Standard multiplex mixtures

Over 75 primer pairs were chosen and a number of multiplex mixtures were designed and used for different purposes. Examples of all multiplex mixes are presented below. *(All unlabeled gel lanes are the marker: 1 kb DNA ladder (GIBCO). At the bottom of each image, the PCR buffer concentration used is also indicated)*

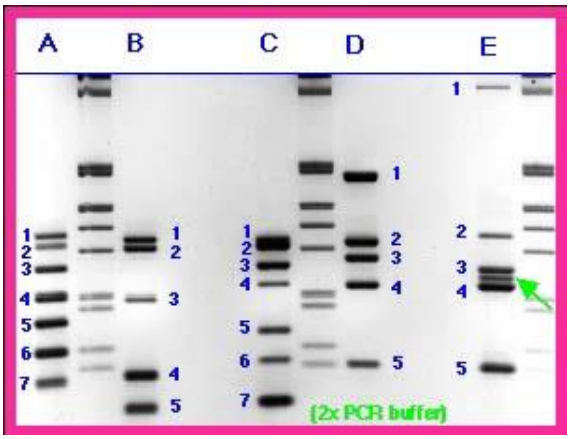

Fig. 1. Mixtures A-E on an a 2.5% agarose gel. Arrow indicates the presence of an unspecific product. Although not desirable, this product did not interfere with the use of mix E in a microdeletion screening project. Occasionally, mix C was used without the primers for loci 4 and 5. In such cases, this mix is called mix C*.

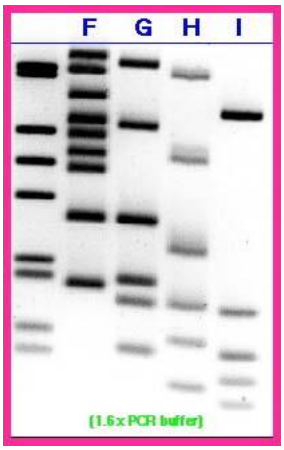

Fig. 2. Mixtures F-I on a 2.5% agarose gel.

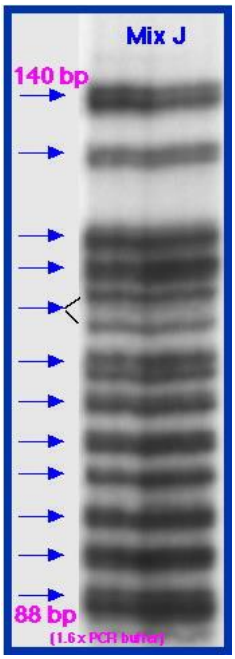

Fig. 3. Multiplex PCR with mix J on four different genomic DNA templates, separated on an a denaturing, 6% polyacrylamide gel. Primers amplify nonpolymorphic loci. The sizes of the longest and the shortest product are also indicated.

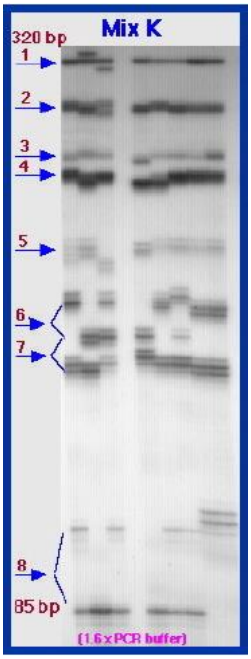

Fig. 4. Multiplex PCR with mix K on eight different genomic DNA templates, separated on an a denaturing, 6% polyacrylamide gel. These primers amplify polymorphic loci, and alleles of different sizes can be observed. The shortest alleles of locus 6 and the longest alleles of locus 7 can, sometimes, overlap, making it difficult to assign precisely their origin. The sizes of the longest and the shortest product are also indicated.

Choosing/designing PCR primers

In designing primers for PCR, the following steps/rules were tested and proven to be useful:

- **length** of individual primers between 18-24 bases. Longer primers (30-35 bp) seem to work in more similar cycling conditions compared with shorter primers, and can make multiplexing easier (see picuters below).
- it is desirable (but not absolutely necessary) that the two primers have a close melting temperature or Tm (say, within 5º C or so). If Tm difference between the two primers is high, the lower Tm can be increased by increasing the length of that primer at the 3' end (this can also keep the size of the amplified locus constant) or the 5' end.
- **purine:pyrimidine content** around 1:1 (maybe 40-60%)
- primer sequence should **start and end** with 1-2 purine bases
- each primer pair should be tested for **primer-primer interactions**. For this purpose a useful Macintosh program is "CPrimer", a freeware available at ftp.bio.indiana.edu. This program also provides the **melting** temperature for the sequences entered, thus helping in designing PCR programs. Very convenient, some web sites offer programs that can be used directly on those sites to do the same functions: (search for optimal primers, melting temperatures).
- primer sequences should be aligned with all DNA sequences entered in the databases (using **BLAST** programs) and checked for similarities with repetitive sequences or with other loci, elwhere in the genome. If two loci are very similar (for example across species) it is useful to design the primers so that at least 1-2 bases at the 3' end are specific for the locus to be amplified
- **cycling** conditions and **buffer** concentrations should be adjusted for each primer pair, so that amplification of the desired locus is specific, with no secondary products (see other pages). If this is not possible, the sequences of the primers should be either elongated with 4-5 bases or simply, changed entirely.

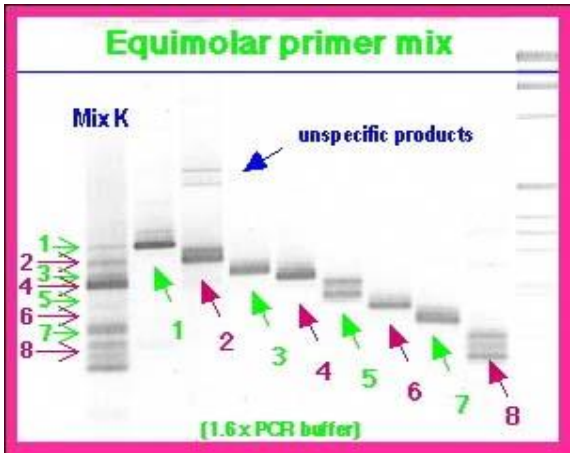

Fig. 7. Multiplex PCR using primers 18-24 bp long. When PCR reaction Eight individual loci are amplified with similar intensities when the primer pairs are used separately. When equimolar amount of these primers are mixed together for a multiplex reaction (Mix K), some of the products are much weaker (#1, #2, #5, #6) than other. In this case, primers had "usual" length, between 18-24bp. (primers used in this case amplify polymorphic loci, explaining the "double" or "triple" bands as seen on a regular agarose gel)

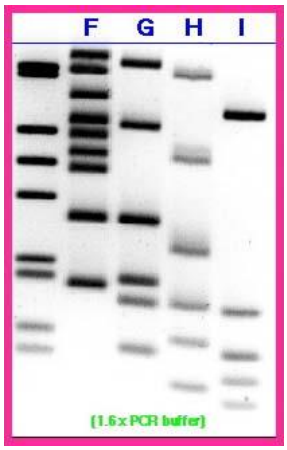

Fig. 8. Multiplex PCR using primers 30-35 bp long. Compared to the figure left, in this case the primers used for multiplexing were longer than 30 bp (up to 37 bp). Equimolar amounts of primer were used and all loci were amplified with comparable intensities in each reaction.

Reaction volume

Q: Does the PCR reaction volume (negatively) influence the outcome?

A: No, especially since the introduction of the small, thin walled, 0.2 µl plastic vials fitting the 96 well metal blocks of the thermocyclers.

A number of observations are worth mentioning:

- if using **older model thermocyclers** (without a heated lid), to run small volume PCR reactions, mineral oil is necessary to cover the reaction mixture. Use of oil increases the volume of liquid in the vial and thus can influence somewhat the outcome. Besides, older thermocyclers require the use of larger plastic vials, with thicker walls, that fit less well in the metal blocks and thus may increase the likelihood of variation in PCr outcome.
- **small, thin-walled plastic vials** designed for the 96 well metal blocks are ideal for running small-volume PCR reactions. Due to the heated lid of the thermocyclers, there are no mineral oil requirements. When tested, reactions yielded similar results, whether the reaction volume was 100, 25 or 5 microliters.
- It is important to mention, that small volume PCRs may be very beneficial when using **small amounts** of DNA template. In general, at a constant amount of template DNA, the yield of PCR product per microliter reaction is higher when the reaction volume is 5 µl compared to 100 µl. This may allow visualisation of the PCR products, sometimes invisible when larger reaction volumes are used.

Multiplexing primer pairs

Single locus PCR. First step in designing a multiplex PCR is choosing the primer pairs which can be combined. One important requirement is to find a PCR program allowing optimal amplification of all loci when taken individually (Fig. 9). This is achieved by adjusting the annealing and extension time and temperature.

