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Troubleshooting the Single-step PCR Site-directed Mutagenesis Procedure Intended to Create a Non-functional *rop* Gene in the pBR322 Plasmid

Lina Jew
Department of Microbiology & Immunology, University of British Columbia

The co-transformation of ColE1-type plasmids, specifically pBR322 and pUC19 results in the exclusion of pBR322 in the transformed cells. The mechanism for this effect is not fully known, but it is speculated that this observed exclusion is due to the molecular differences of the two plasmids. The RNA II in pUC19 has a point mutation that is absent in the RNA II of pBR322. This point mutation causes the RNA I to bind less effectively to RNA II and thus RNA II would not be blocked and can initiate the primer formation. Also, pBR322 has a sequence that codes for a protein called Rop (repressor of primer) and pUC19 does not. Rop has negative regulatory functions and contributes to the inhibition of replication by stabilizing the interaction between RNA I and RNA II. The successful creation of a mutant pBR322 with a non-functional rop gene would give insight into the relative impact of the rop gene in the exclusion effect during co-transformation with pUC19. The non-functional rop gene can be created by inserting stop codons into the rop gene via single-step PCR site-directed mutagenesis. There have been difficulties creating such a mutant. This study investigated and troubleshoots the creation of a non-functional rop gene via single-step PCR site-directed mutagenesis by modifying the previously attempted PCR conditions, varying the primer combinations (3mrop5, 5mrop3, BR299A and BR229B primers) and testing the effect of linear and circular template DNA. It was found that the structural type of template DNA and annealing temperature have relatively small impact on the PCR for site-directed mutagenesis. The addition of the 5mrop3 primer caused the amplification of a product to be inhibited, thus acting as a inhibitor to the PCR. Future experiments include designing a new primer and modify the PCR to include a thermostable DNA ligase.

It has been observed that pUC19 is preferentially selected when *Escherichia coli* DH5α is co-transformed with equal quantities of the following ColE1-type plasmids: pUC19 and pBR322. In order to replicate, these two plasmids rely on the synthesis of a primer precursor called RNA II and its interaction with DNA at the origin of replication. Replication is initiated when RNA II undergoes RNase H cleavage and provides a 3' OH for DNA polymerase to start DNA synthesis. RNA I is another molecule that plays a role in plasmid replication; it is complementary to RNA II and therefore can bind to RNA II and stop the primer formation. As a result, this interaction inhibits the initiation of replication.

A protein called Rop (repressor of primer) is also an important factor in the replication process because Rop stabilizes the complex formed by RNA I and RNA II by negatively regulating the origin of replication. The *rop* gene is present in pBR322 and lacking in pUC19; and because of the impact of *rop* on replication, it is speculated to be a cause of the exclusion of pBR322 during co-transformation with pUC19. Since pUC19 does not contain a *rop* gene, the RNA I/RNA II would not be stabilized and as a result, the RNA II would be able to form a primer and DNA polymerase would begin replication more frequently.

Another speculation to explain why pUC19 was more dominant in this specific co-transformation is the mutation on the RNA II in pUC19 that is absent in the RNA II in pBR322 (1). The pUC19 contains a G→A point mutation in the RNA II gene; and its folding and activation may be temperature dependent. Because of this, RNA I binds less effectively to RNA II and thus cannot exhibit its full negative regulatory effects on DNA synthesis; RNA II is able to form primers more often in pUC19 than pBR322.

There have been multiple attempts to either remove or mutate the rop gene to test the role of the Rop protein in the exclusion effects of pBR322 during co-transformation with pUC19. For instance, Komlienovic (5) attempted to create a mutant pBR322 that had decreased rop expression via site-directed mutagenesis by introducing a single mutation in the sequence of the ribosomal binding site upstream of *rop*. However, when the mutant pBR322 was co-transformed with the wild type pBR322, the results did not parallel with what was

