A mutant screening method by critical annealing temperature-PCR for site-directed mutagenesis

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Abstract

Background: Distinguishing desired mutants from parental templates and undesired mutants is a problem not well solved in Quikchange™ mutagenesis. Although Dpn I digestion can eliminate methylated parental (WT) DNA, the efficiency is not satisfying due to the existence of hemi-methylated DNA in the PCR products, which is resistant to Dpn I. The present study designed a novel critical annealing temperature (Tc)-PCR to replace Dpn I digestion for more perfect mutant distinguishing, in which part-overlapping primers containing mutation(s) were used to reduce initial concentration of template DNA in mutagenic PCR. A Tc-PCR with the same mutagenic primers was performed without Dpn I digestion. The Tc for each pair of the primers was identified by gradient PCR. The relationship between PCR-identified Tc and Tm of the primers was analyzed and modeled with correlation and regression.

Results: Gradient PCR identified a Tc for each of 14 tested mutagenic primers, which could discriminate mismatched parental molecules and undesired mutants from desired mutants. The PCR-identified Tc was correlated to the primer’s Tm (r = 0.804, P<0.0001). Thus, in practical applications, the Tc can be easily calculated with a regression equation, Tc = 48.81 + 0.253*Tm.

Conclusions: The new protocol introduced a novel Tc-PCR method for mutant screening which can more efficiently and accurately select against parental molecules and undesired mutations in mutagenic sequence segments.

Keywords: Mutant screening, Dpn I digestion, Critical annealing temperature-PCR, Site-directed mutagenesis

Background

PCR-based one-step site-directed mutagenesis (SDM) is an efficient and rapid method to generate gene mutation(s) for study of protein structure-function relationship, identification of gene expression and modification of vector [1-3]. Since established by Agilent Technologies according to Papworth et al. [4] and Nelson et al. [5], the QuikChange™ Site-Directed Mutagenesis System has been modified by a number of authors to make its procedure more simple and operable [6-11]. The principle of this technique is taking the double-stranded DNA of a target gene as the template and using a pair of overlapping oligonucleotide primers containing desired mutation to produce mutants by PCR. There are two concerns about this technology. Firstly, the number of cycles during PCR should be reduced to prevent undesired mutations; the resultant lower product yield can be offset by increasing the starting template concentration. Secondly, the parental (WT) DNA existing in final PCR products must be removed. For this, a mutant selection by digestion of Dpn I endonuclease is usually performed after PCR. However, although Dpn I (target sequence 5-GAAMTC) can quickly digest fully methylated DNA (parental strands from bacterial strains), its reaction with hemi-methylated DNA (parental strand combined with PCR-generated strand) is 60-fold slower. Thus the final PCR mixture inevitably contains small amount of parental molecules. In addition, Dpn I digestion cannot select against undesired mutations. Here, the present study designed a novel critical annealing temperature (Tc)-PCR method for more perfect mutant distinguishing, which can be used without Dpn I digestion. The Tc is derived from a regression equation, which can select against parental molecules up to a rate of 100% and undesired mutations located in mutagenic sequence segments.
Methods

Plasmid template

The plasmids used as templates for PCR were constructed using pET20b (+) (69739-3; Novagen, Germany) and pcDNA3.1(+) (V790-20, Invitrogen). The cDNA of different genes with different length, including human growth hormone (hGH) without signal peptide (NM_000515.3; 573 bp), hGH 2 variant isoform 3 precursor (hGH2V3, NM_022558.3; 738 bp), hGH receptor (hGHR; NM_001634.3, 1920 bp), porcine GH binding protein (pGHBP, NM_214254.2, 570 bp) or hGH binding protein (hGHBP, NM_000163.4, 714 bp), was inserted into either pET20b (+) between Nde I and Hind III, or pcDNA3.1 (+) between Pst I and Eco RI. The plasmids were propagated in E. coli DH5alpha cells (Agilent Technologies, CA) and isolated using Qiagen miniprep kits (Qiagen, Germany).

