8. **Kijas, J.M.H., J.C.S. Fowler, C.A. Garbett and M.R. Thomas.** 1994. Enrichment of microsatellites from the citrus genome using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles. BioTechniques *16*:656-662.
9. **Lyall, J.E.W., G.M. Brown, R.A. Furlong, M.A. Ferguson-Smith and N.A. Affara.** 1993. A method for creating chromosome-specific plasmid libraries enriched in clones containing (CA)n microsatellite repeat sequences directly from flow-sorted chromosomes. Nucleic Acids Res. *21*:4641-4642.
10. **Ma, Z.Q., M. Roder and M.E. Sorrells.** 1996. Frequencies and sequence characteristics of di-nucleotide, tri-nucleotide and tetra-nucleotide microsatellites in wheat. Genome *39*:123-130.
11. **Macas, J., G. Gualberti, M. Nouzová, P. Samec, S. Lucretti and J. Doležel.** 1996. Construction of chromosome-specific DNA libraries covering the whole genome of field bean (*Vicia faba* L.). Chromosome Res. *4*:531-539.
12. **Panaud, O., X. Chen and S.R. McCouch.** 1996. Development of microsatellite markers and characterisation of simple sequence length polymorphism (SSLP) in rice (*Oryza sativa* L). Mol. Gen. Genet. *252*:597-607.
13. **Powell, W., G.C. Machray and J. Provan.** 1996. Polymorphism revealed by simple sequence repeats. Trends Plant Sci. *7*:215-222.
14. **Rohrer, G.A., L.J. Alexander, J.W. Keele, T.P. Smith and C.W. Beattie.** 1994. A microsatellite linkage map of the porcine genome. Genetics *136*:231-245.
15. **Wang, Z., J.L. Weber, G. Zhong and S.D. Tanksley.** 1994. Survey of plant short tandem DNA repeats. Theor. Appl. Genet. *88*:1-6.
16. **Weissenbach, J.A.** 1993. A second generation linkage map of the human genome based on highly informative microsatellite loci. Gene *135*:275-278.

*This work was supported by research Grant No. 521/96/K117 from the Grant Agency of the Czech Republic. We thank Prof. D.W. Galbraith (University of Arizona, Tucson) for critical reading of the manuscript. Address correspondence to Andrea Koblížková, Institute of Plant Molecular Biology, Branišovská 31, České, Budějovice, CZ-37005, Czech Republic. Internet: andrea@genom.umbr.cas.cz*

Received 29 January 1998; accepted 30 March 1998.

**Andrea Koblížková, Jaroslav Doležel[1] and Jiří Macas**
*Institute of Plant Molecular Biology*
*České, Budějovice*
*[1]De Montfort University*
*Norman Borlaug Centre for Plant Science*
*Olomouc, Czech Republic*

## Influence of Magnesium Ion Concentration and PCR Amplification Conditions on Cross-Species PCR

*BioTechniques 25:38-42 (July 1998)*

The identification of conserved polymerase chain reaction (PCR) primers from a "map-rich" species to amplify homologous loci in a related species can save considerable time and effort over cloning species-specific DNA markers (9). However, successful application of cross-species PCR requires resolving the effects of sequence divergence of borrowed primers. Mismatches at the 3′ ends of primers reduce both annealing specificity (8) and PCR product yield (5). The effects of annealing temperature (7), number of cycles (2) or magnesium concentration (3) on PCR specificity or yield have not been studied in cross-species applications. We studied the influence of magnesium concentration and duration of annealing and extension steps on locus-specific amplification and product yield using human short tandem repeat (STR) primers to amplify nonhuman primate DNA.

PCR primers for human STR loci were obtained commercially (Research Genetics, Huntsville, AL, USA). Standard PCRs were prepared (4), except $MgCl_2$ concentration varied between 1.0 and 2.5 mM (Table 1). We compared 5 different PCR programs, each preceded by a 5-min denaturation at 94°C and followed by a 10-min extension at 72°C. The annealing temperature was 55°C for each program (Table 1). Five microliters of PCR product were electrophoresed through 8% nondenaturing polyacrylamide gels (Mini-PROTEAN® II; Bio-Rad, Hercules, CA, USA) for 2 h at 70 V. Alleles were visualized with an extremely sensitive silver-staining technique (1). Gels were evaluated qualitatively for yield (judged by band intensity) and locus specificity (absence of nonspecific PCR product).