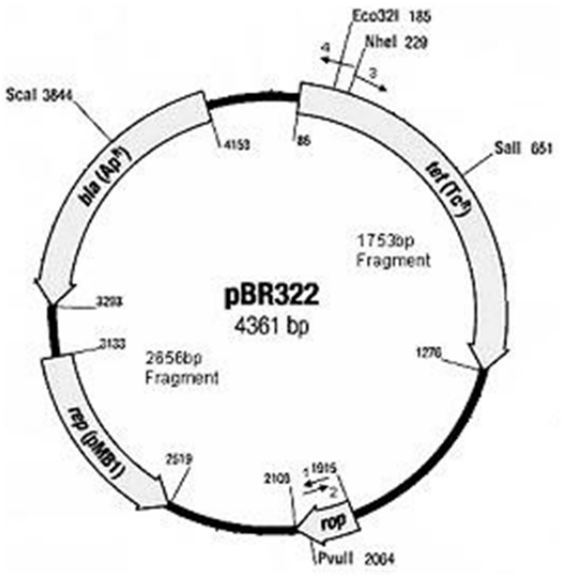

FIG. 1. The restriction map of wild type pBR322. The location and direction of each of the primers (1, 2, 3 and 4) are shown on the map. Primers 1, 2, 3 and 4 are 5mrop3, 3mrop5, BR229A and BR229B, respectively. 5mrop3/3mrop5 are complementary and they overlap; and BR229A/BR229B anneal in the area of the *Nhe*I restriciton site.

expected (6). In theory, the wild type pBR322 should have been excluded during co-transformation, but instead, it was dominant. The results suggested that either the mutation did not work or that a decrease in *rop* expression was not sufficient to cause the exclusion effect.

As an alternative to studying the role of *rop*, Fang (3) attempted to create a non-functional *rop* via a single-step PCR site-directed mutagenesis. Single-step PCR site-directed mutagenesis is a method to create slight variation in a plasmid by designing primers with the desired mutations and amplifying the entire plasmid. It is a simple way to substitute, delete or insert several bases directly into a plasmid. However, no amplified product was evident when Fang (3) attempted this method and as a result, she designed a partial PCR site-directed mutagenesis. Fig. 1 shows the amplification scheme on the restriction map of wildtype pBR322.

The purpose of this study was to troubleshoot the PCR strategies that Fang (3) used and find the cause for the unsuccessful single-step site-directed mutagenesis, specifically by [1] varying the combination of four primers in the PCR, [2] modifying the PCR parameters (annealing temperature), and [3] testing the use of circular and linearized pBR322 template DNA because other researchers have been successful with the single-step PCR site-directed mutagenesis. Once the problem is known, a non-functional *rop* gene in pBR322 can be created and used to test the relevance of *rop* in determining copy number during co-transformation with pUC19.

MATERIALS AND METHODS

Primers. The following four primers were previously designed by Fang (3) and were used in the PCRs: [1] 3mrop5 = 5'-TTAATGTCTAGCTTCAGAGGTACCGGGCCATGT-3' [2] 5mrop3 = 5'-CATGGCCCGGTACCTCTGAAGCTAGACATTAA-3' [3] BR299A = 5'-CGTGCTGCTAGCGCTATATGCGTTGA-3' [4] BR299B = 5'-AGCGCTAGCAGCACGCCATAGTGACT-3'(3). Primers 3mrop5, 5mrop3, BR299A and BR299B bind to base pairs 1944 – 1975, 1944 – 1975, 228 – 253 and 203 – 228 on pBR322, respectively. The unique *Nhe*I restriction site is in the primer BR299B. 3mrop5 and 5mrop3 are complementary to one another; and BR299A and BR229B are reverse primers of one another.

Restriction endonuclease digest conditions. The plasmid DNA pBR322 (Fermentas LOT# 00035700) was linearized at either the *Nhe*I, *Sca*I or *Sal*I restriction sites. *Nhe*I digest: 0.5 µg (1 µl) pBR322, 1 µl Fast digest *Nhe*I enzyme (Fermentas LOT# 00060755), 2 µl 10X fast digest buffer, and 16 µl dH_2O for a total reaction volume of 20 µl. The reaction was incubated at 37°C for 45 minutes and then heat inactivated at 65°C for 15 minutes. *Sca*I digest: 0.5 µg (1 µl) pBR322, 1 µl *Sca*I enzyme, 2 µl 10X REact 6 buffer, 16 µl dH_2O. *Sal*I digest: 0.5 µg (1 µl) pBR322, 1 µl *Sal*I enzyme, 2 µl 10X REact 10 buffer, 16 µl dH_2O. Both *Sca*I and *Sal*I reactions were incubated overnight at 37°C and then heat inactivated at 65°C for 20 minutes.

