quences, indicating that the target fragment was not self-ligated but trapped a small sequence (TCGAATGGAAATCCATCTTTCAATGGGAAAGGAATGGG-AATCATCGCATAGAATTCGA) that belongs to a known gene (GenBank® Accession No. 56662). It is very unlikely that the same fragment can be ligated into the transposon ends more than once, suggesting that this protocol is so efficient that a single-ligated target sequence can be cloned out of a complex genome. We then tested whether or not different flanking sequences can be cloned from cell pools. The two plasmids, pRP466-NEO and pCEP4, were delivered into human KB cells by electroporation. If the transposon jumps in human cells, integrating in the genome, it will have different flanking sequences depending on the transposition sites (6). The cells containing various G418-resistant clones were counted and harvested. The genomic DNA was extracted and digested by TaqI. After the regular phenol/ethanol precipitation procedure, the digested DNA from approximately 1000 cells was ligated at 12°C overnight in a 50-µL volume. The nested PCR described above was performed in 10–20 parallel reactions each containing 0.05–2 µL ligated product. As shown in Figure 1B, in most reactions in which the template DNA was from 1–4 cells, only one band was detected, and a few tubes gave no signal after electrophoresis on 2% agarose gel and visualization by UV. The amplified fragments were recovered from the gel and directly sequenced with primer 4. The primers were then designed, and all of the 8 amplified fragments we sequenced proved to be the flanking sequences of the Tc1 transposon integrated or transposed into the human genome. When the amount of the template was increased, several expected fragments can be amplified in one reaction. The 2 fragments of approximately 200 and 90 bp, respectively, were amplified with high frequency, indicating that these cells represent the major groups in cell pools. This result is consistent with the clonal analysis.

In this paper, we report a protocol to clone the flanks of a known sequence from cell pools. This procedure eliminates many tedious works, including the amplification of a large number of cell clones, extraction and ligation of a large number of DNA samples, as well as the use of an isotope. It is also particularly useful to clone the various flanking sequences of a transposon or a virus when there are multiple copies of either one in a cell. Compared to the classical method of cloning the flanking sequences, this modified procedure is easy, economic and fast. This method can be used to identify random integration sites in different clones or a population of clones. It is also a quick method to identify either transposition frequency or a preferential site.

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Detection of Frame-Shifts within Homopolymeric DNA Tracts Using the Amplification Refractory Mutation System (ARMS)

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Analysis of point mutations is routinely performed following site-directed mutagenesis for the purpose of selecting mutated clones or when screening natural alleles for the presence of specific mutations. Several different techniques have been developed for such screens, e.g., single-strand conformational polymorphism (SSCP) and heteroduplex analysis (4), denaturing gradient gel electrophoresis (DGGE) (6) and constant denaturant gel electrophoresis (CDGE) (3). Among the polymerase chain reaction (PCR)-based methods, the amplification refractory mutation system (ARMS) is easiest to perform and does not need specific PCR materials or special detection equipment (1,2). ARMS is based on the principle that a perfect match at the 3’ end of PCR primers is needed for the efficient extension of the primers by Taq DNA polymerase. Primers used in ARMS cover the mutated nucleotide on their respective 3’ ends. Primer pairs representing either the wild-type or the mutated nucleotide are applied to distinguish both versions by quantitative and qualitative differences in the performance of the PCRs (5).

This standard method is inadequate for the detection of single-nucleotide...
mutations that occur within homopolymeric nucleotide tracts. Such homopolymeric tracts tend to mutate with higher frequencies than regions without repeats due to the problems they present to DNA-replication enzymes. In such cases, diagnostic (or analytical) primers designed with repetitive bases at their 3′ ends do not discriminate between different lengths of homopolymeric base stretches. Such primers can overrun mismatches by folding and shifting the mismatch into internal regions of the annealed primer/DNA template duplex, while providing the perfectly matched 3′ end that is obligatory for an efficient PCR. However, the mismatch-detection principle of ARMS can be applied to the detection of point mutations that occur within homopolymeric tracts by designing diagnostic primers that have several non-repetitive bases on the 3′ end. We have found that ending primers with the second or third non-repetitive nucleotide following the homopolymer tract works well to prevent mismatches during primer annealing without compromising the sensitivity of the ARMS technique. However, since the discrimination is not absolute in any case, analytical PCR should be performed in pairs with both primer sets.

