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Optimizing the generation of random amplified polymorphic DNAs in chrysanthemum

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Abstract. Many conditions of the RAPD reaction procedure may influence the result. This paper presents rapid detection of influential factors with a fractional factorial experiment. A more extensive study of these factors is also presented. Polymerase brand, thermal cycler brand, annealing temperature, and primer, are important factors in obtaining good DNA yields and optimal fragment patterns. Each primer has its optimal annealing temperature, and this is not correlated with the GC content of the primer. Optimal species-primer combinations have to be found by trial and error.

Key words: RAPDs – Experimental design – Fractional factorial – Chrysanthemum – PCR

Introduction

Recently Williams et al. (1990) developed a new technique to detect genetic polymorphisms in a relatively simple way. The technique, named RAPD (Random Amplified Polymorphic DNA) is based on the amplification of DNA with random primers using a Polymerase Chain Reaction (PCR)-based methodology. With this technique neither prior knowledge of DNA sequences nor specific DNA probes are needed. The DNA fragment patterns generated discriminate between individuals, strains, or species (Hardrys et al. 1992). In some cases the inheritance of polymorphic fragments and the localization of the amplified DNA fragment in the genome was examined (Williams et al. 1990; Martin et al. 1991). Therefore, RAPDs can be used for a wide variety of studies using genetic markers (Hadrys et al. 1992).

It is known that reaction conditions need to be well-defined to obtain reproducible patterns. The present study is a succinct investigation of influential factors using statististical methodology. Two experiments were performed. The first (I for short) was on the combined influence of thermal cycler, annealing temperature, enzyme brand, magnesium concentration, primer, pH and gelatin concentration on the yield and quality of DNA. In the second experiment (II; suggested by the results of I), the influence of annealing temperature, magnesium concentration, type of primer, and DNA source, were studied more closely.

Materials and methods

DNA extraction

Chrysanthemum and maize leaves were ground in liquid nitrogen and dried under vacuum. The dry powder was kept at −20 °C. DNA was extracted from the dry powder based on a method described by Saghai-Maroof et al. (1984). In experiment I we used DNA (C2) from one randomly chosen chrysanthemum (*Dendranthema grandiflora*). In II we used DNA from three individuals: C1 and C2 are DNAs from two randomly chosen chrysanthemum cultivars and M is DNA from a randomly chosen maize individual.

Amplification reaction conditions

The random polymorphic markers are amplified in conditions similar to a normal PCR, with the exception that only a single primer is used. The nucleotide order of the primer is chosen randomly, with the only restriction being a G + C content of at least 50 % (Williams et al. 1990). Oligodeoxynucleotide primers were prepared using a DNA synthesizer 381A from Applied Biosystems. In I the primers used were: 3 (5′-TCG TCA CTG A-3′), 4 (5′-TGC TCA CTG A-3′), 14 (5′-CGG CCC CTG T-3′) and

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20 (5′-AGG AGA ACG G-3′). These have GC contents of 50, 50, 80 and 60%, respectively. In II the primers were: 2 (5′-AGG TCA CTG A-3′), 12 (5′-CGG TCA CTG T-3′), 13 (5′-CGG CCA CTG T-3′), 26 (5′-TGC TGG GCG G-3′), and 15 (5′-CGG CCC CGG T-3′), with GC contents of 50, 60, 70, 80, 90%, respectively.