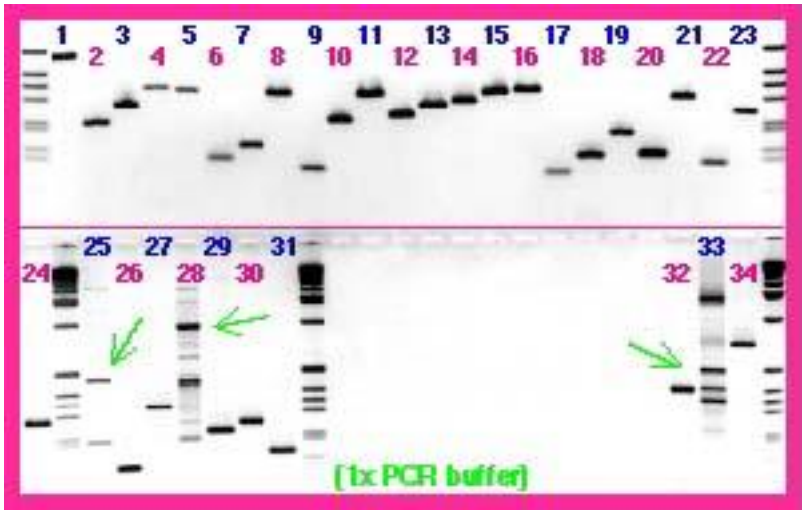

Fig. 9. Single-locus PCR with 34 different primer pairs using the same cycling conditions. Arrows indicate position of the specific products in lanes 25, 28 and 33, in which other unspecific products also appear. Such primer pairs are difficult to use both by themself and in multiplex PCR. However, even though some unspecific products still appeared, primer pair 28 was multiplexed in mixture 5 (Figure 1) and used in a microdeletion screening project. The unspecific products did not interfere with data interpretation. Examples of multiplex reactions using these primers are shown in Fig. 1.

Multiplexing equimolar primer mixtures. The next step is combining the desired primer pairs in multiplex mixture(s), using equimolar amounts of each primer. PCR amplification of the multiplex mixtures can be performed, first using exactly the same PCR program as with individual primer pairs. Very often, this will results in preferential amplification of some loci. Such a situation will require further adjustment in cycling conditions and primer concentration. Although, sometimes unspecific products can be seen in single-locus PCR (yellow arrow in PCR product # 2), these unspecific products usually become invisible when the multiplex reaction is performed. This is probably due to the concurrent ampification of many

specific loci, which overwhelms the unspecific products (although they are probably still present in small quantities).

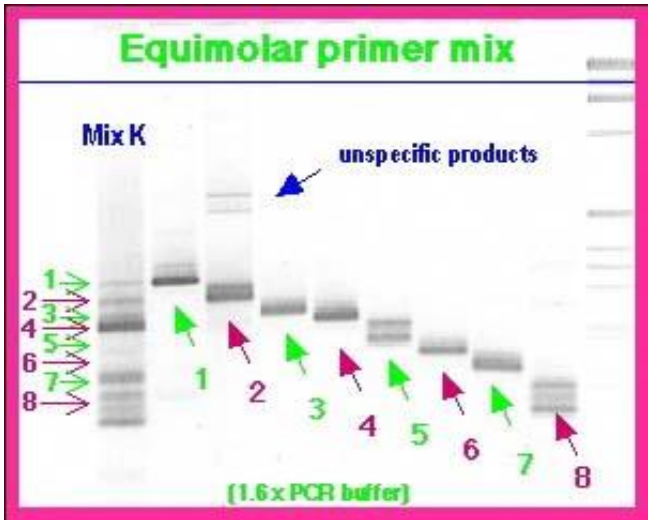

Fig. 7 (duplicate). Single locus PCR and multiplex PCR with equimolar amounts of primers from mixture K, performed in the same cycling conditions. In Some products of mixture K become weak or invisible, requiring further adjustment of primer amount(s) and of cycling conditions. Primers used in mixture K amplify polymorphic loci, explaining the appearence of multiple bands on a nondenaturing agarose gel.

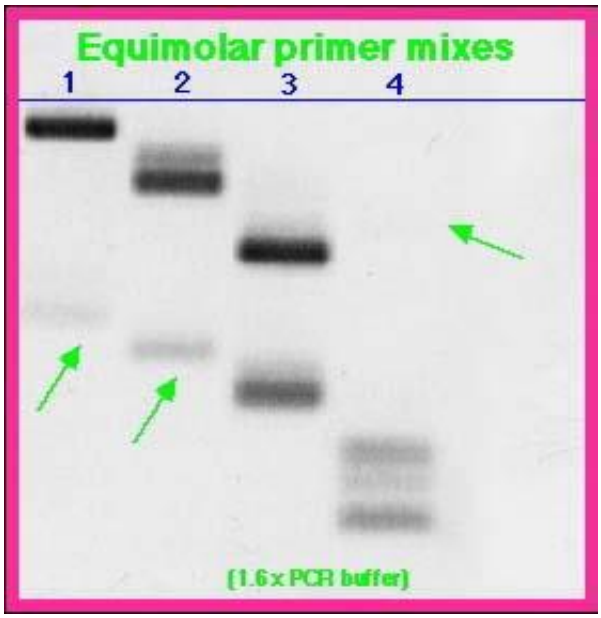

Fig. 10. Equimolar amounts of the same primers used for mixture K (see also Fig. 7 above), where amplified in pairs. In lanes 1, 2 and 4, one locus was amplified less efficiently than the other one (arrows). As mentioned before, amplification of the "weaker" loci can be improved increasing the amount of primers or adjusting the reaction conditions.

Adjustment of cycling conditions

- Annealing time and temperature
- extension time and temperature

For example, figure 11 illustrates the influence of the extension temperature. Equimolar primer mixtures A-D were amplified using two different PCR programs, one at 65º C (yellow lanes) and the other at 72º C (green lanes) extension temperature. In general, there is a higher yield of PCR products for A, B and D when program A was used. This shows that the 72º C extension temperature, negtively influenced amplification of some loci (pink arrows),while also making some unspecific products visible (yellow arrows). It is likely that, for the short PCR products used in these examples (below 500 bp), the higher annealing temperature is probably detrimental to the stability of the DNA helix, so less strands of DNA have the chance to become "copied" by the polymerase after annealing.

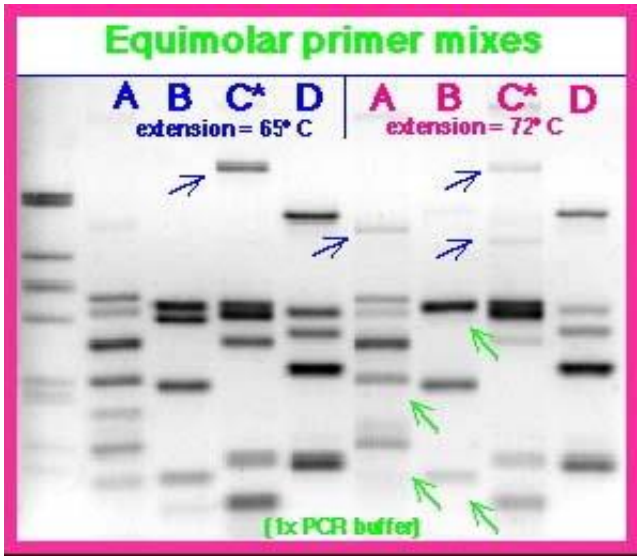

Fig. 11. Example of the influence of extension temperature. Multiplex PCR with mixtrues A-B using two different PCR programs. Reactions on the right side (green) were performed in identical cycling conditions with Fig. 9, whereas reactions on the left side (yellow) were performed using cycling conditions in which extension temperature was dropped from 72 º C to 65 º C. Reaction worked more efficiently with the lower extension temperature (pink arrow show missing products, yellow arros show unspecific products).

Primer amount and buffer concentration. To improve the amplification of some of the DNA products from Fig. 11 above, the amount of primers was increased 2-5x for those loci. At the same time, the PCR buffer concentration was increased to 2x. These modifications allowed a much more efficient and reproducible amplification, with no unspecific products.

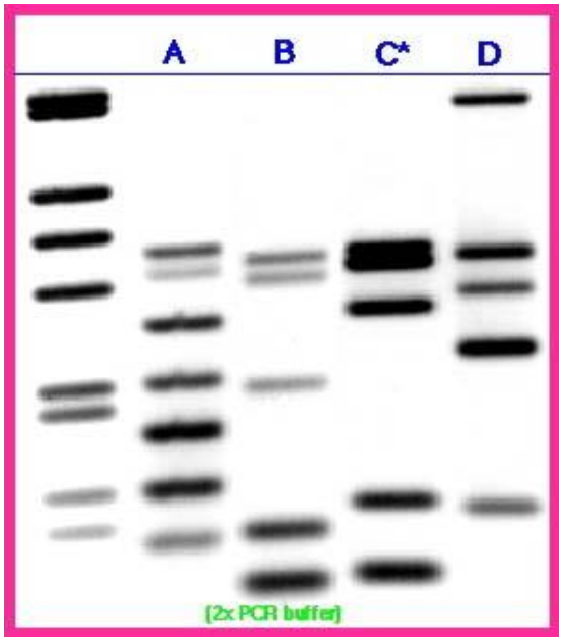

Fig. 12. Multiplex PCR with mixtures A-D, in cycling conditions similar to the ones on the left side of Fig. 11 above (annealing at 65 º C), but using 2x PCR buffer. The amount of primer pairs was increased for some of the weak products from Fig. 11. Cleaner and more efficient amplifications were obtained.

Primer amount in PCR

Absolute value of primer concentration in multiplex PCR. The amount of DNA primer(s) available during the PCR reaction influences the results. Primer concentration taken in a common PCR reaction (for example when amplifying a single locus) is about 100-500 nM each primer. *(Primers can be purchased from various sources at concentrations between 10-25 µM each. Usually, 0.5-1µl primer solution is sufficient for a 25-100 µl PCR reaction)* In a multiplex PCR test using equimolar primer mixtures (Fig. 13), individual primer concentrations were varied between 500 and 15 nM each primer. Given that mixture A used 14 primers (7 loci) and mixture B 10 primers (5 loci), the final primer concentration varied between 7000 and 200 nM (mixture A) and between 5000 and 150 nM (mixture B). Although equimolar primer mixtures did not usually provide optimal amplification of all loci, this test allowed the observation that too high and too low primer amounts may need to be avoided.Too high primer concentrations may inhibit the multiplex reaction whereas too low amounts may not be sufficient.

