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Pre-PCR Gel-Loading Buffer that Increases Specificity

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The improvement of PCR efficiency and specificity is vital when the DNA amount is limiting, as often is the case in forensic and ancient DNA applications (3). In routine diagnostic and forensic applications, there is a danger of contaminating the laboratory during post-

PCR handling of products, for example, when mixing the reaction with viscous gel-loading buffer before gel electrophoresis. To eliminate contamination-prone post-PCR handling procedures for electrophoresis, we routinely added the non-ionic polysaccharide Yellow Sub™ gel-loading buffer (Geneo Bioproducts, Hamburg, Germany) to the PCR mastermix before thermocycling (2). Unexpectedly, we observed that Yellow Sub also strongly decreases non-specific products while retaining the yield of the desired product. To systematize this observation, we varied four PCR parameters in duplicate experiments (with and without Yellow Sub): a 119-bp segment of the mitochondrial DNA control region was amplified in a gradient thermocycler using a series of annealing temperature ranges with six different polymerases, six different PCR buffer pH values and seven different Mg²⁺ concentrations. Furthermore, the annealing temperature experiments were reproduced on two other templates to exclude template-specific artifacts.

The presented hypothesis is that the inclusion of Yellow Sub in the reaction does not change the optimal parameters of the reaction. To test this hypothesis, we performed several reactions, each time dividing the mastermix into two groups: one that received 10% Yellow Sub before the reaction and one that received Yellow Sub after the reaction. The reactions were performed using 1 pg human DNA, 10× PCR buffer, pH 8.75, 10 mM dNTPs (both from Geneo Bioproducts), generally 1 U polymerase (various suppliers, see Table 1), 2 µL Yellow Sub, 1 µM each primer (MWG Biotech, Milton Keynes, UK) and ultrapure water in a total volume of 20 µL. The precision of the thermocycler temperature control is ± 0.2°C. Our standard primer pair, MVF3 and MVF4, and two other primer pairs amplified 96–119-bp segments of the human mitochondrial DNA control region (1): MVF3, nucleotide position (np)16133–np16151; MVF4, np16251–np16234; MVF5, np16228–np16248; MVF6, np16355–np16336; MVF7, np16321–np16341; and MVF8, np16431–np16412. Thirty-three cycles were performed with an initial denaturation step (0–10 min as recommended by suppliers), a denaturation step of 92°C for 30 s, a range of anneal-

ing temperatures for 20 s and an extension step of 72°C for 20 s. The final extension step was 10 s. The PCR products were separated electrophoretically on 4% agarose gels and visualized at 312 nm using ethidium bromide staining. The DNA amount was estimated using defined amounts of the pUC/HpaII length marker (Geneo Bioproducts) or SmartLadder SF (Eurogentec, Seraing, Belgium). Annealing temperatures were tested in the range 49.1°C–67.3°C or 43.2°C–61.4°C with increments of 1°C–3°C using a Mastercycler® gradient thermocycler (Eppendorf Netheler Hinz GmbH, Hamburg, Germany).

Different polymerases were tested using primers MVF3 and MVF4 with DynAzyme™ EXT (Flowgen, Staffordshire, UK), AmpliTaq Gold™ (PE Biosystems, Foster City, CA, USA), Eurogentec Goldstar™ DNA Polymerase (Eurogentec), BioTaq™ DNA Polymerase (BioLine, London, UK) and an unpublished polymerase with buffers, magnesium solutions and cycling conditions as recommended by the suppliers at the gradient annealing temperature shown in Figures 2 and 3. One unit of each enzyme was used, except for Eurogentec Goldstar for which 0.2 U are recommended by the manufacturer.

Different pH values for the PCR buffer were tested with primers MVF3 and MVF4 at increments of 0.25 as shown in Figure 3 at the optimized annealing temperature of 56°C and with an optimized Mg²⁺ concentration of 1.75 mM. Different Mg²⁺ concentrations were tested with primers MVF3 and MVF4 at a range of 1.0–3.5 mM as shown in Figure 4 using the optimized annealing temperature of 56°C and the recommended pH of 8.75.