The reactions were performed in a volume of 50 µl containing 20 mM TRIS-HCl, pH 8.3 (set at room temperature) or pH 7.7 (set at 74 °C), 50 mM KCl, 1.5, 2, 3 or 4 mM $MgCl_2$, 0.01% or 0.001% gelatin, 100 µM each of dATP, dCTP, cGTP and dTTP, 0.2 µM primer, 25 µg of genomic DNA, and 1 U of *Taq* polymerase (Gibco-BRL in I, Amplitaq from Perkin Elmer Cetus in I and II). The mixture was overlaid with two drops of mineral oil. In I a Biophase (BP), Bioexcellence (BE), or Perkin Elmer 480 (PE) were used as thermal cycler, in II a Perkin Elmer 9600 (PE 9600) thermal cycler was used. After 5 min heating at 94 °C 45 cycles were run. Each cycle consisted of 1 min at 94 °C, 1 min at the annealing temperature (35, 36, 37.5 or 39 °C) and 2 min at 72 °C. This was followed by 4 min at 72 °C. For BE, BP and PE 480 heating and cooling rates were the maximum possible, resulting in a cycle time of approximately 9, 9.5 and 7 min, respectively. For PE 9600 a ramp time between annealing temperature and 72 °C of 2 min was programmed. Amplified DNA fragments were separated by electrophoresis in a 1.4–2% agarose gel with a TBE buffer system. Gels were stained with ethidium bromide and fragment patterns were photographed for further analyses. Yields were estimated by comparison with a standard, known to contain 1 µg of DNA.

Statistical design of experiments

Both experiments presented here use a statistical design. Experiment I is based on a 1/8 fraction of a two-level factorial design with eight factors (32 observations; Box et al. 1978). Two of the two-level factors were used as pseudofactors (McLean and Anderson 1984) for the study of four primers. Table 1a lists the factors and their levels.

The main effects of thermal cycler and temperature are inherently confounded with runs. We deliberately confounded a third effect (ACD, see Table 1). As the generalized interactions of the three effects are also confounded with runs (Box et al. 1978), we reached a division in seven between-runs and 24 within-runs effects. Influential effects, as pointed out with half-normal plots (Daniel 1959) of each set, were used to calculate descriptive regression equations. Non-influential effects were used to calculate standard errors between and within runs, respectively. The runs with the BP thermal cycler were duplicated with the PE. Thus we were able to separate aliased effects (see Results and discussion).

In experiment II, a randomized block design was employed with thermal cycler runs as blocks. The factors studied were Mg concentration (2, 3, and 4 mM), annealing temperature (36, 37.5, 39 °C), primer (GC content 50, 60, 70, 80, and 90%, respectively) and DNA template (two from chrysanthemum, one from *Zea mays*). The number of samples required for one replicate of the design (135) enabled a precise study of effects. Therefore, we did not duplicate the experiment. The results were studies with an analysis of variance (error term: four-factor interaction).

All analyses of the results deal with the square-root of yield and the logit of the score, defined as log [score/(10 − score)], in view of stabilization of the variance (Box et al. 1978).

Results and discussion

Summaries of the results are given in Tables 1–3; the raw data of both experiments are available on request from the first author. Regression equations for experiment I are given in Table 1. The BP/BE part was constructed such that the annealing temperature by pr2 interaction (BF) could not be distinguished from the thermal cycler by enzyme brand interaction (AC). Other effects were also aliased. The runs with the PE thermal cycler were used for de-aliasing (Schoen and Wolff, in preparation). Table 1 bears on the de-aliased effects. The effect of DNA polymerase is conclusive.

Table 2A illustrates the BF interaction. The second pseudofactor for primer contrasts primers 3 and 4 with

Table 1. Regression equations describing results of the first experiment

a. Coding of experimental variables

	Code −1	Code +1
Thermal cycler (A)	BE	BP
Annealing temperature (B)	35 °C	36 °C
Enzyme brand (C)	Amplitaq	Gibco BRL
Magnesium concentration (D)	1.5 mM	2 mM
Pseudofactor pr1 (E)	(Primer 3, primer 14)	(Primer 14, primer 20)
Pseudofactor pr2 (F)	(Primer 3, primer 4)	(Primer 4, primer 20)
pH (G)	7.7	8.3
Gelatin concentration (H)	0.01%	0.001%

b. Square root of yield

$0.97 + 0.21\,B - 0.40\,C - 0.16\,BF - 0.12\,BH - 0.07\,ABH$

Standard errors: 0.10 (intercept, B; 6 degrees of freedom) or 0.057 (remaining coefficients; 20 degrees of freedom)