Primers and site-directed mutagenesis

The primers for mutagenesis by PCR were designed basically according to the manufacturer (QuickChange™ Mutagenesis kit; Agilent Technologies, CA) but a modification was made according to Braman, et al. [4] and Liu, et al. [6] to reduce initial concentration of template DNA. Briefly, each pair of primers contained a non-overlapping sequence at the 3'-terminus and a primer-primer complementary (overlapping) sequence at the 5'-terminus. The non-overlapping sequence was significantly larger than the overlapping to make the melting temperature of the former higher than that of the latter. The designed primers listed in Table 1 were used for mutagenesis of the target residues D112G, D112K, I4V, L45D, E56D, R64D, R64M, N109Y and D116F in hGH molecule. All mutation sites were located in the complementary region, 3-7 bases

Table 1 The primers for site-directed mutagenesis

| Primer         | Sequence                                      | Length | Mismatches | Tm  |
|----------------|-----------------------------------------------|--------|------------|-----|
| D112G(F)       | CTATGgCCTCTAAAGGACCTAGAGGAAG                  | 29     | 1          | 76.0|
| D112G(R)       | GGAGGcCATAGACGTTGCTGACAG                    | 26     | 1          | 75.3|
| D112K(F)       | CTAaAaCTCTAAAGGACCTAGAGGAAG                  | 29     | 2          | 68.3|
| D112K(R)       | GGAGTtrATAGACGTTGCTGACAG                    | 26     | 2          | 66.8|
| I4V(F)         | CAACCgTTCCCTTATCCAGGCTTTTG                 | 27     | 1          | 72.5|
| I4V(R)         | GAAcGGTGGAGAGAACATATGTATATCTCTCTCTCT       | 32     | 1          | 73.9|
| L45D(F)        | CATTCgatCAGAACCCCCAGACCTCTCTCTTGTTCT       | 36     | 3          | 76.1|
| L45D(R)        | GGGTGCTgcATGATGACTTCTTGCTCTTTCGGAT          | 37     | 3          | 71.8|
| E56D(F)        | TCGAgcTCTATCCGACACCTCCTCAAC                | 27     | 1          | 74.1|
| E56D(R)        | GAAAGAgTCTGAGAAACAGAGGAGAGTCTCG            | 31     | 1          | 76.3|
| R64D(F)        | CTCACAcgAGGAGAAAACACAC                          | 25     | 3          | 63.8|
| R64D(R)        | CTCgtrGTGGAGGGTGTCGGAAAG                    | 27     | 3          | 69.7|
| N109Y(F)       | CAGCgAgtCTATGACCTCTTAAAGGAGCTAGAGGAAG      | 38     | 2          | 77.9|
| N109Y(R)       | GCAcTaGcCTGAGACGAGGCCCTACACC                | 30     | 2          | 76.9|
| D116F(F)       | AAAGttCTAGAGGAGAGCATACACACAC                | 33     | 2          | 73.6|
| D116F(R)       | CTCACACgATACGAGGAGAAAACACAC                          | 38     | 2          | 77.9|
| R64M(F)        | TCCACACgAGGAGAAAACACAC                          | 24     | 1          | 68.0|
| R64M(R)        | TCCaTGTGGAGGGTGTCGGAAAG                    | 25     | 1          | 71.8|
| hGH2V3-P233S(F)| TGGaCTGAGGAgGgTGCTACTCCAGTGGCCACAGCC         | 36     | 3          | 80.6|
| hGH2V3-P233S(R)| GAGTTACgCCTTCACgCAGGAGGAGGACTTGGG           | 35     | 3          | 79.4|
| hGHBp-S237(C(F)| GATGkGCATAGAATTGCGGCTCTGGCAAA               | 31     | 1          | 76.3|
| hGHBp-S237(C)(R)| TTACGCATCTGGAGGAGGTCTACATAGAGCC            | 34     | 1          | 76.8|
| pGHBp-S237(C(F)| GATGkGCATAGAATTGCGGCTCTGGCAAA               | 31     | 1          | 76.3|
| pGHBp-S237(C)(R)| TTACGCATCTGGAGGAGGTCTACATAGAGCC            | 34     | 1          | 76.8|
| hGHR1-255R235C(F)| AACAAAttgAACTCTGGGAAATTATGGGC          | 26     | 3          | 59.8|
| hGHR1-255R235C(R)| TTGATgTTGGGGGCTCTACACA                | 21     | 3          | 58.7|
| hGHR1-638R235C(F)| AACAAAttgAACTCTGGGAAATTATGGGC          | 26     | 3          | 59.8|
| hGHR1-638R235C(R)| TTGATgTTGGGGGCTCTACACA                | 21     | 3          | 58.7|

\(T_m\) is the melting temperature of mutagenic oligonucleotide primers annealing with the WT template to 50%.

