experiments. The expression of purified GST was significantly greater in IPTG-induced than uninduced lysates by Coomassie blue staining, as expected, as well as by Western blot analysis with anti-GST antibodies (Figure 3A). Thus, the anti-GST antibodies prepared by depletion of the immune serum are also capable of detecting the appropriate antigen. It is likely, however, that this preparation contains antibacterial antibodies. In contrast, while affinity-purified anti-FAC antibodies detected two different GST-FAC fusion proteins, these antibodies failed to detect GST (Figure 3B). Thus, the anti-FAC antibody preparation can detect the fusion partner of GST and appears to be largely free of anti-GST antibodies. Finally, these antibodies were also evaluated for their ability to immunoprecipitate FAC from transfected COS-1 cells. Incubation of cytosolic lysates successively with anti-FAC antibodies (from both rounds of purification) and protein A agarose (Affi-Gel®; Bio-Rad) followed by SDS-PAGE and autoradiography showed the expected 63-kDa antigen (Figure 3C). Similar quantities of anti-GST antibodies failed to immunoprecipitate FAC. These data demonstrate that the affinity-purified anti-FAC antibodies are monospecific and bind to soluble FAC in mammalian cells.

In summary, this protocol obviates the need for covalent coupling of the antigen to reactive matrices and can be completed in less than one day. The elution conditions preserve the integrity of the antibodies for many applications without the need for dialysis of the elution buffer. Although the subsequent binding capacity of protein-coated glutathione agarose is reduced considerably, affinity-purified antibodies obtained from the second round of purification remain comparable in purity and function to those obtained from the first round.

REFERENCES


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Differential Amplification Kinetics for Point Mutation Analysis by PCR

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Detection of single-base mutations in DNA is a frequent task in many fields of biology and medicine. Polymerase chain reaction (PCR) has greatly facilitated obtaining fragments of DNA containing such critical nucleotide positions. However, the error-prone activity of Taq DNA polymerase (i.e., mismatch extension and nucleotide misincorporation), while advantageous for broadly specific amplification using partially mismatched primers, presents an obstacle when the presence or absence of an amplification product is used as an indicator for detection of point mutations. Mismatches at the 3′ ends of primers, caused by critical base changes in the target DNA, do not prevent amplification of such targets because of the ability of Taq DNA polymerase to extend 3′ mismatched primers.

Two main strategies are used to make PCR applicable to analysis of point mutations. Some methods use very low dNTP concentrations (each at 2–4 μM), which virtually prevent mismatch extension but generate little amplification product. Thus, sensitive methods are required for PCR product visualization instead of simple agarose gel electrophoresis (4,8). Other methods artificially introduce destabilizing mismatches close to the 3′ ends of primers so that an additional 3′ mismatch of the primer will prevent detectable amplification even after 30–40 amplification cycles (1,3,5,9). This second approach requires extensive optimization of primers and is inherently unstable because of the possibility of additional mutations in the target DNA.

It is also strongly dependent on an optimal PCR annealing temperature because the stability of primer hybridization to the target critically affects primer extension.

The kinetics of the appearance of an amplification product are influenced by any mismatch at the 3′ end of a primer. During the first few cycles of a
PCR, extension from 3′ mismatched primers will be very inefficient. Only later in the PCR, when perfectly matched products of these initial extensions serve as templates, exponential amplification will commence. After 25 or more thermal cycles, at the saturation plateau of the PCR, similar amounts of DNA fragment will be visualized by gel electrophoresis for matched as well as mismatched original target DNAs, thus making allelic discrimination impossible.

Recently, we developed a hemi-nested PCR method for allele discrimination of the omp1 gene of the bacterial genus Chlamydia (7). Saturated positive PCRs for amplification of a portion of the chlamydial omp1 locus were diluted 1:100 in distilled H2O. Five microliters of the dilutions, containing approximately 10⁹ molecules of the omp1 PCR product, were amplified in 25-µL allele-specific PCR mixtures. These mixtures contained one of the previously used Chlamydia genus-specific primers and one allele-specific primer. The reaction buffer was 20 mM Tris-HCl, pH 8.3, 25 mM KCl, 2 mM MgCl₂, 0.05% Nonidet® P-40 (NP40), 0.05% Tween® 20 and 0.01% bovine serum albumin, supplemented with 200 µM each dNTP, 0.2 µM each primer and 0.8 U Taq DNA polymerase (Promega, Madison, WI, USA).

Initially, we performed these PCRs similarly to the method described by Bein et al. (2), using annealing temperatures of about 60°C, which are close to the calculated melting temperature (Tₘ) of the allele-specific primers. These primers were designed with several mismatches close to and at the 3′ ends (Figure 1). 10-µL aliquots of the PCR mixtures were restricted with HaeIII (Promega). In subsequent experiments, lowering the annealing temperature to 50°C or less did not negatively affect the allelic discrimination in 15 PCR cycles through undesirable amplification of non-cognate alleles but increased the product yield in the cognate alleles (data not shown). Thus, even if partially mismatched primers hybridized well to the template at annealing temperatures of 10°C or more below the calculated Tₘ, they were not able to prime a high-level generation of PCR products in 15 cycles if they were 3′ mismatched. We concluded that the efficiency of primer extension, but not of hybridization, drove the generation of a visible product from cognate alleles in a PCR of only 15 thermal cycles.