PCR Conditions. The PCR conditions programmed and used on the Biometra Tgradient PCR machine are shown in Table 1. The annealing temperature was either 48°C or 51.4°C. The mastermix of the PCR included 1X Taq buffer with $(NH_4)SO_4$, 0.5 mM dNTPs, 1.5 mM $MgCl_2$, and dH_2O; after aliquoting the mastermix into PCR tubes, 50 ng of either cut or uncut template pBR322 DNA, and the different primer (2 pmol/µl) combinations were added to their respective PCR tubes. The following primer combinations were tested: [1] All four primers, [2] 3mrop5 and BR299A, [3] 5mrop3 and BR299B, [4] 3mrop5, BR299A and either 5mrop3 or BR229B, [5] no primers, and [6] BR299A and BR299B. On top of the various primer combinations, plasmid DNA cut with either NheI, ScaI or *Sal*I and uncut plasmid DNA was used to test the effect of using linear and circular DNA as template.

Electrophoresis gel. A 0.8% gel was made with 1X TAE and agarose (Fermentas LOT# 00044610) and ran with 1X TAE. 6 µl of the following mix was loaded as the molecular ladder: 0.5 µg GeneRuler 1kb DNA ladder (Fermentas LOT# 00033708), dH_2O and 1X DNA loading dye; and 10 µl of the following was loaded: 5 µl sample, 1X DNA loading dye, and dH_2O. The electropheresis gels were run for approximately 1 hour at 100V.

TABLE 1. The PCR conditions used to attempt and troubleshoot site-directed mutagenesis of *rop* in pBR322. * indicates the annealing temperatures that were varied. The total of 40 cycles was run; and the total runtime was approximately 5 hours and 48 minutes.

Condition	Steps				
	Initial denaturation	Denaturation	Annealing	Elongation	Final elongation
Temp. (°C)	95	95	48 or 51.4	72	72
Time (sec)	60	45	60	360	600
Repeats	1		39		1

TABLE 2. The expected amplified product size when the following primer pairs are put together in the PCR reaction: 3mrop5/5mrop3; 3mrop5/BR229A; 5mrop3/BR229B; and BR229A/BR229B.

Primer Combinations	Expected product size (bp)
3mrop5/5mrop3	4361 (Entire plasmid)
3mrop5/BR229A	1753 (Part of the plasmid)
5mrop3/BR229B	2656 (Part of the plasmid)
BR229A/BR229B	4361 (Entire plasmid)

RESULTS

PCR site-directed mutagenesis with all four primers. When all four primers were present in the PCR, none of the expected bands resulted. Table 2 shows the expected product size of the various primer combinations. If the 5mrop3 and 3mrop5 primers had worked, then the entire plasmid would have been amplified resulting in a 4361 bp band that should have been relatively more intense than the negative control (Fig. 2, lanes 2 and 3). However, as shown in Fig. 2, this was not the case – lanes 6, 9, 12 and 15 in Fig. 2 did not yield any positive products. There was nothing in lanes 6 and 9 except for an intense blob at the bottom of the gel; and lanes 12 and 15 resembled the negative control in lane 3 suggesting that the plasmid was not amplified.

PCR site-directed mutagenesis with 3mrop5 and BR229A. The expected 1753 bp fragment was always produced whenever the only primers present in the reaction were 3mrop5 and BR229A. The 1753 bp product was also formed whenever BR229B was added to the said primer set (as shown in Fig. 2).

PCR site-directed mutagenesis with 5mrop3 and BR229B. When 5mrop3 and BR229B were used in the PCR, a product size of 2656 bp was expected. In Fig. 2, when 5mrop3 and BR229B were the only primers in the reaction coupled with linearized DNA, the result was a smear with no distinct 2656 bp fragment (lanes 5 and 8); and when circular DNA was used, a thick band at approximately 2700 bp resulted (lanes 11 and 15).