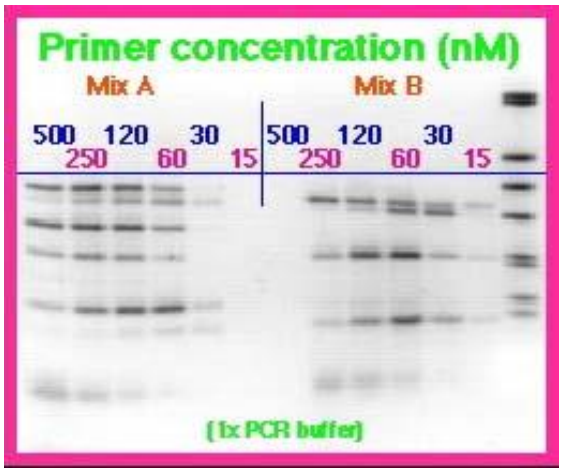

Fig. 13. Multiplex PCR with mixtures A and B (see also Fig. 1). Numerical values indicate the concentration of each primer in the final reaction. Mixture A includes 14 primers and mixture B includes 10. Reactions work best at around 200 nM (each primer) in mixture A and 60 nM (each primer) in mixture B.

Primer and template concentrations. Within limits, increasing primer concentration may improve the outcome of the PCR reaction, and should be considered as a way to optimize PCR reactions. This is exemplified in Fig. 14 below, in which increasing primer and template DNA concentrations, both improve the results.

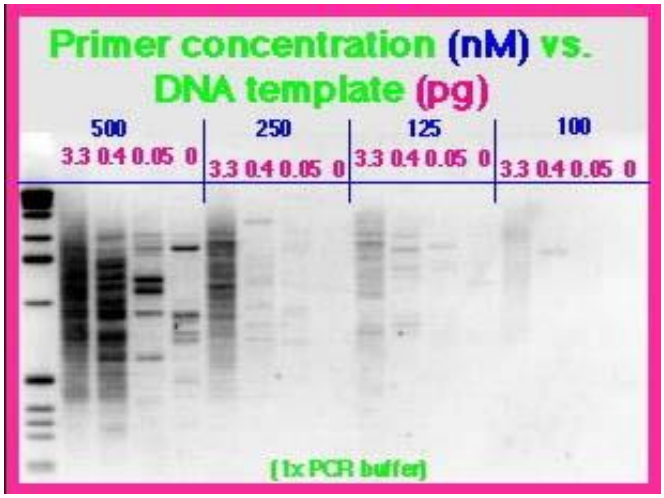

Fig. 14. PCR with various concentrations of genomic DNA template and various concentrations of a degenerate primer. At each primer concentration, reaction improves with increasing amount of template. Reaction also improves with increasing amount of primer. DNA contamination is visible in the "no-DNA" reaction at 500 nM primer concentration.
(For comparison, the amount of DNA in a diploid human cell is about 6.6 pg)

Salt (KCl) concentration

For the successful PCR or multiplex PCR amplification of many loci (especially products between 100-1000 bp) raising the buffer concentration to 1.4x-2x (or only the KCl concentration to about 70-100mM) dramatically improves the efficiency of the reaction. In fact the effect of the KCl concentration was more important than any of the adjuvants tested (DMSO, glycerol or BSA). Generally, many primer pairs producing longer amplification products worked better at lower salt concentrations, whereas many primer pairs producing short amplification products worked better at higher salt concentrations. This is illustrated in the three figures below in which there is a realtive shift in the intensity of the products from the longer one towards the shorter ones as the ionic strength increases. An increase in salt concentration makes longer DNA denature slower than shorter DNA, so shorter molecules will be amplified preferrentially. Some primers, however, worked well over a wide range of buffer/salt concentrations. Examples of multiplex reactions at different buffer concentrations are shown in Fig. 16, 17 and 18.

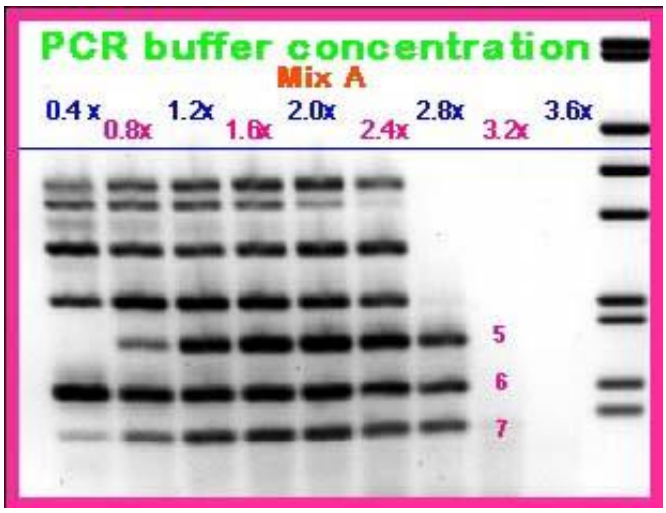

Fig. 16. Multipex PCR amplification of mixture A at increasing buffer (salt) concentrations.

In **Fig. 16**, both primers for locus 6 have a melting point around 58° C whereas primers for locus 5 have a melting point around 52° C. At the same ionic strength (1x buffer) and annealing temperature (54° C), amplification of locus 6 will be favored over 5. To increase binding of primers for locus 5 while keeping the annealing temperature the same, stringency of the PCR buffer needs to be decreased. This can be easily done by increasing the KCl (or buffer) concentration. A different example is locus 7, where both primers have a similar melting point with primers for locus 6 (58° C). They are not as well amplified in 1x buffer, but respond well to increase in the salt concentration. In this case, the explanation may be that the entire product 7 has a lower GC content than product 6. This makes the DNA helix of product 7 less stable when exposed to the extension temperature. Some of the new strands may detach from the template, before the polymerase fully amplifies them. Decreasing the stringency of the buffer (1.6x-2x) might "stick" the newly synthesized strands better to the template, allowing the polymerase to finish its task.

PCR reactions in which only KCl or Tris-HCl concentrations were varied, showed that the described effect is due to the salt (KCl). Tris-HCl concentration did not influence the outcome of the reactions over a large range of concentrations (from 0.75x to 5x) whereas $MgCl_2$ concetrations have a somewhat different effect (detailed on page 14).

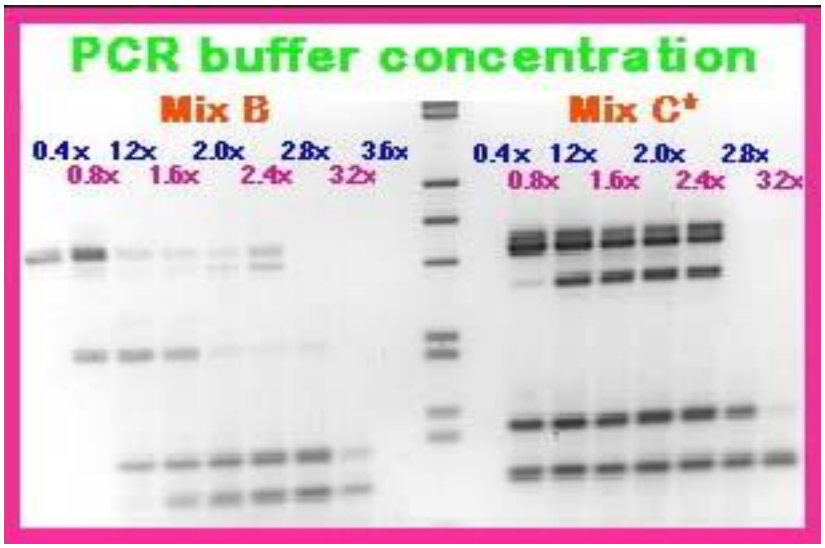

Fig. 17. Multipex PCR amplification of mixtures B and C* at increasing buffer (salt) concentrations.

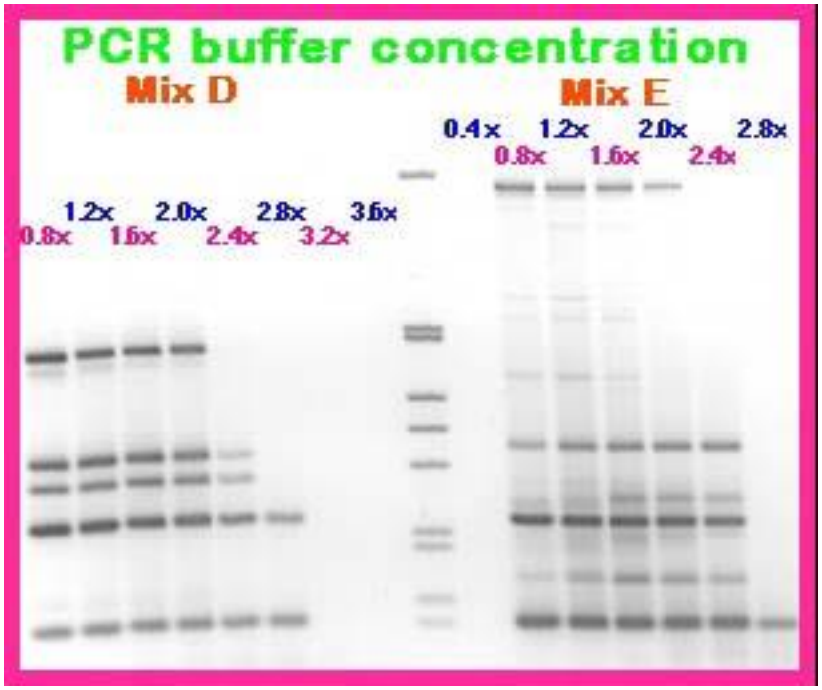

Fig. 18. Multipex PCR amplification of mixtures D and E at increasing buffer (salt) concentrations.

