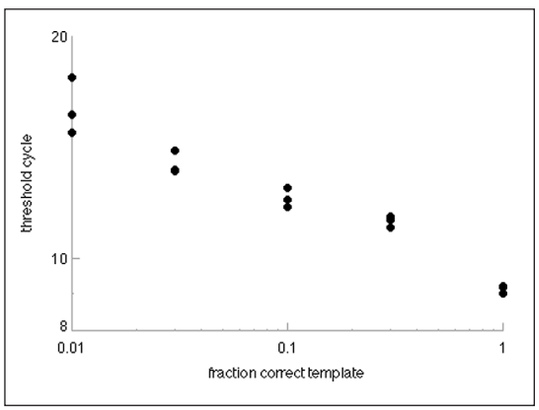

Figure 3. Accuracy of the assay. The threshold cycle was determined as described in the legends to Figures 1 and 2, with *Pgk1* templates and primers for detection of an AT base pair vs. a G·T mismatch. The fraction correct (AT29) template was 0.01, 0.03, 0.1, 0.3, or 1, in triplicate, as shown.

Applications of the Assay

The assay we describe here should be particularly applicable to the analysis of imprinting and allelic exclusion of small amounts of material, including single cells, as it shares many of the strengths of the single nucleotide primer extension assay that has been commonly used for this purpose (2, 14,15). As with the radioactive single nucleotide primer extension assay, the assay can also be adapted for absolute quantification if a synthetic mismatched template is added at known concentration at the original amplification step (14). The accuracy, sensitivity, simplicity, and relative low cost of the assay, as well as its potential to be automated, should make it a valuable method for all of these applications.

REFERENCES

1. **Gilliland, G., S. Perrin, K. Blanchard, and H.F. Bunn.** 1990. Analysis of cytokine mRNA and DNA: detection and quantitation by competitive polymerase chain reaction. Proc. Natl. Acad. Sci. USA *87*:2725-2729.
2. **Shen, S.I., C. Gao, and J. Singer-Sam.** 1998. Use of a reverse transcriptase-polymerase chain reaction assay to analyze allele-specific expression in individual hippocampal neurons. Mol. Genet. Metab. *63*:96-102.
3. **Bleicher, K.B., T.R. Pippert, W.E. Glaab, T.R. Skopek, J.F. Sina, and D.R. Umbenhauer.** 2001. Use of real-time gene-specific polymerase chain reaction to measure RNA expression of three family members of rat cytochrome P450 4A. J. Biochem. Mol. Toxicol. *15*:133-142.
4. **Fujii, K., Y. Matsubara, J. Akanuma, K. Takahashi, S. Kure, Y. Suzuki, M. Imaizumi, K. Iinuma, et al.** 2000. Mutation detection by TaqMan-allele specific amplification: application to molecular diagnosis of glycogen storage disease type Ia and medium-chain acyl-CoA dehydrogenase deficiency. Hum. Mutat. *15*:189-196.
5. **Pals, G., C. Young, H.S. Mao, and M.J. Worsham.** 2001. Detection of a single base substitution in a single cell using the LightCycler™. J. Biochem. Biophys. Methods *47*:121-129.
6. **Vandesompele, J., A. De Paepe, and F. Speleman.** 2002. Elimination of primer-dimer artifacts and genomic coamplification using a two-step SYBR Green I real-time RT-PCR. Anal. Biochem. *303*:95-98.
7. **Waterfall, C.M. and B.D. Cobb.** 2002. SNP genotyping using single-tube fluorescent bidirectional PCR. BioTechniques *33*:80-90.
8. **Garavito, G., D. Klein, M. Denis, A. Pugliese, C. Ricordi, and R.L. Pastori.** 2002. Real-time sequence-specific primer polymerase chain reaction amplification of HLA class II alleles: a novel approach to analyze microchimerism. Transplantation *73*:822-825.
9. **Hiratsuka, M., Y. Agatsuma, and M. Mizugaki.** 1999. Rapid detection of CYP2C9*3 alleles by real-time fluorescence PCR based on SYBR Green. Mol. Genet. Metab. *68*:357-362.
10. **Park, J.G. and V.M. Chapman.** 1994. CpG island promoter region methylation patterns of the inactive-X-chromosome hypoxanthine phosphoribosyltransferase (Hprt) gene. Mol. Cell. Biol. *14*:7975-7983.
11. **Eckert, K.A. and T.A. Kunkel.** 1990. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. Nucleic Acids Res. *18*:3739-3744.
12. **Wu, D.Y., L. Ugozzoli, B.K. Pal, and R.B. Wallace.** 1989. Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. Proc. Natl. Acad. Sci. USA *86*:2757-2760.
13. **Karsai, A., S. Muller, S. Platz, and M.T. Hauser.** 2002. Evaluation of a homemade SYBR® Green I reaction mixture for real-time PCR quantification of gene expression. BioTechniques *32*:790-796.
14. **Buzin, C.H., J.R. Mann, and J. Singer-Sam.** 1994. Quantitative RT-PCR assays show *Xist* RNA levels are low in mouse female adult tissue, embryos, and embryoid bodies. Development *120*:3529-3536.
15. **Szabó, P.E. and J.R. Mann.** 1996. Maternal and paternal genomes function independently in mouse ova in establishing expression of the imprinted genes Snrpn and Igf2r: no evidence for allelic trans-sensing and counting mechanisms. EMBO J. *15*:6018-6025.
16. **Boer, P.H., H. Potten, C.N. Adra, K. Jardine, G. Mullhofer, and M.W. McBurney.** 1990. Polymorphisms in the coding and noncoding regions of murine Pgk-1 alleles. Biochem. Genet. *28*:299-308.
17. **Komura, J., S.A. Sheardown, N. Brockdorff, J. Singer-Sam, and A.D. Riggs.** 1997. *In vivo* ultraviolet and dimethylsulfate footprinting of the 5′ region of the expressed and silent *Xist* alleles. J. Biol. Chem. *272*:10975-10980.

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