PCR site-directed mutagenesis with 3mrop5, BR229A and either 5mrop3 or BR299B. Since the primers (3mrop5 and BR229A) are necessary to produce the 1753 bp fragment, then the 1753 bp was expected to be produced in the presence of the 3mrop5, BR229A and either 5mrop3 or BR299B. For both experiment with either linearized template DNA or circular template DNA, the expected 1753 bp fragment was produced in the presence of BR299B, but the 1753 bp fragment was absent when 5mrop3 was also part of the reaction. Fig. 2 shows the amplified 1753 bp fragment (lanes 17, 19, 21, 23) and it was the most intense when the template DNA was linearized (lane 17).

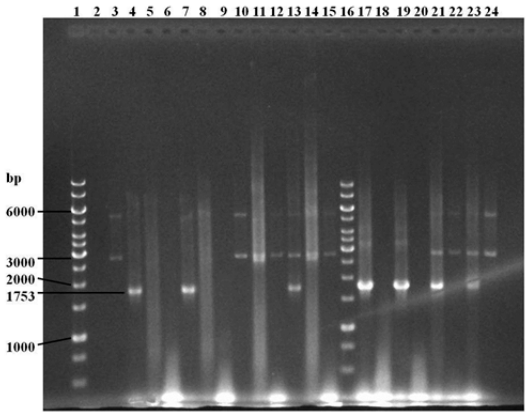

FIG. 2. The PCR products affected by the annealing temperature of the PCR, the structural type of DNA used as template, and the different primer combinations. Lanes 1 and 16 are the 1 kb molecular weight ladder. This figure corresponds to Table 3. Refer to Table 3 for the specifics of each lane.

PCR with template DNA linearized with either *Sca*I or *Sal*I restriction endonuclease. Template DNA was also linearized with either *Sca*I or *Sal*I to help see which primer combination could form the expected products. The primer combinations tested were: BR229A/BR229B, 3mrop5/BR229A, and 5mrop3/BR229B; and the annealing temperature used was 51.4°C. In the PCR consisting of pBR322 digested with *Sca*I, there should be no 2656 bp fragment because the restriction cut disrupts the fragment; and for the PCR consisting of pBR322 digested with *Sal*I, there should be no 1753 bp fragment because the digest disrupts it.

As expected, when the template DNA was cut with *Sca*I, the 1753 bp fragment was significantly amplified and no 2656 bp fragment was produced (Fig. 3, lane 4). The results obtained using the template DNA that was cut with *Sal*I, suggested that the digestion was incomplete. There should be no 1753 bp fragment, but evidently it was produced (Fig. 2, lane 8); it was not amplified as much as the 1753 bp fragment (Fig. 2, lane 4) in the PCR with *Sca*I, which supports the possibility of an incomplete digestion. Interestingly, when the PCR reactions were prepared and run again, the 1753 bp fragment was not formed and amplified (Fig. 3, lane 9). However, the rest of the results were consistent between the tests. Moreover, whether linear or circular plasmid DNA was used, the amplification of the entire plasmid was unsuccessful when the BR229A/BR229B primer combination was used.

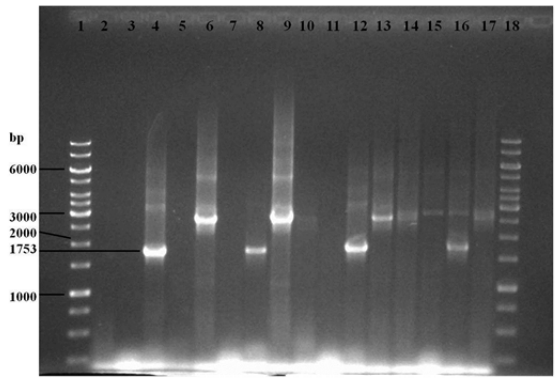

FIG. 3. The PCR products that were produced with either circular (unmodified) template DNA or linear (cut with either *Sca*I, *Sal*I or *Nhe*I) template DNA; and at an annealing temperature of 51.4°C. Lanes 1 and 18 is the 1kb molecular weight ladder. No fragment at 2656 bp is expected to be produced when DNA cut with *Sca*I is used in the PCR; and no 1753bp fragment is expected when DNA cut with SalI is used. Refer to Table 4 for the specifics of each lane.