Annealing time and temperature

Annealing time

An annealing time of 30-45 seconds is commonly used in PCR reactions. Increase in annealing time up o 2-3 minutes did not appreciably influence the outcome of the PCR reactions. However, as the polymerase has some reduced activity between 45 and 65º C (interval in which most annealing temperature are chosen), longer annealing times may increase the likelihood of unspecific amplification products (data not shown)

Annealing temperature (see also figure 18)

Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially template DNA). For example, the PCR product depicted in Fig.

22 could be amplified easily at annealing temperatures of 55 º C in the presence of 1-100 ng genomic DNA template. Below this limit, there was no detectable PCR product on agarose gels *(this primer pair amplifies a polymorphic locus, explaining the two bands seen on non-denaturing agarose gels)*. It was observed that the specific product can be detected again, even in the presence of very low DNA template concentrations, if the annealing temperature is also decreased. In the reactions depicted in figure 22, the DNA template amount was decreased to 3.1 pg (which is about half the DNA content of a diploid human cell). Remarkably, only one allele was preferentially amplified when the template DNA was approximately 6.6 pg. To achieve these results, reaction was performed at 45 º annealing temperature (a 10 degrees drop from usual). No unspecific products are seen. However, if the same reaction is performed in the presence of a higher amount of DNA template, the low annealing temperature results in the appearance of many unspecific secondary products. Thus, it appears that by decreasing the amount of DNA template, the number of potentially unspecific sites is also decreased, making possible the drop in annealing temperature.

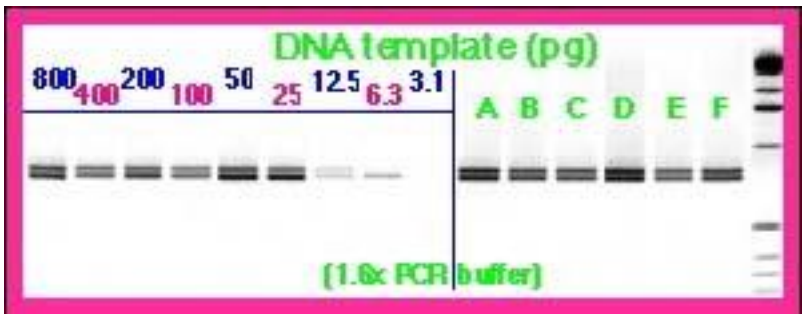

Fig. 22. PCR amplification of a polymorphic locus in the presence of decreasing, low amounts of genomic template DNA and at an annealing temperature 10 º C lower than normal.
Lanes A-F show slight variation in the amount of product, when vials with identical reaction mixture were placed in different position in the metal block of a thermocycler. Amount of template was 800pg/reaction.

Polymorphisms and annealing temperature

Annealing temperature is important in finding and documenting polymorphisms. Slight mismatches, (even 1 base-pair mutations) in one of sequences bound by the two primers used to amplify a DNA locus, can be detected by slight variations in annealing temperature and/or by multiplex PCR. In Fig. 23 such a polymorphism on human Y chromosome is detected in a few DNA samples by amplifying that locus along with other ones using multiplex mixture C (see also Fig. 1). In Fig. 24, same polymorphism is detected by performing PCR reaction only with the specific primer pair, but increasing the stringency of the annealing temperature.

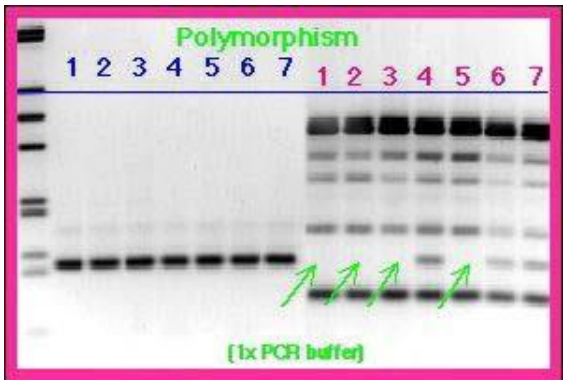

Fig. 23. Single-locus PCR on 7 different template DNAs with a primer pair amplifying a polymorphic locus (yelow). Multiplex PCR of the same templates when the primer pair is part of mixture C. Reactions were performed in the same cycling conditions (annealing at 54 º C). The slight mismatch in primer binding (polymorphism) is detected only in the multiplex reaction by the lack of the amplification product (magenta arrows).

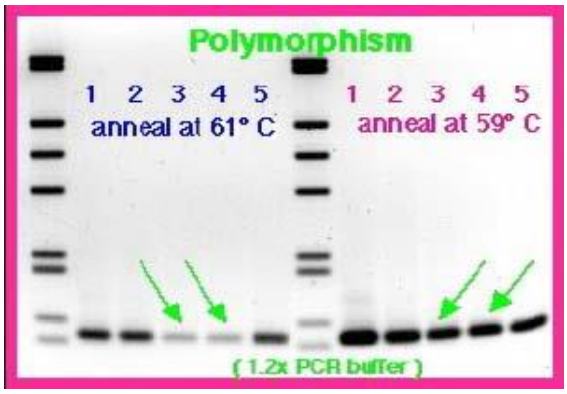

Fig. 24. Same primer mismatch described above can be detected by single-locus PCR reactions after increasing the stringency of the annealing temperature. Samples 3 and 4 show a decrease of product at 61 º C annealing temperature but have a "normal" appearance at 59 º C annealing temperature (magenta arrows).

Extension time and temperature

Extension time

In multiplex PCR, as more loci are simultaneously amplified, the pool of enzyme and nucleotides becomes a limiting factor and more time is necessary for the polymerase molecules to complete synthesis of all the products. Extension time will play an important role in adjusting the outcome of the PCR reaction. This is illustrated in the experiments depicted in two figures below. In one experiment, multiplex mixtures A-D (see also fig. 1) were amplified using PCR programs with 1 and 2 minutes extension times, respectively. Higher yields of PCR products were obtained in all four mixtures when the longer extension time was used. Optimal amplification of all loci will require further adjustments in other factors influencing the reaction (buffer concentration, amount of individual primers). A somewhat lower reproducibility of the results between Fig 11 and Fig 25 was most probably due to a combination of small pipetting differences and the fine balance between buffer, dNTP and MgCl2 concentration (see those topics). Within the same experiment, however, results were reproducible and the effect of various parameters could be studied (Fig. 25).

In the other experiment (Fig. 26) increasing the extension time in the multiplex PCR increased the amount of longer products, at the "expense" of the shorter ones.

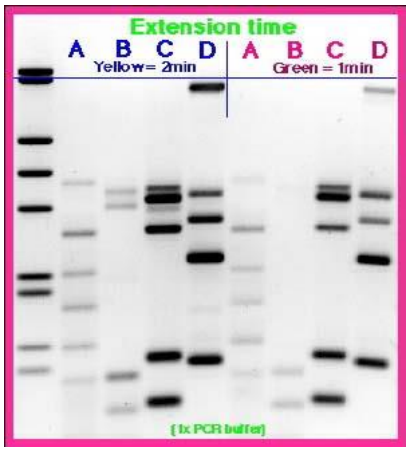

Fig. 25. Multiplex PCR of mixtures A-D comparing PCR programs with 2 (green) and 1 (yellow) minute extension time at 54° C annealing temperature. Comparison of equivalent lanes shows an improvement in yield when extension time is 2 minutes. Some faint unspecific bands appear, possibly due to the low buffer concentration (1x).

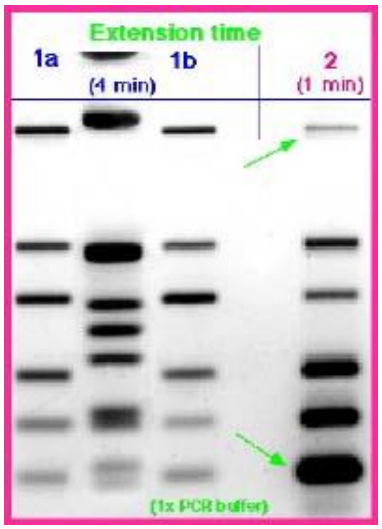

Fig. 26. Same multiplex mixture was amplified on PCR programs differing only in their extension time (1 and 4 minutes). Shorter amplification products are preferentially amplified with short extension times (1 minute) whereas the longer products become more visible as the extension time increases (arrows). At the same time, at 4 minutes, the shorter products lose much of their intensity. Reactions in lanes 1a and 1b are identical (different DNA templates only).

Extension temperature

Figure 11 illustrates the influence of the extension temperature. Equimolar primer mixtures A-D were amplified using two different PCR programs, one at 65º C (yellow lanes) and the other at 72º C (green lanes) extension temperature. In general, there is a higher yield of PCR products for A, B and D when program A was used. This shows that the 72º C extension temperature, negtively influenced amplification of some loci (pink arrows),while also making some unspecific products visible (yellow arrows). It is likely that, for the short PCR products used in these examples (below 500 bp), the higher annealing temperature is probably detrimental to the stability of the DNA helix, so less strands of DNA have the chance to become "copied" by the polymerase after annealing.