For all primers, the mutagenesis oligonucleotides are denoted in lowercase.
away from the 5'-terminus. Thus, both forward and reverse primers shared the mutagenic region, but held a 9-17 base-long overlapping sequence. The total length of the primers varied from 21 to 38nt. The melting temperature ($T_m$) was calculated using the formula provided by the manufacturer Agilent Technologies: $T_m = 81.5 + 0.41(\% GC) - 675/N - \% mismatch$. Here, $N$ is the primer length in bases. All the primers were synthesized by Generay Biotechnology (Shanghai, China). Mutagenic reaction was performed in 50 ul of PCR mix containing 2 ng of pET20b (+)-hGH (WT) as template, 200 nM primer and 2.5 U Pfu DNA polymerase (Fermentas, Canada). The PCR temperature profile was: an initial denaturation at 94°C for 3 min, followed by 30 cycles with each at 94°C for 30 sec, annealing at the desired temperature for 30 sec, extension for 2 kb/min and a final extension at 72°C for 5 min. The PCR products of SDM were transformed into E. coli DH5alpha competent cells.

**Mutant screening by gradient PCR**

For a more efficient mutant screening, a $T_c$-PCR with the SDM-generated mutants as templates and the same mutagenic primer pairs was designed to replace the traditional *Dpn I* digestion. The $T_c$, which can select against mismatched parental and undesired mutant templates from completely matched desired mutants was identified by a set of gradient-PCR with the generated clones from SDM and the same primer pairs listed in Table 1. Five clones were randomly collected from the transformants of each mutant and underwent PCR with the WT template as criteria. Six different annealing temperatures were designed according to the $T_m$ of the primers bound to the WT template. Among these 6 tested temperatures, the annealing temperature at which there was still substantial amplification of the mutant template but no detectable products of the WT template was identified as the $T_c$. The PCR was performed in a final volume of 20 μl PCR mix containing 20 ng template, 200 nM of each primer, 2 μl 10×reaction buffer (37.5 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 2 mM Mg²⁺, 0.01% Tween20) and 2U Taq DNA polymerase. The temperature profile was: initial denaturation at 94°C for 3 min followed by 35 cycles with each at 94°C for 30 sec, annealing at the desired temperature for 30 sec, extension for 2 kb/min and a final extension at 72°C for 5 min. The PCR products of SDM were transformed into E. coli DH5alpha competent cells.

**Results**

**Critical annealing temperature identified by gradient-PCR**

Figure 1A showed the results of mutant screening by gradient PCR using D112K forward and reverse primers which contained two designed mutations. At the gradient annealing temperatures from 64 ~ 69°C, the WT template, as well as two clones (D112K-1 and D112K-3) out of five randomly collected from the transformants without *Dpn I* digestion, showed detectable products only at annealing temperatures of 64 and 65°C, whereas the other three clones (D112K-2, D112K-4 and D112K-5) maintained detectable up to 67°C or higher. Thus the $T_c$ of D112K was identified as 66°C. Sequence analysis (Figure 1B) demonstrated that the two clones, D112K-1 and D112K-3, which lost detectable products at $T_c$ (66°C) were WT molecules and the other three were the desired mutants (GAC was changed into AAA).

Figure 1C showed the gradient PCR screening for a single-site-mutant, D112G. The WT template and three clones (D112G-1, D112G-3 and D112G-5) lost detectable products at 68°C, whereas the other two (D112G-2 and 4) maintained detectable over 68°C; this annealing temperature was therefore identified as the $T_c$ of D112G. Similarly, DNA sequence analysis demonstrated that the three clones becoming undetectable at the $T_c$ were WT molecules and the other two (D112G-2 and 4) still detectable at or over $T_c$ were desired mutants.
The gradient-PCR identified T_c for all tested primers were listed in Table 2.