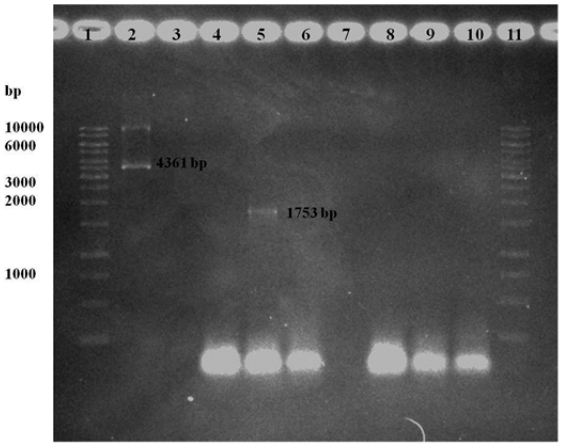

FIG. 4. The PCR products that were produced with linear (cut with either *Sca*I or *Sal*I) template DNA; and at an annealing temperature of 51.4°C. Lanes 1 and 11 are the 1 kb molecular weight ladder. Lane 2 is the wild type pBR322. Lanes 3-6 are PCR product from template DNA cut with *Sca*I; lane 3 contains no primers (negative control), lane 4 has primers BR229A/BR22B, lane 5 has primers 3mrop5/5mrop3, and lane 5 has primers 5mrop3/BR229B. Lanes 7-10 are template DNA cut with *Sal*I; lane 7 contains no primers (negative control), lane 8 has primers BR229A/BR229B, lane 9 has primers 3mrop5/5mrop3, and lane 10 has primers 5mrop3/BR229B.

The effect of changing the annealing temperature of the PCR. Two temperatures (48°C and 51.4°C) that were near the melting temperatures of the primers were tested to determine whether temperature played a significant role in the site-directed mutagenesis PCR. Regardless of whether the template DNA was linearized (Fig. 2, lane 6 and 9) or circular (Fig. 2, lane 12 and 15), when all four primers were present, temperature did not seem to affect the reaction. However, when only the 3mrop5 and BR299A were present in the reaction and the template was linearized, the expected 1753 bp fragment was present and it was two times more intense at the higher (51.4°C) annealing temperature than the lower (48°C) temperature. When unmodified (circular) template DNA was used, the higher temperature resulted in the expected 1753 bp fragment, but at the lower temperature, the said expected band was absent (Fig. 2, lanes 13 and 10, respectively).

DISCUSSION

Regardless of the changes in annealing temperature and the type (linear or circular) of template DNA used, the 1753 bp fragment was consistently amplified in all the reactions that contained 3mrop5 and BR229A. The only time when the 1753 bp fragment was not produced and amplified was when 5mrop3 was also present in the PCR; there were no problems when BR229B was present in the reaction. This suggests that 5mrop3 somehow interferes with the amplification process; a plausible explanation is that 5mrop3 has a higher affinity to its complimentary primer, 3mrop5, than to the template DNA. The sequences of 3mrop5 and 5mrop3 overlap and are completely complementary to one another and thus are likely to bind together and form primer dimers. The absence of product amplification is consistent with the data found by Zheng *et al* (7) when complete-overlapping primer pairs were used. This explanation is also supported by the accumulation of primer dimers at the bottom of the gel (Fig. 2, lanes 18, 20, 22 and 24). The primer 5mrop3 could also be interfering by annealing to different parts of the template DNA, thus hindering the amplification process.

Lanes 11 and 14 in Fig. 2, shows the potential amplification of the 2656 bp fragment. The DNA used as the template was circular and it has been shown that uncut circular plasmid DNA is mostly in supercoiled form (4). Supercoiled DNA migrates faster than relaxed DNA when run on an electrophoresis gel which explains why the fragment for the template is located at approximately 2800 bp instead of at 4361 bp. The 2656 bp fragment looks thick because it is located near the band of the template DNA.

The annealing temperature of 51.4°C caused an increase in the amplification of the 1753 bp fragment – the band on the electrophoresis gel was more intense. Also, when circular DNA was used as the template, the 1753 bp fragment was not produced at 48°C, but it was at 51.4°C. This suggests that a higher annealing temperature works better when circular template DNA is used instead of linear template DNA.

TABLE 3. The tested combinations of temperature, linear and circular template, and primers that corresponds to the particular PCR products in Figure 2. The 'X' indicates the presence of the item.