The corollary of this observation is that the kinetics of amplification with a 3′ perfectly matched primer versus a 3′ mismatched primer can be used for detection of single-base mutations. A

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**Figure 1. DNA sequences of the primers used in this study, the amplified cognate and selected non-amplified, non-cognate alleles.** The primers AR388 and Koala were used in this study to demonstrate PCR cycle dependence of amplification kinetics-based PCR typing. They differ in the 3′-terminal base, which is cognate for the respective allele. The remaining oligonucleotide contains degeneracies to allow for efficient hybridization to both C. pneumoniae AR388 and Koala alleles. The chlamydial alleles listed below are examples from other chlamydial species that are not amplified by the AR388 and Koala primers (7). Lower-case letters of the nucleotide sequences indicate mismatches to the AR388 primer. Degenerate positions are indicated as follows: R = A or G; S = C or G; Y = C or T.

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**Figure 2. Titration of optimal thermal cycle number for cognate amplification in allele-specific PCRs.** Amplification products of Chlamydia genus-specific PCRs (ca. 10⁹ templates in 5 µL of a 1:100 dilution of the genus-specific PCR) of the chlamydial strains (omp1 alleles) AR388 and Koala (AR388 or Koala template) were re-amplified in both AR388 and Koala allele-specific PCRs. These PCRs contained one identical genus-specific primer and one of the allele-specific AR388 or Koala primers. The allele-specific primers differed by one base at the 3′ ends (Figure 1). 10-µL aliquots of the PCR mixtures were analyzed by 1.5% agarose gel electrophoresis and ethidium bromide staining. Specific products for cognate template-primer combinations are visible after 12 thermal cycles, while products in the non-cognate PCRs begin to appear after 18 (Koala template in AR388 PCR) and 24 cycles (AR388 template in Koala PCR). The molecular size marker is φX174 DNA restricted with HaeIII (Promega).
simple design of such allele-specific PCRs that does not require primer and PCR optimization is possible. First, a DNA fragment containing the site to be analyzed is amplified to saturation. Then, a portion of this PCR containing approximately 10^8 amplified molecules is amplified for a low number of thermal cycles using one of the primers of the first PCR and one allele-specific primer. The allele-specific primer must incorporate the critical site at the 3′ end and should contain enough nucleotides to ensure efficient annealing.

We tested this hypothesis in PCRs for discrimination of the 2 closely related chlamydial omp1 alleles, C. pneumoniae AR388 and Koala. Two degenerate primers (Figure 1) with the same sequence [AAT CA(G/C) TAG TAG (A/G)(CA ATG (C/T)TG T(G/C)G], which is present in both alleles, were synthesized; however, they had either C (cognate for AR388) or T (cognate for Koala) as a 3′-end base. These primers were used in allele-specific PCRs together with the degenerate Chlamydia genus-specific primer 201CHOMP (7) from the first amplification. A hot-start PCR was performed in a PCR System 2400 Thermal Cycler (Perkin Elmer, Norwalk, CT, USA) for 12, 15, 18, 21 or 24 cycles for 30 s each at 95°C, 30 s at 46°C and 30 s at 72°C. Figure 2 demonstrates the validity of our assumption; clearly visible, correct amplification products of the cognate alleles can be observed after 12 cycles, while products of the non-cognate alleles begin to appear after 18 (AR388 PCR with Koala template) or 21 cycles (Koala PCR with AR388 template).

These data underscore the robustness, ease and specificity of allele identification by allele-specific PCR of products of previous PCRs. We have applied this approach to many different bacterial (7) and vertebrate DNA templates (S.J. Ewald, personal communication) and found that it typically detects point mutations without any optimization of the reaction parameters. The low annealing temperature combined with low complexity and high template number in allele-specific PCR facilitates efficient amplification of cognate alleles. Fifteen thermal cycles are typically sufficient to clearly visualize the amplification products by agarose gel electrophoresis, while no traces of non-cognate amplification can be observed. Our results suggest that this method is generally applicable to point mutation analysis by PCR.

REFERENCES


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Nonelectrophoretic Genotyping Using Allele-Specific PCR and a dsDNA-Specific Dye

The ability to rapidly and inexpensively genotype large numbers of individuals has become increasingly important in a wide range of applications, from genomics and linkage mapping to population genetics research. Several methods are available that make use of the polymerase chain reaction (PCR) to rapidly assay base substitution polymorphisms. Examples include restriction enzyme digestion, allele-specific oligonucleotide hybridization, ligation capture, allele-specific PCR (ASPCR) and others (1). We focus on an alternative detection method for ASPCR (4,9).

The method we describe uses a double-stranded (ds)DNA-specific fluorescent dye (PicoGreen®; Molecular Probes, Eugene, OR, USA). Under the conditions of this assay, PicoGreen is substantially more sensitive to double-stranded than to single-stranded DNA. Consequently, there is no need to separate the products electrophoretically nor to precipitate, spin dialyze or bind the product to a streptavidin-coated plate as is typically done to eliminate unincorporated primers. In the procedure described here, amplified products are transferred to microplates containing the dye and the plates scanned with a fluorescence plate reader. Emissions values are logged directly to a spreadsheet, and genotypes are inferred from the emissions values according to empirically established criteria. For studies seeking to use ASPCR to assay biallelic base substitution or insertion/deletion polymorphisms, this method represents a dramatic increase in throughput and a reduction in cost over previous methods.

We have developed an ASPCR assay for a GA transition in the p53 protooncogene of chinook salmon (Oncorhynchus tshawytscha). As an illustration of the use of dsDNA-specific dye in ASPCR, we genotyped 32 individuals in a blind sample for the p53-