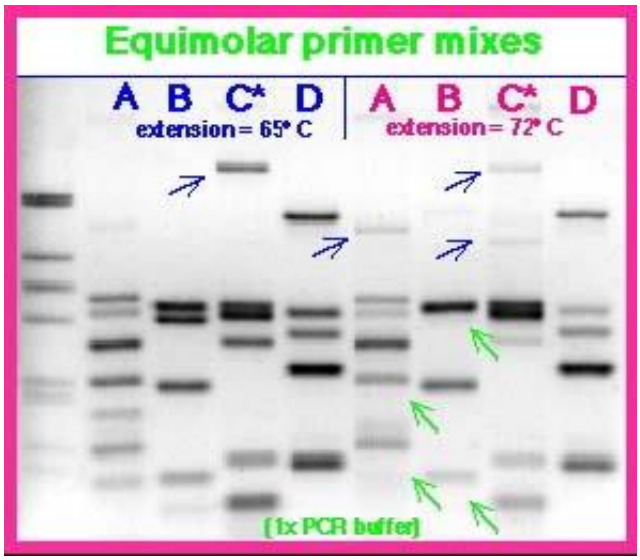

Fig. 11 (duplicate). Example of the influence of extension temperature. Multiplex PCR with mixtrues A-B using two different PCR programs. Reactions on the right side (green) were performed in identical cycling conditions with Fig. 9, whereas reactions on the left side (yellow) were performed using cycling conditions in which extension temperature was dropped from 72 º C to 65 º C. Reaction worked more efficiently with the lower extension temperature (pink arrow show missing products, yellow arros show unspecific products).

DNA template

All multiplex reactions performed in this laboratory used human genomic DNA as a template. From both multiplex and single-locus PCR reactions, results showed that the amount of DNA template strongly influences the outcome of the reaction. In conditions in which the amount of DNA available is very low, reaction or cycling conditions can be adapted and modified to allow reaction to work efficiently.

The following five images provide examples illustrating the importance of the DNA template concentration.

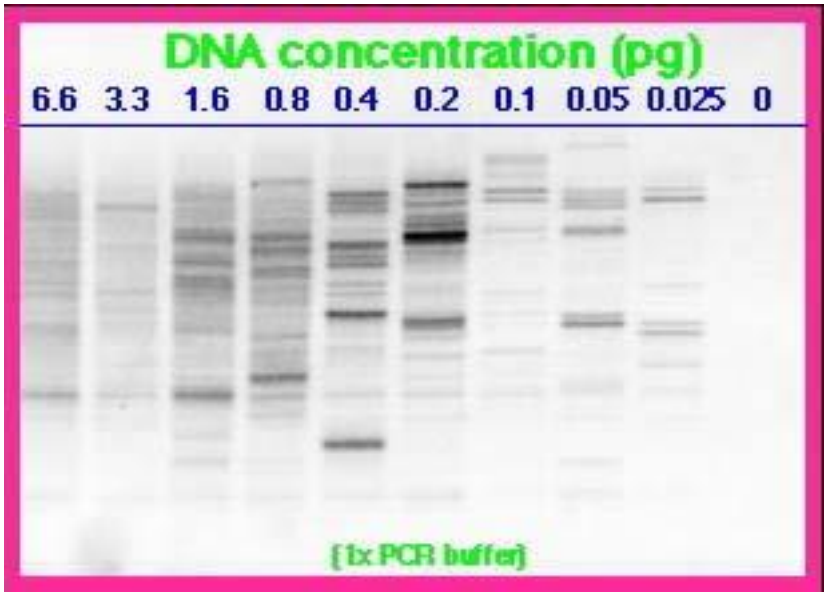

Fig. 27. PCR amplification of very low amounts of genomic DNA using a degenerate primer. Amount of PCR product decreases with the decreasing amount of template.

Fig. 28. Multiplex PCR using primer mixture A in 1x PCR buffer. As the amount of template drops, most products become gradually weaker. Cycling conditions were identical. Arrow indicates the presence of an unspecific product.

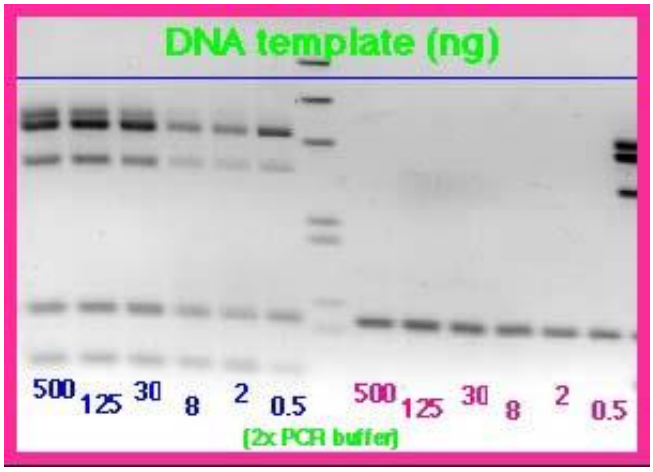

Fig. 29. Multiplex PCR with mixture C* and single-locus PCR with one of the primer pairs form the same mixture. As the DNA template decreases, some bands become weaker in the multiplex reaction. Over the same range of concentrations, this effect is not so visible when only one primer pair is used.

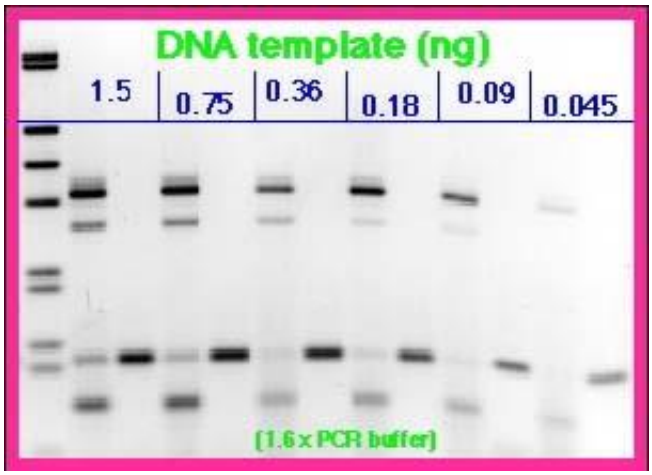

Fig. 30. Multiplex PCR with mixture C* and PCR amplification using only one of the primer pairs from the same mixture. Very low template DNA concentrations were used (0.045 is the amount of DNA from 6 diploid cells). Again, the amount of PCR product decreases with the reduction in template DNA but less so when only one primer pair is used. PCR program used has a lower annealing temperature (about 5º C lower) than the program used for the reactions in Fig. 29.

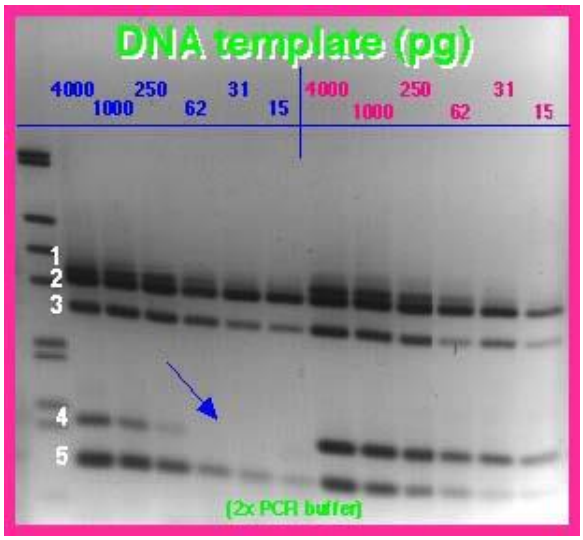

Fig. 31. Multiplex PCR with mixture C* on two genomic DNA temlpates, one (yellow) carrying a polymorphism for one primer binding site and another one (green) with perfect match. As in Fig. 30 above, to amplify such reduced amounts of DNA template, the same program with low annealing temperature had to be used. Arrow indicates that the polymorphism at locus 4 is detected with the decrease in DNA template amount.

Taq polymerase

Different concentrations of a Taq polymerase were tested using primer mixture C (Fig. 32). The most efficient enzyme concentration seemed to be around 0.4µl or 2 Units/25µl reaction volume. Too much enzyme, possibly because of the high glycerol concentration in the stock solution, resulted in an unbalanced amplification of various loci and a slight increase in the background. too little enzyme resulted in the lack of some of the amplification products.

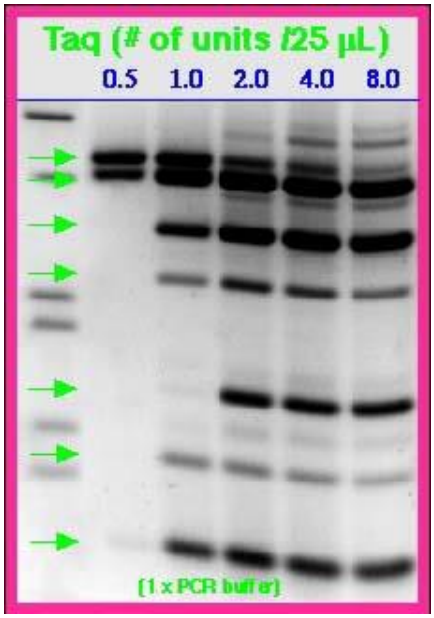

Fig. 32. Amplification products of mixture C, using 0.5 Units/25µl, 1 Unit/25µl, 2Units/25µl, 4 Units/25µl and 8 Units/25µl reaction volume are shown. Arrows indicate the expected positions of the amplification products. The most appropriate enzyme concentration was between 1-2 Units/25µl.