Lane #	Template	Temp.	Added Primers				Observed PCR Product (bp)	
			3mrop5	BR229A	5mrop3	BR229B	1753	2656
2 (No Taq)	cut	51.4						
3 (No Taq)	uncut	51.4						
13	uncut	51.4	X	X			X	
4	cut	48	X	X			X	
7	cut	51.4	X	X			X	
17	cut	48	X	X		X	X	
19	cut	51.4	X	X		X	X	
21	uncut	48	X	X		X	X	
23	uncut	51.4	X	X		X	X	
11	uncut	48			X	X		X
14	uncut	51.4			X	X		X
5	cut	48			X	X		
6	cut	48	X	X	X	X		
8	cut	51.4			X	X		
9	cut	51.4	X	X	X	X		
10	uncut	48	X	X				
12	uncut	48	X	X	X	X		
15	uncut	51.4	X	X	X	X		
18	cut	48	X	X	X			
20	cut	51.4	X	X	X			
22	uncut	48	X	X	X			
24	uncut	51.4	X	X	X			

To confirm that the template DNA was actually linearized by *Nhe*I, another experiment was done, where the pBR322 was linearized by either a *Sca*I or *Sal*I digestion instead. By doing so, the amplification patterns created by the different primer combinations observed previously could be tested for any inconsistencies. The PCR with template DNA that was digested with *Sal*I showed inconsistent results. In the case where the pBR322 was linearized by *Sal*I, the 1753 bp should not have been amplified because the cut prevents the complete replication of the 1753 bp fragment. Based on Fig. 3, lane 8, it was evident that the 1753 bp was amplified, however this might have been due to incomplete digestion.

Single-step whole plasmid PCR site-directed mutagenesis has been successfully accomplished by other researchers. Zheng *et al* (7) were successful after they restricted their primers pairs to 25-45 bases in length with a melting temperature greater than 78°C; this method helped reduce primer dimer formation and increased primer-template annealing. The design of the primer is a critical factor to the overall success of the PCR as it should contain at least eight non-overlapping bases at the 3' end of the primer, contain targeted mutation(s) in both primers, include a silent mutation in at least one of the primers, and have a least one G or C at each terminus (7). The primers designed by Fang did not fully meet the said conditions.

Based on all the results obtained, the cause of the unsuccessful attempts at using single-step site-directed mutagenesis to create a non-functional rop gene might have been largely due to the 5mrop3 primer. The 3mrop5 primer anneals properly, but the 5mrop3 acted as an inhibitor and interfered with the amplification process. The other modifications did not have significant impact on the amplification process. Single-step PCR site-directed mutagenesis might be

TABLE 4. The tested combinations of linearized and unlinearized template and primer that produced particular PCR products observed in Figure 3. The 'X' indicates the presence of the item.

Lane #	Cut	Added Primers				PCR Product (bp)
		3mrop5	BR229 A	5mrop3	BR229B	1753bp
2	ScaI					
6	SalI					
10	NheI					
14						
4	ScaI	X	X			X
8	SalI	X	X			X
12	NheI	X	X			X
16		X	X			X
3	ScaI		X		X	
7	SalI		X		X	
11	NheI		X		X	
15			X		X	
5	ScaI			X	X	
9	SalI			X	X	
13	NheI			X	X	
17				X	X	

accomplished by redesigning the primer to avoid dimer pairing.

FUTURE EXPERIMENTS

Future experiments include designing a new reverse primer for the 3mrop5 primer. The overlapping complementary primers were a problem because the chance of the primers annealing together and forming primer dimers was high; and the 5mrop3 primer proved to cause some kind of interference in the PCR. A technique called 'ligation-during-amplification' can be applied to site-directed mutagenesis and it will still be a single-step PCR. In ligation-during-amplification, a thermostable DNA ligase is added to the PCR reaction; and it should be noted that closed circular DNA is used as the template (2). After the extension of the primers on the circular template, the ligase closes the gap and forms a double-stranded DNA (2). After the denaturation step, the two circular DNA strands serve as templates for the next round of extension and ligation and the DNA is amplified exponentially. This way, the newly designed primer does not have to overlap with 3mrop5; and the mutations to create a non-functional *rop* gene can be incorporated into circular pBR322; and the final product will still be closed circular DNA.

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