### Mutant selection against WT and undesired mutants by critical annealing temperature PCR

Figure 2 showed the results of PCR screening with the gradient-PCR-identified T_c as annealing temperature and sequence analysis on 16 transformants of D112K and D112G after SDM without Dpn I digestion. At annealing temperature of T_c (66°C for D112K and 68°C for D112G), the clones that showed substantial PCR products were all desired mutants as demonstrated by DNA sequencing; whereas those that lost detectable products were either WT or undesired mutants that had “mistake” located in mutagenic sequence segments.

T_c-PCR screening and sequence analysis on all tested clones of D112K and D112G were shown in Table 3.

### Reliability of critical annealing temperature PCR at different initial concentrations of template

To test the reliability of T_c-PCR at different initial concentrations of template, both WT and mutant plasmids (10-80 ng in 50 ul reaction volume) of D112K and D112G were used for PCR with T_c as annealing temperature. As shown in Figure 3, in the WT templates no detectable products could be observed at all tested concentrations, but in the mutant templates the PCR bands were detectable at 10 ng, and enhanced with concentrations from 10 to 50 or 60 ng.

### Relationship between gradient PCR-identified T_c and T_m

The correlation analysis on relationship of gradient PCR-identified T_c to T_m and other features of the primers (Table 2) showed that the PCR-identified T_c of 9 tested primer pairs of hGH mutants were correlated with their T_m (r = 0.804, P<0.0001). The relationship between T_c and T_m were statistically modeled with the stepwise linear regression. Taking T_m as the explanatory variable, the linear regression model for T_c and T_m was expressed as the following regression equation:

\[ T_c = 48.81 + 0.253 \times T_m \]

Here, T_m is the melting temperature of mutagenic primer pairs with WT template.

To test the repeatability of the regression equation-derived T_c,a series of gradient PCR were run with different target genes (hGH-D112K, hGH-D112G, hGH2V3-P233S, hGHR-R255C, hGHBP-S237C and pGHBP-S237C). As a representative, hGH2V3-P233S contained 3 designed mutations. According to the regression equation above, the predicted T_c should be: T_c_F (forward primer) = 69.21°C and T_c_R (reverse primer) = 68.91°C. Thus, a set of annealing temperatures were selected from 68 to72°C. As shown in Figure 4 The WT template (pcDNA3.1-hGH2V3) lost detectable products at 70°C, whereas the mutant template (pcDNA3.1-GH2V3-P233S) maintained
Table 2 Features of the primers and their gradient-PCR identified-\(T_c\)

| Primer               | Length | Mismatches | %GC | Nt3* | Nt5Δ | \(T_m\) | \(T_c\) |
|----------------------|--------|------------|-----|------|------|---------|---------|
| D112G(F)             | 29     | 1          | 51.7| 23   | 5    | 76.0    | 68      |
| D112G(R)             | 26     | 1          | 57.7| 20   | 5    | 75.3    | 68      |
| D112K(F)             | 29     | 2          | 41.4| 22   | 4    | 68.3    | 66      |
| D112K(R)             | 26     | 2          | 46.2| 19   | 4    | 66.8    | 66      |
| I4V(F)               | 27     | 1          | 48.1| 21   | 5    | 72.5    | 67      |
| I4V(R)               | 32     | 1          | 40.6| 28   | 3    | 73.9    | 67      |
| L45D(F)              | 36     | 3          | 52.8| 28   | 5    | 76.1    | 67      |
| L45D(R)              | 37     | 3          | 40.5| 27   | 7    | 71.8    | 67      |
| E56D(F)              | 27     | 1          | 51.9| 21   | 5    | 74.1    | 67      |
| E56D(R)              | 31     | 1          | 48.4| 23   | 7    | 76.3    | 67      |
| R64D(F)              | 25     | 3          | 52.0| 15   | 7    | 63.8    | 65      |
| R64D(R)              | 27     | 3          | 59.3| 20   | 4    | 69.7    | 65      |
| N109Y(F)             | 38     | 2          | 47.4| 31   | 4    | 77.9    | 69      |
| N109Y(R)             | 30     | 2          | 60.0| 24   | 3    | 76.9    | 69      |
| D116F(F)             | 33     | 2          | 45.5| 27   | 4    | 73.6    | 69      |
| D116F(R)             | 38     | 2          | 52.0| 29   | 7    | 77.9    | 69      |
| R64M(F)              | 24     | 1          | 47.4| 16   | 7    | 68.0    | 67      |
| R64M(R)              | 25     | 1          | 40.5| 21   | 3    | 71.8    | 67      |
| hGH2V3-P233S(F)      | 36     | 3          | 63.9| 19   | 3    | 80.6    | 70      |
| hGH2V3-P233S(R)      | 35     | 3          | 62.9| 17   | 4    | 79.4    | 70      |
| hGHBP-S237C(F)       | 31     | 1          | 48.4| 26   | 4    | 76.3    | 69      |
| hGHBP-S237C(R)       | 34     | 1          | 44.1| 28   | 5    | 76.8    | 69      |
| pGHBP-S237C(F)       | 31     | 1          | 48.4| 26   | 4    | 76.3    | 69      |
| pGHBP-S237C(R)       | 34     | 1          | 44.1| 28   | 5    | 76.8    | 69      |
| hGHR1-255R235C(F)    | 26     | 3          | 38.5| 18   | 5    | 59.8    | 60      |
| hGHR1-255R235C(R)    | 21     | 3          | 38.1| 16   | 2    | 58.7    | 60      |
| hGHR1-255R235C(F)    | 26     | 3          | 38.5| 18   | 5    | 59.8    | 60      |
| hGHR1-255R235C(R)    | 21     | 3          | 38.1| 16   | 2    | 58.7    | 60      |