Five native Taq polymerases, from five different sources, were used to amplify multiplex mixture D in 1.6x PCR buffer using 2Units enzyme/25µl reaction (Fig. 33). In the same buffering conditions, all these enzyme performed similarly.

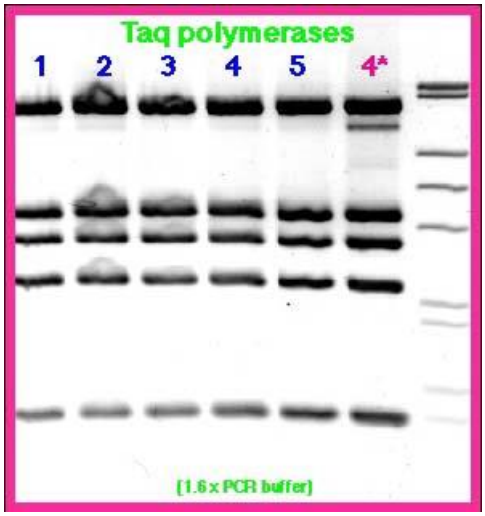

Fig. 33. Multiplex PCR of mixture D in 1.6x PCR buffer using Taq polymerases from five sources. Lanes 1 to 5 indicate that all enzymes work similarly at the same concentration. Lane 4* (green) shows the products obtained when the enzyme from lane 4 was used in the buffer provided by the vendor. An unspecific product appeared, indicating that buffer composition influences the results.

Nucleotides (dNTP)

dNTP "instability"

One important observation, coming from experiments with multiplex PCR, is that dNTP stocks are very sensitive to cycles of thawing/freezing. After 3-5 such cycles, multiplex PCR reactions usually did not work well. To avoid such problems, **small aliquots** (2-5 µl) of dNTP (25 mM each), lasting for only a couple of reactions, can be made and kept frozen at -20º C. However, during long-term freezing, small amounts of water evaporate on the walls of the vial changing the concentration of the dNTP solution. Before using, it is essential to **centrifuge** these vials at high speed in a microfuge.

This low stability of the dNTP is not so obvious when single loci are amplified.

Relationship between $MgCl_2$ and dNTP concentration

dNTP concentrations of about 200µM each are usually recommended for the Taq polymerase, at 1.5mM $MgCl_2$ (Perkin Elmer Cetus). In a 25 µl reaction volume, theoretically these nucleotides should allow synthesis of about 6-6.5 µg of DNA. This amount should be sufficient for multiplex reactions in which 5 to 8 or more primer pairs are used at the same time. To work properly (besides the magnesium bound by the dNTP and the DNA), Taq polymerase requires free magnesium. This is probably the reason why small increases in the dNTP concentrations can rapidly inhibit the PCR reaction (Mg gets "trapped")whereas increases in magnesium concentration often have positive effects.

The relationship between the concentration of magnesium and that of the dNTPs was investigated by performing PCR with a degenerate primer in reactions that contained 200, 400, 600 and 800 µM each dNTP, combined with 1.5, 2, 3, 4 or 5 mM $MgCl_2$ (Fig. 34). This test confirmed that any increase in dNTP concentration requires an increase in the concentration of magnesium ions in order for the reaction to work. At 200 µM each dNTP, reaction worked at all magmesium concentrations, but for this primer it worked better at 3 mM (which is about double the recommended magnesium concentration for the amount of dNTP). At 800 µM each dNTP, reaction worked only aboove 3 mM magnesium.

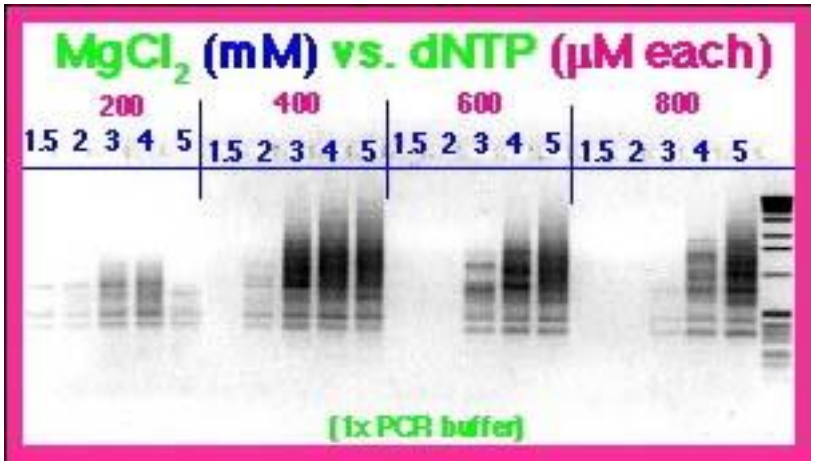

Fig. 34. PCR with a degenerate primer at different Mg and dNTP concentrations. Each of the Mg concentrations (1.5, 2, 3, 4, 5 mM) were combined with each of the following dNTP concentrations (each): 200µM, 400µM, 600µM and 800µM. Results indicate that increasing dNTP concentrations require increasing Mg concentrations for the PCR reactions to work.

Common dNTP use in PCR and multiplex PCR

In another test aimed at examining the proper dNTP concentration, a multiplex PCR using primer mixture D was performed. The $MgCl_2$ concentration was kept constant (3mM) while the dNTP concentration was increased stepwise from 50 to 100, 200, 400, 600 and 1200 µM each deoxynucleotide (Fig. 35). The best results were achieved at 200 and 400 µm dNTP; reaction was rapidly inhibited after these values. Lower than usual dNTP concentrations still allowed PCR amplification, but with somewhat less efficiency (lane "50").

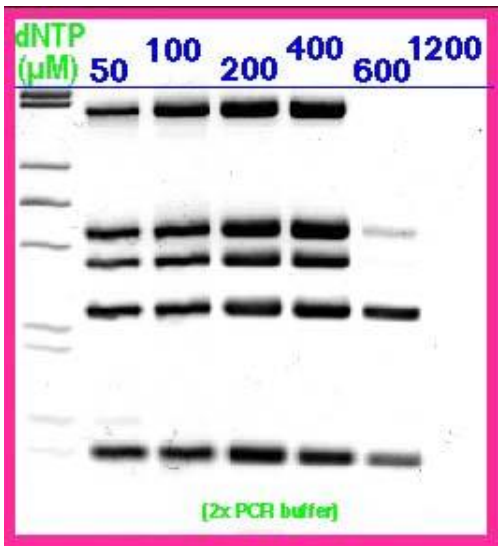

Fig. 35. Multiplex PCR amplification of mixture D in 2x PCR buffer (3 mM Mg) using increasing concentrations of dNTP (50mM, 100mM, 200mM, 400mM, 600mM and 1200mM each). Most efficient amplification is seen at concentrations of 200-400µM each dNTP. Further increase in the dNTP concentration inhibits the reaction when $MgCl_2$ is kept constant.

$MgCl_2$ concentration

Relationship between $MgCl_2$ and dNTP concentration (also on page 13)

dNTP concentrations of about 200µM each are usually recommended for the Taq polymerase, at 1.5mM $MgCl_2$ (Perkin Elmer Cetus). In a 25 µl reaction volume, theoretically these nucleotides should allow synthesis of about 6-6.5 µg of DNA. This amount should be sufficient for multiplex reactions in which 5 to 8 or more primer pairs are used at the same time. To work properly (besides the magnesium bound by the dNTP and the DNA), Taq polymerase requires free magnesium. This is probably the reason why small increases in the dNTP concentrations can rapidly inhibit the PCR reaction (Mg gets "trapped")whereas increases in magnesium concentration often have positive effects.

The relationship between the concentration of magnesium and that of the dNTPs was investigated by performing PCR with a degenerate primer in reactions that contained 200, 400, 600 and 800 µM each dNTP, combined with 1.5, 2, 3, 4 or 5 mM $MgCl_2$ (Fig. 34). This test confirmed that any increase in dNTP concentration requires an increase in the concentration of magnesium ions in order for the reaction to work. At 200 µM each dNTP, reaction worked at all magmesium concentrations, but for this primer it worked better at 3 mM (which is about double the recommended magnesium concentration for the amount of dNTP). At 800 µM each dNTP, reaction worked only aboove 3 mM magnesium.

Relationship between $MgCl_2$ and buffer (or salt) concentration

Two of the most important ingredients influenceing the results of a PCR reaction are the buffer (especially salt) and the magnesium concentrations. To study their relationship, a multiplex PCr was performed using mixture C (Fig. 36, below). Two sets of reactions were performed at two "extreme" concentrations of salt (KCl), 1x (50mM) and 3x (150 mM), and various magnesium concentrations (yellow values). Two other sets of reactions were performed at two "extreme" magnesium concentrations, 1.5 and 10.8 mM and various salt (KCl) concentrations (blue values). The dNTP concentration was kept constant, at 200 μM each deoxynucleotide. The following observation can be drawn:

- at 1x salt concentration and 200 μM each dNTP, reaction worked best at about 1.5 mM magnesium. At higher magnesium concentrations unspecific products appeared, but they gradually decreased in intensity towards 21.6 mM (probably because $MgCl_2$ is a salt, decreasing the stringency of the buffer - same way KCl does).