Amplification results using rhesus monkey (*Macaca mulatta*) DNA varied among the 5 PCR programs and depended on magnesium concentration. Programs with short extension times (1/1/1 and 1/1.25/1.25) (see Table 1 for explanation of notation) gave inconsistent results. Either they failed to amplify at any magnesium concentration (locus IGF2R), or they only amplified in a narrow range of magnesium concentration with no optimum (IID) (Table 1; Figure 1, A–D). Program 1/1.5/1.5, with intermediate extension time, was very sensitive to magnesium concentration and typically lacked an optimal concentration (Figure 1). Program 1/1.5/1.5 did not amplify either locus at low (1.0–1.5 mM) magnesium concentration but did amplify at higher levels (1.5–2.5 mM) (Table 1; Figure 1). Programs with long extension times (1/1/1+2 and 1/2/2.5) were less affected by magnesium concentration, always amplified both loci (though sometimes nonspecifically) and typically had an optimum magnesium level. Low magnesium concentration (1.0–1.5 mM) resulted in nonspecific amplification, while higher concentrations (2.0–2.5 mM) produced strong, locus-specific amplification (Table 1; Figure 1), presumably because primers cannot bind to template DNA in the absence of sufficient magnesium (3). For example, program 1/1/1+2 consistently amplified locus IID, regardless of $MgCl_2$ concentration, with an optimum concentration at 2.0–2.5 mM (Table 1; Figure 1, A–D). Program 1/2/2.5 always amplified locus IID, sometimes nonspecifically, but had no optimum magnesium concentration (Table 1; Figure 1, A and D). In general, shorter annealing and extension times (1/1/1) resulted in more amplification failures, while longer annealing and extensions times (1/2/2.5) resulted in more nonspecific amplification.

We further compared the best PCR program (1/1/1+2) and the two commonly used human programs (1/1/1 and 1/2/2.5) to a touchdown PCR program (6) at another 2 STR loci (ACPP, MYC) in chimpanzees (*Pan troglodytes*), rhesus (*M. mulatta*) and cebus monkeys (*Cebus apella*). Touchdown PCR is intended to reduce the frequency of nonspecific amplification by decreasing an initially high annealing temperature during each of the first 10 cycles, until the touchdown annealing temperature is reached (6). Results again revealed that amplification condi-

**Table 1. Results of 5 PCR Programs and 4 $MgCl_2$ Levels at 2 STR Loci in Rhesus Monkeys**

| Locus | PCR Conditions[a] | $MgCl_2$ Concentration 1.0 mM | 1.5 mM | 2.0 mM | 2.5 mM |
|---|---|---|---|---|---|
| IID | 1/1/1 | - | + | + | - |
| | 1/1.25/1.25 | - | + | + | + |
| | 1/1.5/1.5 | - | ++ | + | + |
| | 1/1/1+2[b] | ns | ns | ++ | ++ |
| | 1/2/2.5 | ns | ns | + | + |
| IGF2R | 1/1/1 | - | - | - | - |
| | 1/1.25/1.25 | - | - | - | - |
| | 1/1.5/1.5 | - | - | + | + |
| | 1/1/1+2[b] | + | + | ++ | + |
| | 1/2/2.5 | ++ | ++ | + | ns |

-: no amplification
ns: non-locus-specific amplification
+: locus-specific amplification
++: optimal locus-specific amplification
[a]Denaturation (in minutes) at 94°C, annealing (in minutes) at 55°C, extension (in minutes) at 72°C.
[b]Denotes a 1-min extension, plus a 2-s auto-extension (2 s added each cycle).

tions affected locus specificity and varied across magnesium concentration levels. Compared to program 1/1/1+2, program 1/1/1 (with short annealing and extension steps) more often completely failed to amplify the locus, while program 1/2/2.5 (with long annealing and extension steps) produced more non-locus-specific amplification and more complete amplification failure. Although we did not systematically study the influence of different annealing temperatures, it was edifying to note that touchdown PCR (6) produced locus-specific amplification in only 8% of all locus/magnesium combinations tested. Considering results for all 4 loci together, program 1/1/1+2 produced locus-specific amplification more often (42% of all locus/magnesium combinations tested) than did 1/1/1 (32%) and 1/2/2.5 (25%), a telling result for 2 programs commonly used to amplify STR loci in humans (10). Complete amplification failure is another problem in cross-species PCR. Overall, program 1/1/1+2 failed to amplify in only 6% of all locus/magnesium combinations tested, compared to 32% failure for 1/1/1, 31% for 1/2/2.5 and 38% for touchdown PCR (Table 1; unpublished data). These differences are due to either annealing or extension times. Excess annealing time in program 1/2/2.5 allows more opportunities for false priming, resulting in nonspecific amplification (3,11). The observation that program 1/1/1 often failed to amplify at a particular magnesium concentration, while program 1/1/1+2 amplified the same locus (e.g., IID and IGF2R in rhesus, ACPP in chimpanzees), can be attributed to the effects of longer extension time. Reducing extension time to 20 s

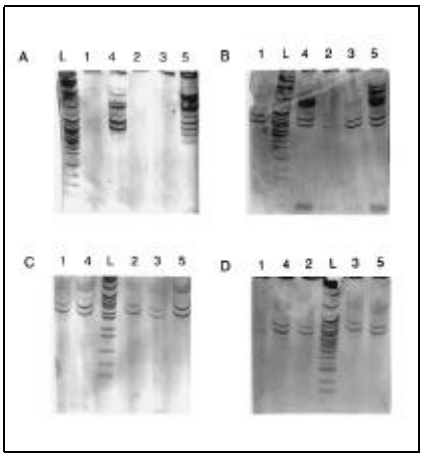


**Figure 1. Results of PCR amplification of locus IID in rhesus monkeys.** Lane 1: program 1/1/1; lane 2: program 1/1.25/1.25; lane 3: program 1/1.5/1.5; lane 4: 1/1/1+2; lane 5: program 1/2/2.5 (denaturation/annealing /extension time in minutes). Lane L represents the molecular weight standard run on each gel (pBR322/MspI). The two locus-specific bands are adjacent to the 180- and 201-bp fragments. (A) 1.0 mM $MgCl_2$, (B) 1.5 mM $MgCl_2$, (C) 2.0 mM $MgCl_2$, (D) 2.5 mM $MgCl_2$.

did not affect product yield in human optimization studies (11), but longer extension times are recommended when primer sequences diverge from template DNA sequence (3), as routinely expected in cross-species PCR.