For example, in the case of the *Drosophila melanogaster* GFAT gene (EMBL Accession No. Y18628), 6 T residues in the genomic sequence (Figure 1A) caused problems for cDNA synthesis in various reactions, resulting in cDNAs containing only 5 T residues at that position. To distinguish between false positives and correctly reverse-transcribed cDNAs (i.e., clones containing 5 vs. 6 Ts), primers A, B (Figure 1A) and C (5′-GATCAAGACTACATTCC-3′) were designed. The melting points of the primers are 50.2° (Primer A) and 49.1°C (Primers B and C), respectively. Primers A and B are separated from primer C by 168 bp of the *Dmel*GFAT cDNA (Figure 1B). A PTC-150 MiniCycler™ (MJ Research, Watertown, MA, USA) with heated lid and *Taq* DNA Polymerase (Cat. No. 18038-018; Life Technologies, Gaithersburg,

![Figure 1. Specification of primers used for the PCR detection of frame-shift mutations. (A) Sequence of the *D. melanogaster Gfat* gene from nucleotide position no. 6188–6217 (EMBL Accession No. Y18627) showing cDNA polymorphism depending on the method used for cDNA synthesis. Primer A: reverse complement to the genomic DNA containing 6 A residues. Primer B: reverse complement to the cDNA containing 5 A residues. Bold character: nucleotide position to be investigated. (B) Schematic drawing of the arrangement of primers with respect to the mutation to be detected. Fragment size: 200 bp, optimal product size for PCR detection of DNA fragments minimizing influences of length, sequence and potential secondary structures. Hatched triangle: localization of frame-shift mutation. Hatched arrows: positions of primers for preliminary PCR of varied kinds of DNA templates.](image)

![Figure 2. PCR detection of frame-shift mutations. PCRs generated with primers B and C (containing 5 T residues) were loaded in even-numbered lanes 2–12. PCRs generated with primers A and C (containing 6 T residues) were loaded in odd-numbered lanes 3–13. The template is frame-shifted *Gfat* cDNA (5 A) or native *Gfat* (6 A) as indicated. Lanes 1 and 14 contain a 1-kb ladder (LTI) size marker. (A) Influence of annealing temperature on PCR performances. Lanes 2–5, 59°C; lanes 6–9, 61°C; lanes 10–13, 63°C. All PCRs contained 1 U *Taq* polymerase. (B) Influence of *Taq* DNA polymerase concentrations on PCR efficiency. Annealing temperature was 61°C. *Taq* DNA polymerase concentrations are shown as U *Taq* polymerase reaction; lanes 2–5, 0.5 U; lanes 6–9, 1 U; lanes 10–13, 2.5 U. (C) Analysis of different *Gfat* cDNA and genomic DNA probes for frame-shift polymorphism. *Taq* DNA polymerase concentration was 1.0 U/50 µL reaction; annealing temperature was 61°C. Templates: lanes 2 and 3, λ3.2 genomic DNA (6 T); lanes 4 and 5, cDNA IV.8 (6 T); lanes 6 and 7, cDNA II.2.4 (6 T); lanes 8 and 9, cDNA V.1 (5 T); lanes 10 and 11, cDNA VI.1, after site-directed mutagenesis (6 T, see text and panel D); lanes 12 and 13, negative controls without template. (D) Analysis of individual mutagenized clones (see text). PCR conditions are as in panel C. Templates: lanes 2 and 3, VI.1 (6 T); lanes 4 and 5, VI.2 (5 T); lanes 6 and 7, VI.3 (5 T); lanes 8 and 9, VI.4 (5 T); lanes 10 and 11, VI.5 (5 T); lanes 12 and 13, VI.6 (5 T).](image)
MD, USA) were used. In the case of comparing DNA of different origins (e.g., cloned cDNAs, isolated genomic DNA or λ-cloned genomic DNA), preliminary PCRs were performed initially with flanking 5’ and 3’ primers (Figure 1B, hatched arrows) to obtain DNA templates of similar size for the analytical PCRs. We used 2 µL of a 1/1000 dilution of the preliminary PCRs as template. All PCRs contained 1× PCR Buffer (Life Technologies), 2 mM MgCl2, 200 µM dNTP, 0.5 µM of each primer and 1 U Taq DNA polymerase per 50 µL volume. To minimize pipetting variations, master mixtures were prepared containing all ingredients except primers and templates. Thirty cycles were performed as follows: 1 min at 94°C, 1 min at the respective annealing temperature and 1 min at 72°C. Ten microliters of each 50 µL PCR were loaded onto a standard TBE 1.2% agarose gel. The concentration of DNA templates, including cDNA, was 1.0 U/50 µL of a 1/1000 dilution of the preliminary PCRs as template. All PCRs contained 1× PCR Buffer (Life Technologies), 2 mM MgCl2, 200 µM dNTP, 0.5 µM of each primer and 1 U Taq DNA polymerase per 50 µL volume. To minimize pipetting variations, master mixtures were prepared containing all ingredients except primers and templates. Thirty cycles were performed as follows: 1 min at 94°C, 1 min at the respective annealing temperature and 1 min at 72°C. Ten microliters of each 50 µL PCR were loaded onto a standard TBE 1.2% agarose gel. The concentration of Taq DNA polymerase was found to be the most crucial parameter for a successful discriminating reaction.