- at 3x salt concentration and 200 μM each dNTP, reaction worked best between 1.5 and 3.5 mM magnesium. As the stringency of the buffer was already lower than usual (due to the high KCl concnentration), further increase in $MgCl_2$ increased the "combined" stringency of teh reaction even more. Thus, fewer long unspecific products were obtained and the reaction was almost completely inhibited towards 21.6 mM magnesium.

- at 10.8 mM $MgCl_2$ and 200 μM each dNTP, reaction worked best around 2x salt (KCl) concetration (mostly specific products amplified). However, it is obvious that overall amount of PCR product is reduced compared to the reactions taking place at 1.5 mM magnesium. In this respect, high magnesium concentrations seem to inhibit the reaction more than high KCl (3x) concentrations. Therefore, it is likely that this magnesium inhibition is more than just a reduction in stringency of the reaction mixture.

- at 1.5 mM magnesium and 200 μM each dNTP, reaction worked best around 2x salt (KCl) concentration (all products amplified, few unspecific products visible). Overall product amount is higher than in the reactions taking place at 10.8 mM magnesium.

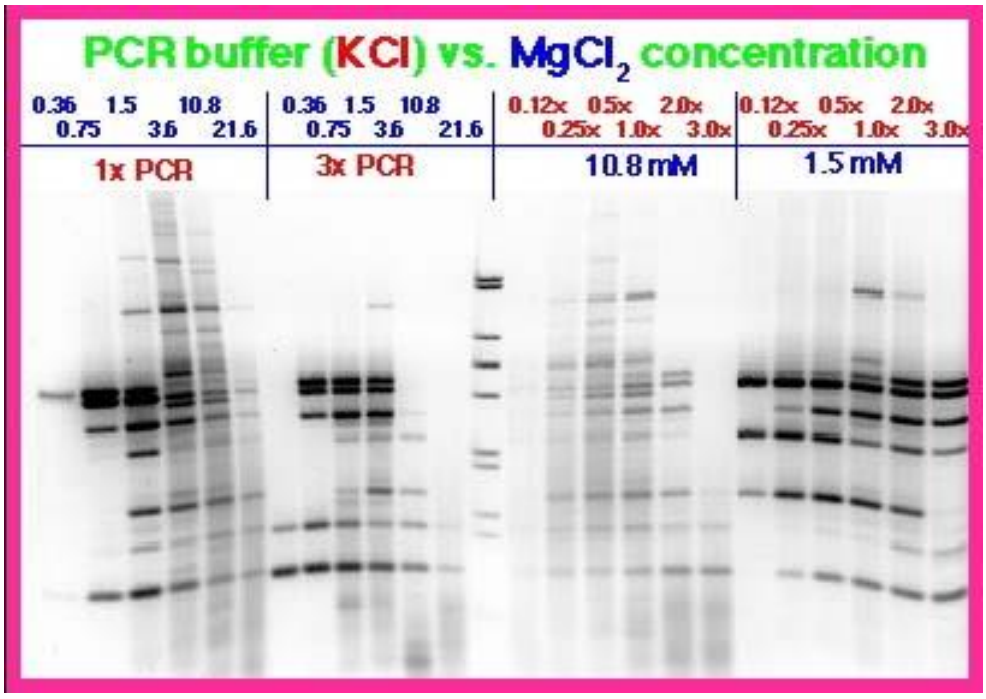

Fig. 36. Realtionship between magnesium and salt (KCl) concentration in PCR reactions. For a detailed description of the figure, please read text above.

Effects of variations in $MgCl_2$ concentration only

A recommended $MgCl_2$ concentration in a standard PCR reaction is 1.5mM, at dNTP concentrations of around 200μM each. To test the influence of $MgCl_2$, a multiplex PCR with mixture C was performed, keeping dNTP concentration at 200μM each and gradually increasing $MgCl_2$ from 1.8 to 10.8 mM (Fig. 37). The overall amplification became gradually more "specific" (unspecific bands disappeared) and the products acquired comparable intensities (at 10.8mM). However, higher concentrations of $MgCl_2$ appeared to inhibit the polymerase activity, decreasing the amount of all products. Taking into consideration the amount of PCR products, the best magnesium concentration should be between 1.8 and 3.6 mM. The large unspecific product (arrow) appeared due to the lower annealing temperature at which the reaction took place.

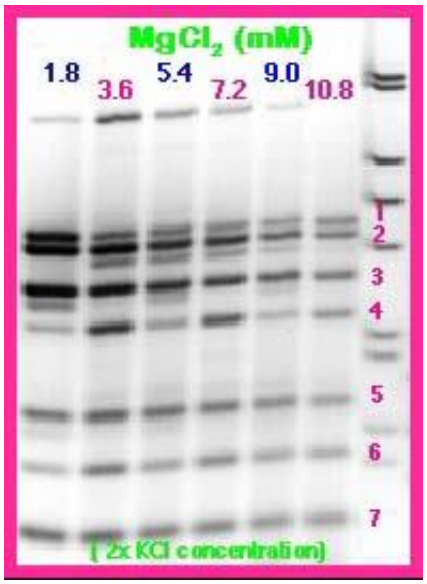

Fig. 37. Multiplex PCR amplification with mixture C at 2x KCl and increasing magnesium concentrations. Overall reaction becomes more specific at 10.8 mM magnesium, but the products are reduced in intensity. The most optimal magnesium concentration is somewhere between 1.8 and 3.6 mM where the PCR product amount is higher. The unspecific product (arrow) appears due to a lower than usual annealing temperature used for this reaction.

Gel electrophoresis

Comparison of agarose type (non-polymorphic loci)

Two types of agarose from the same manufacturer (both in use in this laboratory) were compared for their efficiency in separating the multiplex PCR products (Fig. 38). Multiplex PCR with primer mixtures A (one sample) and F (4 samples) was performed. Same amount of each reaction was loaded on a 3% agarose gel of each type. Electrophoresis time was about 1.6-1.7x longer for the regular (SeaKem LE) agarose gel.

In accordance to the manufacturer's specifications, the NuSieve agarose separates short products better than the regular agarose, and in a reduced amount of time. Although the gels had the same thickness, results also indicate that the "special" NuSieve agarose is more transparent than the regular agarose. Although NuSieve agarose is much more expensive, it provides some cost reduction by requiring less amount of agarose for the same separation power and by requiring less amount of separation time. These particular advantages can make such "specialized" agaroses useful for particular applications.

It is worth mentioning that other agaroses (from different manufacturers) used, perform similarly.

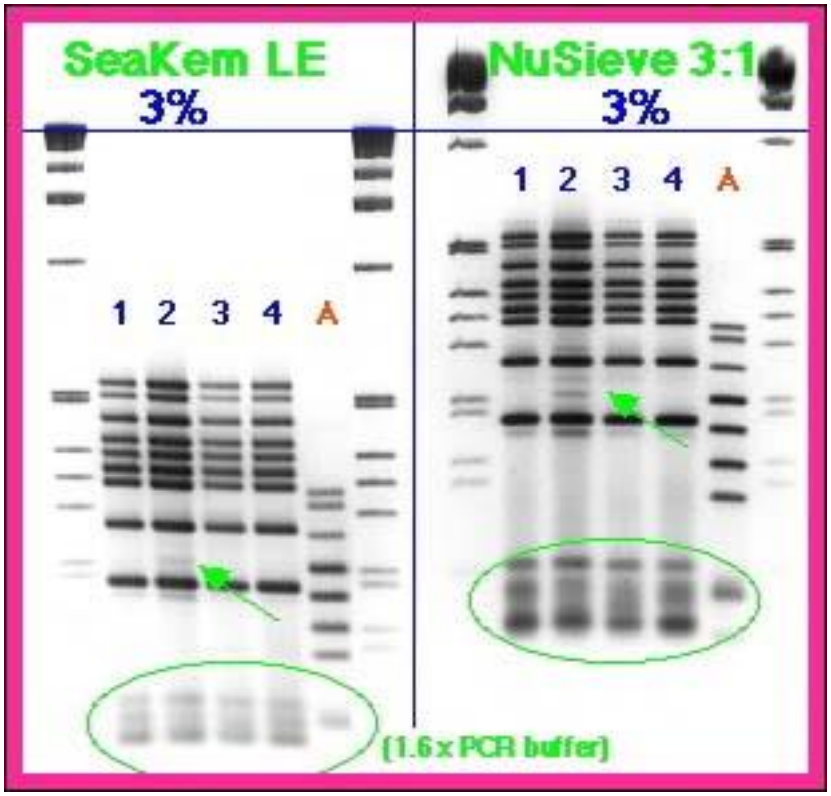

Fig. 38. Separation of the same multiplex products of mixtures A and C (four lanes) on two different agaroses. Arrow indicates a few unspecific products in lane 2 and circle indicates primers (or primer-dimers), both of these being stronger on the NuSieve gel. This shows tthat NuSieve gels have a higher transparency. Also, separation on NuSieve gels was achieved in les amount of time, over a shorter gel length. The unmarked lane(s) is the 1 kb ladder (GIBCO).

Agarose gel (running time)

Agarose gels can be run at various voltages, depending on the separation desired and the available time. It was noticed that, at least for PCR products smaller than 600 bp, separation is better and bands are sharper if gels are run very fast (3-4 hours for a 15-20 cm long 2-3% agarose gel). When the same gel runs at a low voltage overnight (14-16 hours) the products become less separable or "puffy" due to the diffusion in the gel (compare Fig. 39 below with lane C in Fig. 1).