Because short annealing times (≤1 min) and long extension times (>1.5 min) rather consistently produced locus-specific amplification regardless of magnesium level, we designed a program for cross-species PCR, consisting of an initial denaturation for 4 min at 92.5°C followed by 4 cycles of (1/0.75/1.5), then 26 cycles of (0.75/0.75/1.5+2), with annealing set at 55°C. This design was intended to minimize nonspecific priming with a short annealing step, allow ample time for extension and still permit rapid throughput (30 cycles in 2 h 40 min). We used 46 STRs on human chromosome 3 to compare our new program to program 1/1/1. Without optimizing any other factor, our customized program produced locus-specific amplification in 48% of these STRs in rhesus monkeys, compared to only 24% for program 1/1/1. Identifying the important role of the PCR conditions in producing robust, locus-specific amplification allowed us to optimize a program for cross-species PCR. This program has increased our likelihood of identifying STR loci in nonhuman primates, which do not amplify by standard human PCR programs. This is an important consideration, because approximately 1 in 4 human STRs is polymorphic in rhesus monkeys (Reference 4 and unpublished data), while even fewer (ca. 1 in 9) are polymorphic in the more distantly related squirrel monkey, *Samiri boliviensis* (12). Future optimization studies might begin with the results reported here to study the influence of other factors, especially annealing temperature, that are likely to affect amplification success in cross-species PCR.

**REFERENCES**


1. **Bassam, B.J., G. Caetano-Anolles and P.M. Gresshoff.** 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. *196*:80-83.
2. **Bell, D.A. and D.M. DeMarini.** 1991. Excessive cycling converts PCR products to random-length higher molecular weight fragments. Nucleic Acids Res. *19*:5079.
3. **Eeles, R.A. and A.C. Stamps.** 1993. Polymerase Chain Reaction (PCR): The Technique and Its Applications. R.G. Landes Co., Austin.
4. **Ely, J.J., M.L. Campbell, D.L. Gonzalez and W.H. Stone.** 1996. Successful application of PCR-amplified DNA markers for paternity determination in rhesus monkeys (*Macaca mulatta*) and chimpanzees (*Pan troglodytes*). Laboratory Primate Newsletter *35*:1-4.
5. **Kwok, S., D.E. Kellogg, N. McKinney, D. Spasic, L. Goda, C. Levenson and J.J. Sninsky.** 1990. Effects of primer-template mismatches on the polymerase chain reaction: human immunodeficiency virus type 1 model studies. Nucleic Acids Res. *18*:999-1005.
6. **Mellersh, C. and J. Sampson.** 1993. Simplifying detection of microsatellite length polymorphisms. BioTechniques *15*:582-584.
7. **Rychlik, W., W.J. Spencer and R.E. Rhoads.** 1990. Optimization of the annealing temperature for DNA amplification *in vitro*. Nucleic Acids Res. *18*:6409.
8. **Sommer, R. and D. Tautz.** 1989. Minimal homology requirements for PCR primers. Nucleic Acids Res. *17*:6749.
9. **Sun, H.S. and B.W. Kirkpatrick.** 1996. Exploiting dinucleotide microsatellites conserved among mammalian species. Mamm. Genome *7*:128-132.
10. **Weber, J. and P.E. May.** 1989. Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am. J. Hum. Genet. *44*:388-396.
11. **Wittwer, C.T., G.B. Reed and K.M. Ririe.** 1994. Rapid cycle DNA amplification, p. 174-181. *In* K.B. Mullis, F. Ferre and R.A. Gibbs (Eds.), The Polymerase Chain Reaction. Birkhauser, Boston.
12. **Zhong, F., A.G. Brady, J. Shi, C.R. Abee, C.C. McCombs and J.P. Michalski.** 1996. Strategy using pooled DNA to identify 56 short tandem repeat polymorphisms for the Bolivian squirrel monkey. BioTechniques *21*:580-586.


*This work was supported by National Institutes of Health Grants Nos. RR05080, RR04301 and RR08083. We thank Jose Tandoc for technical assistance. Address correspondence to Dr. John J. Ely, Department of Biology, Trinity University, 715 Stadium Drive, San Antonio, TX 78212-7200, USA. Internet: jely@trinity. edu*

Received 29 August 1997; accepted 13 April 1998.

**J.J. Ely, A. Reeves-Daniel, M.L. Campbell, S. Kohler and W.H. Stone**
*Trinity University*
*San Antonio, TX, USA*