* The number of bases from 3'-terminus to the mutation site.
Δ The number of bases from 5'-terminus to the mutation site.

Figure 2 \(T_c\)-PCR screening and sequence analysis on 16 clones of D112K and D112G after SDM. The \(T_c\) is 66°C for D112K and 68°C D112G. The sequencing results are represented by M (desired mutant), W (wild type) and U (undesired mutant), respectively. N.C and W.C represent negative and WT controls, respectively.
substantial products at 70°C. The PCR-identified \( T_c \) (70°C) was very close to the equation predicted (\( T_c = 69.21 \)°C and \( T_{cr} = 68.91 \)°C). Similarly, in the tests using other different genes with different length, the PCR-identified \( T_c \) were also very close to the equation predicted \( T_c \) (shown in Figure 4).

Critical annealing temperature PCR screening with bacterial colonies as templates or with different sources of Taq DNA Polymerase

When using the bacterial colonies containing the desired plasmid (PET20b(+)/hGH-D112K and -D112G) as templates, a \( T_c \) could be identified by a gradient PCR and the PCR-identified \( T_c \) (66°C and 68°C) were also very close to the equation predicted (D112K: \( T_{cf} = 66.09 \)°C and \( T_{cr} = 65.72 \)°C; D112G; \( T_{cf} = 68.04 \)°C and \( T_{cr} = 67.86 \)°C) (Figure 5 and Table 2). However, the present experiment suggests that the initial denaturation time should be increased to 7 min to enhance denaturation of the plasmids in E. coli DH5alpha cells.

A similar result was also obtained when we repeated the study with the enzymes from different batches or different companies, such as recombinant Taq DNA Polymerase (Fermentas, Canada), Taq DNA Polymerase (Takara, Japan), and 2 × Taq master mix (Dsbio, Peking).

Discussion

Distinguishing desired mutants from parental template and undesired mutations in SDM is still a problem not well solved. Although as a favorite method, digestion of \( Dpn \) I can eliminate fully methylated WT DNA (parental strands from bacterial strains), around 20-30% of hemi-methylated WT molecules (parental strand combined with PCR-generated strand) could not be removed [6,8] due to hemi-methylated DNA in the PCR products is more resistant to \( Dpn \) I [3,12]. In addition, \( Dpn \) I cannot select against undesired mutations.

Liu and Naismith [6] recently presented a strategy to minimize the parental molecules by enhancing the PCR efficiency. In their design scheme, each pair of primers contained non-complementary sequences at their 3’ terminus and primer-primer complementary sequences at the 5’ terminus. The mutation sites were placed in the complementary region. The non-overlapping sequences were longer than the complementary sequences so that they have a \( T_m \) higher than that of primer-primer complementary sequences. Thus the primer dimerization was eliminated and the new synthesized PCR products could be used as the templates in the subsequent PCR cycles. This design can significantly increase the PCR efficiency and requires less template DNA.