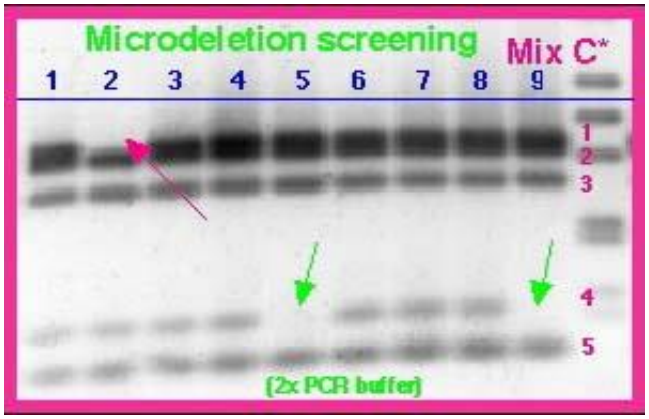

Fig. 39. Multiplex PCR with mix C was performed on 9 DNA samples to screen for microdeletions (chromosome Y loci. Gel separation was performed overnight (14 hours). Products appear diffuse, less intense, and less separable (product 1 and 2 are "fused" together). Green and magenta arrows indicate lack of loci #1 and # 4 (microdeletions) in some of the DNA samples tested. he unmarked lane(s) is the 1 kb ladder (GIBCO).

Agarose gels and polymorphic loci

As depicted also in Fig. 7, agarose gels can be used to separate PCR products of plymorphic loci. In most cases, two or three bands appear, due to heteroduplex formation between the long and short alleles. However, separation of multiplex PCR reaction products of many polymorphic loci (for example mixture K) coud become a problem for nondenaturing agarose gels. In mixture K, products were chosen so they differ by no less than 5 bp and no more than 45 bp. As depicted in Fig. 7 and Fig. 40 (below), agarose gels do not have sufficient separation power. Bands become to overlap and it is difficult or impossible to find and label each band. Denaturing polyacrylamide gels are recommended in such cases (see below).

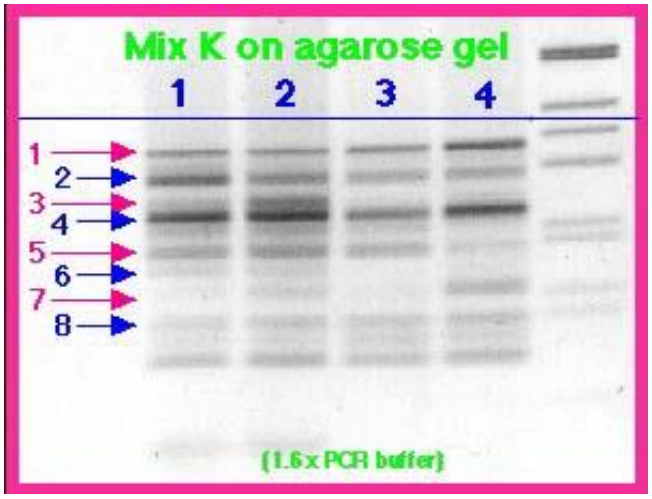

Fig. 40. Multiplex PCR amplification of mixture K, using four different DNA samples. A 2.5% agarose gel was used to separate the products. As depicted also in **Fig. 7,** 2-3 bands become visible for each product. When together, many of these bands start overlapping, making identification of individual products/alleles impossible. The unmarked lane(s) is the 1 kb ladder (GIBCO).

Non-denaturing PAA gels

To separate PCR products differing in only a few bp in length (for example, microsatellite markers), 6-10% PAA gels need to be used. Whereas non-denaturing PAA gels work very well for non-polymorphic loci, unusual bands appear when microsatellites are separated on this type of gels. For example, in an analysis of two polymorphic loci from two hybridomas, each carrying one copy of a human chromosome, for each locus tested there were 2 bands on the non-denaturing PAA gel (Fig. 41). It is unclear where the extra band originates when only one allele is amplified.

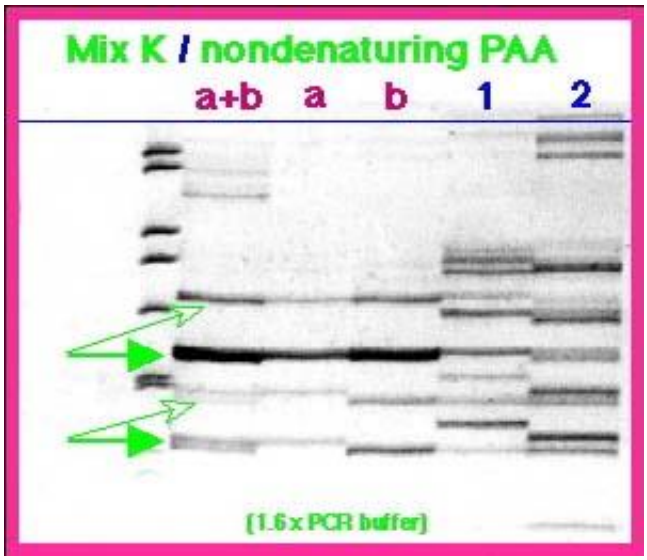

Fig. 41. Multiplex amplification of two loci on DNA from two human-rodent cell lines, each with a different copy of a human chromosome, and their combination (a+b). Although in lanes a and b each locus yields only one allele (i.e. one band), on a non-denaturing polyacrylamide gel each of the two expected products (arrows) was acompanied by another one running slower on the gel (oblique arrows). A similar aspect persisted in lane A+B. Lanes 1 and 2 show separation of products of mixture K on two different genomic template DNAs. The unmarked lane(s) is the 1 kb ladder (GIBCO).

Denaturing PAA gels

Denaturing 6% PAA/7M urea sequencing gel can be easily used to separate radiolabeled multiplex PCR products, whether these are polymorphic or unique. Denaturing PAA gels, however, are more expensive, time consuming and might prove technically more difficult. Figure 3 below, shows separation of the unique products of multiplex mixture J; double bands are visible for some of the loci. Figure 4 below shows separation of the polymorphic loci (microsatellites) of multiplex mixture K; two distinct alleles are visible for many of loci in the 8 DNA samples tested.

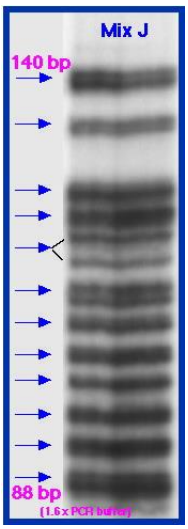

Fig. 3 (duplicate). Multiplex PCR with mix J on four different genomic DNA templates, separated on an a denaturing, 6% polyacrylamide gel. Primers amplify nonpolymorphic loci. The sizes of the longest and the shortest product are also indicated.

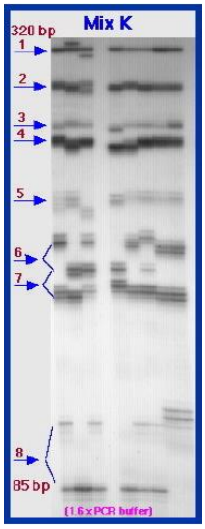

Fig. 4 (duplicate). Multiplex PCR with mix K on eight different genomic DNA templates, separated on an a denaturing, 6% polyacrylamide gel. These primers amplify polymorphic loci, and alleles of different sizes can be observed. The shortest alleles of locus 6 and the longest alleles of locus 7 can, sometimes, overlap, making it difficult to assign precisely their origin. The sizes of the longest and the shortest product are also indicated.

Adjuvants in PCR reactions

Various authors recommend DMSO and glycerol to improve amplification efficiency (higher amount of product) and specificity (no unspecific products) of PCR, when used in concentrations varying between 5-10% (v/v). In the multiplex reaction, however, these adjuvants gave conflicting results. For example, 5% **DMSO** (Fig. 42) improved the amplification of some products, decreased the amount of others whereas some loci were not influenced at all. Similar results were obtained with 5% **glycerol** (data not shown). Therefore, the usefulness of these adjuvants needs to be tested in each case.

BSA, in concentrations of up to 0.8µg/µl (higher than previously described) appeared to increase the efficiency of the PCR reaction much more than either DMSO or glycerol. It should be noted that BSA did not have an inhibitory effect on any of the loci amplified (data not shown).

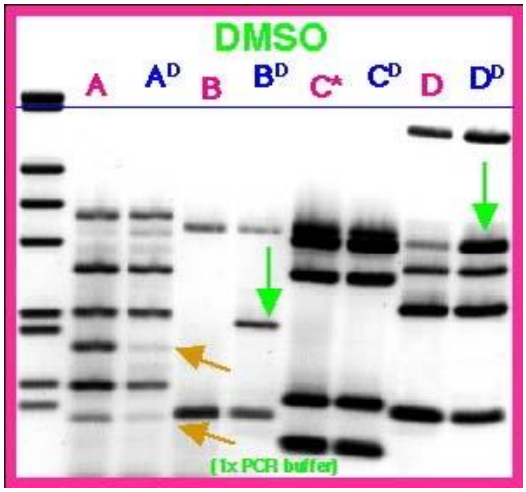

Fig. 42. Comparative multiplex PCR using mixtures A to D with 5% DMSO (superscript D) and without DMSO, in 1x buffer. Some loci from mixture A (blue arrows) are stronger when no DMSO is used. However, DMSO helps amplify (magenta arrows) one locus in mix B and one locus in mixture D. Amplification of PCR products of mixture C* were unaffected by DMSO.