c. Logit of quality

$-0.45 + 0.46\,B - 0.67\,C + 0.41\,E + 0.31\,DE$

Standard errors: 0.23 (intercept, B; 6 degrees of freedom) or 0.16 (remaining coefficients; 21 degrees of freedom)

primers 14 and 20. The former primers show a larger effect of annealing temperature than the latter. It is tempting to ascribe this result to the difference in GC content, but the experiment does not really permit such a conclusion. Table 2B gives means of logit-quality scores for primer by concentration of magnesium, showing the effect of magnesium concentration to differ for some primers. It is again tempting to ascribe the latter interaction to the high GC percentage of primer 14.

Table 2. Selected tables of means from the first experiment, the BE/BP portion[a]. (A) yield (square root): annealing temperature by primer[b]. (B) quality of fragment pattern (logit): magnesium concentration by primer

(A)

Temp.	Primer (GC%)			
	3 (50)	4 (50)	14 (80)	20 (60)
35 °C	0.448	0.709	1.067	0.805
36 °C	1.247	1.402	0.908	1.170

(B)

Mg conc.	Primer (GC%)			
	3 (50)	4 (50)	14 (80)	20 (60)
1.5	−0.86	−0.21	0.77	0.49
2.0	−0.65	0.45	−1.10	0.66
Difference[c]	0.21	0.66	−1.87	0.17

[a] Tables contain means of four values
[b] Standard error of differences: 0.229 when comparing figures within the same row, otherwise 0.282
[c] Standard error of differences between these differences: 0.898

In experiment II, those factors that seemed to be important in optimizing the reaction conditions were studied more closely (see Materials and methods). Analyzing DNA yield it appeared that primer, annealing temperature, DNA template × primer and annealing temperature × primer were important factors (F-values > 40). Remaining effects had F-values < 10; they were not evaluated further.

The above interactions were studied in relation to the GC content of the primer. Primer × temperature interaction was of particular interest as we expected an optimum that might be different for each primer. Figure 1 gives DNA yields for the annealing temperatures and the primers, both as a total and split up for the different DNA templates. No clear pattern is apparent. Primer × temperature interaction, therefore, does not seem to be GC% dependent. Possibly no property of the primer other than the exact nucleotide order can predict yield.

Primer, DNA template, and annealing temperature had a major influence on the score for the quality of fragment pattern: there were significant interactions of annealing temperature by DNA template and of primer by DNA template. A significant three-factor interaction, template by temperature by primer, was