To find the annealing temperature optimum for primers MVF3 and MVF4, we amplified in the range 49.1°C–67.3°C in duplicate (with and without Yellow Sub). The temperature optimum was found to be 56°C. As is seen in Figure 1, we can exclude that Yellow Sub changes the optimal annealing temperature by more than ± 1°C (the difference between temperature steps around 56°C in this and other experiments). The addition of Yellow Sub before the reaction does, however, improve specificity while maintaining the

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Table 1. Comparison of Amplification with Different Polymerases with and without Yellow Sub

Enzyme ^a & Buffer	Amplification with Yellow Sub				Amplification without Yellow Sub			
	Yield	Primer Dimers	Smear	Dropouts	Yield	Primer Dimers	Smear	Dropouts
unpublished polymerase	high	yes	no	no	high	more	yes	yes
BioTaq Polymerase	high	yes	no	no	high	more	yes	yes
BioTaq Diamond	high	no	no	yes	high	more	no	yes
Eurogentec Goldstar	medium	no	no	no	medium	more	no	no
DyNAzymeEXT	low	yes	no	no	lower	more	no	yes
AmpliTaq Gold	low	yes	no	yes	low	more	no	yes

^a1 U enzyme, except for Eurogentec Goldstar: 0.2 U as recommended by supplier

yield of the reaction. Figure 1 shows an example of a reaction based on the same mastermix, except that Yellow Sub had been added to half of the mastermix before the reaction, while Yellow Sub was added to the other half after thermocycling. The nonspecific products that resulted from the lowest temperatures dis-

appear when the reaction is performed with Yellow Sub (Figure 1). This result was obtained to a greater or lesser degree with the six commercially available polymerases tested here. Figure 2 shows an example using DyNAzyme EXT in duplicate (with and without Yellow Sub) with primers

MVF3 and MVF4. As with the other polymerases, Yellow Sub improves specificity while maintaining the yield of the reaction. All enzymes showed higher specificity when amplified with Yellow Sub (Figure 2 and Table 1), and amplification dropouts were more frequent without Yellow Sub. Only Euro-

Figure 1. Amplified products of primers MVF3 and MVF4 with Yellow Sub added before (top) and after (bottom) the reaction was performed in a Mastercycler gradient thermocycler. The Yellow Sub is seen as a fast-migrating dark stain beyond 34 bp in UV light. The left lanes contain 750 ng pUC/*Hpa*II length marker. Note that the two right lanes contain no products and no primer dimers (normally located at 34 bp). Also note the smear of non-specific product at the low temperature range without Yellow Sub addition.

Figure 2. Products amplified with polymerase DyNAzyme EXT with Yellow Sub added before (bottom) and after (top) the reaction was performed in a Mastercycler gradient thermocycler. The left lanes contain 750 ng pUC/*Hpa*II length marker. Note that the two right lanes at the top contain no products but do contain primer dimers, while the two right lanes at the bottom contain products with weak primer dimers. All primer dimers seen at the bottom are weaker than those seen at the top. Yield of product with Yellow Sub is higher than it is without Yellow Sub.

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Figure 3. Products amplified at various PCR buffer pH values with Yellow Sub added before (top) and after (bottom) the reaction. The left lanes contain SmartLadder SF length marker. The pH lanes were run in duplicate.

Figure 4. Products amplified at various Mg^{2+} concentrations with Yellow Sub added before (bottom) and after (top) the reaction. The left lanes contain pUC/HpaII length marker. The Mg^{2+} concentrations were run in duplicate. Note that amplification without Yellow Sub failed at 1.75 mM, the optimal concentration with Yellow Sub. Also note that successful amplification without Yellow Sub was attained at 2.0 mM.

gentec Goldstar never failed to amplify a product in the absence of Yellow Sub (Table 1). An increase in the yield of product with Yellow Sub was seen for DyNAzyme EXT (Table 1). No increase was seen for AmpliTaq Gold, which showed the lowest yield for all enzymes, nor for the unpublished polymerase, BioTaq Polymerase, or BioTaq Diamond, the latter showing the highest yields for all enzymes (Table 1).

Additionally, primers MVF3 and MVF4 were used to test whether Yellow Sub influences the pH value and Mg^{2+} concentration. The pH values tested, in duplicate and with and without Yellow Sub, were 8.0, 8.25, 8.5, 8.75, 9.0, 9.25 and 9.5. The addition of Yellow Sub does not affect the optimal pH value as shown in Figure 3. However, the optimal Mg^{2+} concentration may appear to increase up to 0.25 mM (Figure 4), but this difference may lie within the pipetting error. The results were reproduced using two additional primer pairs (described above) for the mitochondrial control region. In summary, these experiments derived from the same mastermixes show that Yellow Sub consistently reduces nonspecific PCR products while retaining equivalent yields, without significantly changing the optimal annealing temperature, pH value or Mg^{2+} concentration.

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