Interestingly, the frame-shift discrimination works over a broad annealing temperature range, although to variable extent (Figure 2A). However, the appropriate annealing temperature has to be tested in accordance with the calculated melting temperature of the respective primers before the ARMS application. The Taq DNA polymerase concentration that gave the most consistent success in discriminating between T and T residues in a variety of DNA templates, including cDNA and genomic clones, was 1.0 U/50 µL reaction (Figure 2B). The use of higher concentrations of Taq DNA polymerase diminished the discriminating capacity of the ARMS technique (Figure 2B, lanes 10–13). Using optimized conditions, sequences of cDNA containing 5 T residues (V.1) were distinguished from cDNA and genomic clones containing 6 T residues (Figure 2C; λ.3.2, IV.8 and II.2.4). The PCR results in Figure 2C have been confirmed by DNA sequencing.

Clones obtained from site-directed mutagenesis experiments of cDNA V.1 (5T) using the MORPH™ Mutagenesis Kit (5 Prime→3 Prime, Boulder, CO, USA) and primer GFAT12-T6 (5’-CG-ACGTCGCTTTTATATCGCAGT-CCG-3’) were analyzed under the same conditions (Figure 2D). Analytical PCR was performed with both primer sets (Figure 2D). Clone VI.1 was found positive for 6 T residues, which was confirmed by DNA sequence analysis. Clones VI.2–VI.6 contained 5 T residues.

The method presented here distinguishes wild-type and mutant DNA sequences within homopolymeric nucleotide tracts by means of their different performances as PCR templates. Mutations in homopolymeric DNA tracts can be detected using the ARMS technique; however, diagnostic (analytical) primers must be designed so that the mutated nucleotide to be detected does not lie at the 3’ end of the primer, as in the standard method, but approximately 2–4 bases towards the 5’ end. However, analytical primers have to be designed very carefully in respect to the properties of the homopolymeric tract: short [3–4 nucleotides (nt)] and/or G/C homopolymeric tracts require longer extensions (3–4 nt) beyond the homopolymeric tract, while longer (>4 nt) and/or A/T homopolymeric tracts require short (2–3 nt) 3’ extensions of the primers. Having 2–4 non-repetitive bases at the 3’ end of the primer worked consistently in the experiments reported here. We have also successfully applied this method for discriminating poly(G) tracts of different lengths (data not shown). The method presented expands the possible applications of the ARMS. However, the discrimination of both the wild-type and the mutated templates is only quantitative in most cases. Therefore, PCRs using both primer sets must be performed in parallel. High template concentrations may enable some amplification from the “wrong” primer pair.

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Empirical Midpoint Dissociation Temperature (Tₐ) Determination for Oligonucleotide Probes Using a PCR Thermal Cycler

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Investigators in applied microbiology and molecular microbial ecology use oligonucleotide probes to quantify nucleic acids from specific microbial