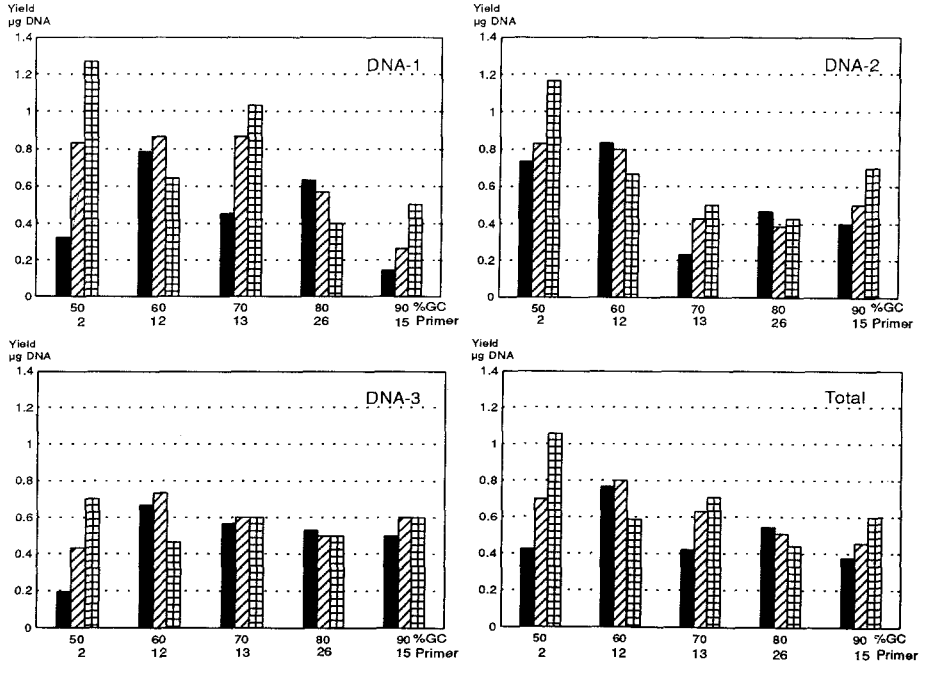

Fig. 1. The influence of annealing temperature (■ 36 °C, ▨ 37.5 °C, ⊞ 39 °C) and primer GC content on the DNA yield (retransformed values) obtained in all DNAs together and the three DNAs separately (experiment II)

Table 3. Interactions on logit-quality of fragment pattern in the second experiment[a]. (A) ranking of primer according to DNA template. (B) ranking of DNA template according to annealing temperature. Primers (A) or DNA templates (B) connected by a *line* do not differ significantly (LSD test, 5% level of significance). Mean values are given between brackets

(A)

DNA template	Primer				
C1	26 80% (0.14)	13 70% (0.22)	12 60% (0.27)	2 50% (0.40)	15 90% (0.71)
C2	26 80% (0.22)	12 60% (0.27)	13 70% (0.46)	2 50% (0.55)	15 90% (1.16)
M	26 80% (0.00)	2 50% (0.28)	13 70% (0.70)	12 60% (0.85)	15 90% (0.88)

(B)

Temperature	DNA template		
36 °C	C1 (0.25)	C2 (0.60)	M (0.65)
37.5 °C	C1 (0.42)	C2 (0.54)	M (0.59)
39 °C	C1 (0.39)	M (0.39)	C2 (0.46)

[a] Table contains means of 9 (A) or 15 (B) values. Standard error of differences 0.112 (A) or 0.087 (B; not applicable to comparisons between temperatures)

mainly due to the values of maize for primer 15 at 36 °C. Means involving these values are, therefore, not conclusive.

The quality of fragment pattern in maize shows a different ranking of the primers from chrysanthemum (Table 3A). The effects of DNA template, primer and their interaction are a direct consequence of the fact that each combination of individual and primer gives a different pattern; they do not reflect a relation between the GC content of a primer and the quality of fragment pattern. Which primer is best for which species must be determined by trial and error. A difference in quality and yield is probably partly determined by whether the fragment amplified is from single-copy DNA or whether the same fragment can be amplified from repeated DNA. Future work will possibly resolve this. The interaction between DNA template and annealing temperature (Table 3B) points to the conclusion that the optimum temperature differs for the three DNA templates.

Mg concentration appeared to be important for the exact pattern obtained, but had only minor influence on DNA yield. It had no influence on quality scoring in II, either as a main effect or as an interaction with other factors. The primer × Mg concentration interaction found in I might therefore be a characteristic of primer 14 only. We conclude that Mg concentration and annealing temperature should be optimized for each species and primer combination separately.

The influence of annealing temperature on the fragment pattern is only minor.

Optimizing PCR-based technology is a laborious task as many components can be altered in PCR reactions and not all processes and mechanisms are fully understood. In the present study DNA template concentration and polymerase concentration were not varied. In contrast to some other studies (Williams et al. 1990; Arnold et al. 1991) experiments with chrysanthemum showed that the limits to DNA template and polymerase concentration are not narrow (1 to 500 ng for DNA template and 0.6 to 2U for *Taq* polymerase per 50 µl) and that the patterns obtained are relatively constant (Wolff, unpublished results). The inheritance and genetic variation of RAPD fragment patterns in chrysanthemum is described in Wolff and Peters-Van Rijn (1993).

The present study has shown clearly that using a factorial design can, with only few samples lead to important conclusions. From these conclusions a more specific experiment can be set up in which the most interesting factors are then studied in more detail.

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