Chapter 5

Tailor-Made Protein Tyrosine Phosphatases: In Vitro Site-Directed Mutagenesis of PTEN and PTPRZ-B

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Abstract

In vitro site-directed mutagenesis (SDM) of protein tyrosine phosphatases (PTPs) is a commonly used approach to experimentally analyze PTP functions at the molecular and cellular level and to establish functional correlations with PTP alterations found in human disease. Here, using the tumor-suppressor PTEN and the receptor-type PTPRZ-B (short isoform from *PTPRZ1* gene) phosphatases as examples, we provide a brief insight into the utility of specific mutations in the experimental analysis of PTP functions. We describe a standardized, rapid, and simple method of mutagenesis to perform single and multiple amino acid substitutions, as well as deletions of short nucleotide sequences, based on one-step inverse PCR and DpnI restriction enzyme treatment. This method of SDM is generally applicable to any other protein of interest.

Key words Site-directed mutagenesis, PCR, Protein tyrosine phosphatase, PTEN, PTPRZ-B, PTPRZ1

1 Introduction

The directed alteration of cDNA sequences to modulate protein function in in vitro or in vivo experimental settings constitutes one of the basic approaches to establish, at the molecular and cellular level, protein structure-activity relationships governing biological processes. Together with structural and functional studies, SDM applied to relevant proteins has emerged as an indispensable technique to test and validate experimental hypothesis and to characterize intrinsic protein biological activities, as well as to define the mechanism of action of specific drugs. Site-directed mutagenesis is especially well suited for studying the biological activities of enzymes, since protocols for the readout of activity are usually available. In the case of PTPs, different experimental approaches exist to study in vitro PTP biological properties, including catalytic activity towards different substrates, sensitivity to inhibitors or redox regulatory agents, and binding to ligands, substrates, and

Rafael Pulido (ed.), *Protein Tyrosine Phosphatases: Methods and Protocols*, Methods in Molecular Biology, vol. 1447,
DOI 10.1007/978-1-4939-3746-2_5,

regulatory proteins, among others [1–9]. In this regard, mutations at the PTP active site have been used to engineer binding sites for specific inhibitors [10], as well as to obtain antibodies that specifically recognize the oxidized conformation of the PTP and keep the enzyme in its inactive state [11].

All Cys-based PTPs follow a two-step enzymatic mechanism that starts with a nucleophilic attack on the phospho-substrate by the catalytic Cys (located on the CxxxxxR signature motif-containing catalytic P-loop), which is aided by a general acid donating-proton residue, generally an Asp (located in the WPD-loop), to release the dephosphorylated substrate. This generates a characteristic phospho-enzyme intermediate. In a second step, a water molecule is deprotonated by the catalytic Asp (which now acts as a general base) and the remaining hydroxyl group attacks the P-O thiol-phosphate bond from the phospho-enzyme intermediate, releasing the phosphate moiety [12–16]. The conservative substitution of the catalytic Cys by a Ser (C/S mutation) is the standard enzyme-inactivating mutation used in PTP studies [17]. Importantly, C/S PTP mutations may act as dominant negative PTP variants, likely by binding to substrates (without dephosphorylating them) or by competing with effectors [18–22]. The substitution of the catalytic Asp by an Ala (D/A mutation) renders an enzyme with very low activity, which usually binds to substrates with higher affinity than the C/S mutation, being referred as a substrate-trapping mutation [23]. Substrate-trapping D/A mutations have been used successfully to identify *bona fide* substrates of different PTPs, alone [24–28] or in combination with the C/S mutation [29]. A list of residues important for catalysis of PTPs, using PTPN1/PTP1B as a model, is provided in reference [30].