On the basis of Liu and Naismith, we designed a modified protocol in which the \( Dpn \) I digestion was replaced by a \( T_c \)-PCR that could select against parental molecules and undesired mutations in mutagenic sequence segments at an accuracy of 100%. In this PCR the primers were the same used to introduce the mutations and the templates were SDM-generated clones. Thus theoretically the primers completely match the desired mutant and should have an annealing temperature to the mutant higher than to any other mismatched templates such as parental molecules and undesired mutants. Since DNA chemosynthesis is much more error-prone than DNA replication by polymerase [13-16], it is important to eliminate the undesired mutants generated by mistake primers. The key for this PCR screening is a

![Figure 3](http://www.biomedcentral.com/1472-6750/13/21)
critical annealing temperature ($T_c$) that can discriminate mismatched templates from the matched desired mutant. Using a gradient PCR, we identified the $T_c$ for each mutant template and demonstrated that it was correlated to the primer’s $T_m$. Thus, in practical applications, the $T_c$ can be easily calculated with a regression equation, $T_c = 48.81 + 0.253 \times T_m$.

The reliability of the $T_c$-PCR screening was confirmed by using different vectors harboring different genes (ranging from 573 bp to 1920 bp) and by employing Taq DNA polymerase from different sources.

**Figure 4** Verification of regression equation-derived $T_c$ by gradient PCR with different genes. As showed in the figure, the $T_c$ identified by gradient PCR (hGH2V3-P233S: 70°C, pGHBP-S237C: 69°C and hGHR-R235C: 60°C) were very close to those derived from the regression equation (hGH2V3-P233S: $T_{cF} = 69.21$ and $T_{cR} = 68.91$; pGHBP-S237C: $T_{cF} = 68.54$°C and $T_{cR} = 68.31$°C; and hGHR-R235C: $T_{cF} = 59.81$°C and $T_{cR} = 60.53$°C). Although hGHR-R235C was inserted into pcDNA3.1(+) (5428 bp) differing from pET20b(+) (3716 bp), the $T_c$ from PCR was almost same as that from the equation predicted.

**Figure 5** $T_c$-PCR screening with bacterial colonies as templates or with Taq DNA polymerase from different sources. A. Colony gradient PCR performed at indicated annealing temperatures using bacterial colonies carrying hGH WT or hGH mutant (D112K or D112G) plasmid to detect their $T_c$. B. Twenty colonies screened at $T_c$ after SDM. The sequencing results are represented by M (desired mutant) and W (wild type), respectively. C. Gradient PCR at the indicated annealing temperatures with Taq DNA Polymerases from different companies, such as Fermentas and Takara.
polymerases from different sources. Furthermore, the feasibility of the $T_c$-PCR as a colony PCR was tested with bacterial colonies containing the desired plasmid. These suggest that the $T_c$-PCR can have a wider usage.

Conclusions

In summary, the new protocol presented in the present study introduced a $T_c$-PCR screening to replace the $Dpn$ I digestion, which can more efficiently and accurately select against parental molecules and undesired mutants which have a “mistake” in mutagenic sequence segments.

Abbreviations

WT: Wild type; SDM: Site-directed mutagenesis system; $T_c$-PCR: Critical annealing temperature-PCR.

Competing interests

The authors have declared that no competing interests exist.

Authors’ contributions

Y L designed the study, carried out all experiments, acquired and analyzed the data, drafted and revised the manuscript; T W analyzed the data and performed the statistical analysis; X C and Y Z carried out the site-directed mutagenesis and gradient PCR; J S participated in the design of the study; Y L designed the study, carried out all experiments, acquired and analyzed the data, drafted and revised the manuscript; Y W designed the study, analyzed the data, drafted and revised the manuscript; T W analyzed the data and revised the manuscript, supervised and supported the study. All authors read and approved the final manuscript.

Acknowledgements

This study was funded by The National Natural Science Foundation of China (30870924, Wan Yu).

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Received: 23 August 2012 Accepted: 19 February 2013 Published: 11 March 2013

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