Protein-protein interactions constitute a major regulatory mechanism in the control of PTP biological activity, not only by conferring substrate specificity but also by regulating the compartmentalization and subcellular location of PTPs. This is aided by the existence of a domain modular organization on many PTPs that specifically orchestrate their differential binding to effectors and regulators [31–33]. In addition, linear C-terminal amino acid motifs that represent canonical binding sites for PDZ protein domains are present in some PTPs, including PTEN (-Thr-Lys-Val; -TKV motif) and PTPRZ-B (-Ser-Leu-Val; -SLV motif), as well as in some myotubularins. PDZ domains exist in many scaffolding and regulatory proteins, and are considered as major elements in the compartmentalization of the cell at the molecular level, with important implications in human disease [34, 35]. PDZ-domain binding to protein partners can be experimentally manipulated by PDZ-domain rearrangements, as well as by mutation of the residues from the PDZ-binding motif on the protein partners [36, 37].

PTEN is a lipid- and protein-phosphatase that controls cell homeostasis. PTEN behaves as a major tumor-suppressor protein

in humans by dephosphorylating PIP3, the product of the oncogenic PI3K, and PTEN functional alterations are strongly associated with human disease, especially cancer. *PTEN* gene is a frequent target of mutations in human tumors, and patients with PTEN hamartoma tumor syndrome (PHTS) or with autism spectrum disorders (ASD) carry *PTEN* germ-line mutations [38–42]. Thus, the potential to introduce PTEN mutations for experimental purposes is required to study PTEN functionality or effects of PTEN disease-associated mutations [43, 44]. PTPRZ-B is a receptor-type PTP that is encoded by gene *PTPRZ1* and that is mainly (but not exclusively) expressed in the nervous system. *PTPRZ1*-encoded protein isoforms are involved in the regulation of cell-cell and cell-extracellular matrix interactions, and display both tumor suppressive and oncogenic properties [45–50]. In addition, *PTPRZ1* isoforms are involved in myelination processes [51–53], and they have been proposed as a target for Parkinson's disease and schizophrenia [54, 55]. Both PTEN and PTPRZ-B bind PDZ domains through their C-terminal PDZ-binding motifs, which impacts on their stability, subcellular location, and function [36, 56–59].

Mutations useful to study the functions of PTEN and PTPRZ-B in any cell system or experimental setting are illustrated in Table 1. These include specific mutations affecting the catalysis or the binding to PDZ domains of PTEN and PTPRZ-B, and may be extended to other PTPs. Using PTEN and PTPRZ-B cDNAs in different plasmid backgrounds, we describe a standardized, rapid, and simple mutagenesis method to obtain single and multiple amino acid substitutions, as well as short-length nucleotide deletions. The method is based on one-step inverse PCR followed by DpnI restriction enzyme treatment and direct *E. coli* transformation, and can potentially be applied to any other cDNA of interest.

Table 1
Amino acid substitution mutations of utility to study PTEN and PTPRZ-B function

Mutation	Location	Functional consequence	References
PTEN[a]			
C124S	PTP domain (P-loop)	Catalytically inactive (full)	[61–63]
G129E	PTP domain (P-loop)	Loss of lipid phosphatase activity	[64]
Y138L	PTP domain	Loss of protein phosphatase activity	[65]
D90A	PTP domain (WPD-loop)	Catalytically inactive; substrate trapping	[66]
V403A	C-terminal tail	Loss of PDZ-domain binding	[59]
PTPRZ-B[b]			
C1073S	PTP D1 domain (P-loop)	Catalytically inactive (full)	[57, 67]
D1041A	PTP D1 domain (WPD-loop)	Catalytically inactive; substrate trapping	[68]
S1453A	C-terminal tail	Loss of PDZ-domain binding	[58]
V1455A	C-terminal tail	Loss of PDZ-domain binding	Predicted

[a]Amino acid numbering corresponds to the major human PTEN isoform (NP_000305)
[b]Amino acid numbering corresponds to the short human PTPRZ-B isoform (NP_001193767)

2 Materials

All solutions are prepared in double-distilled, RNase-free water. Plasticware is autoclaved or sterilized by ethylene oxide.

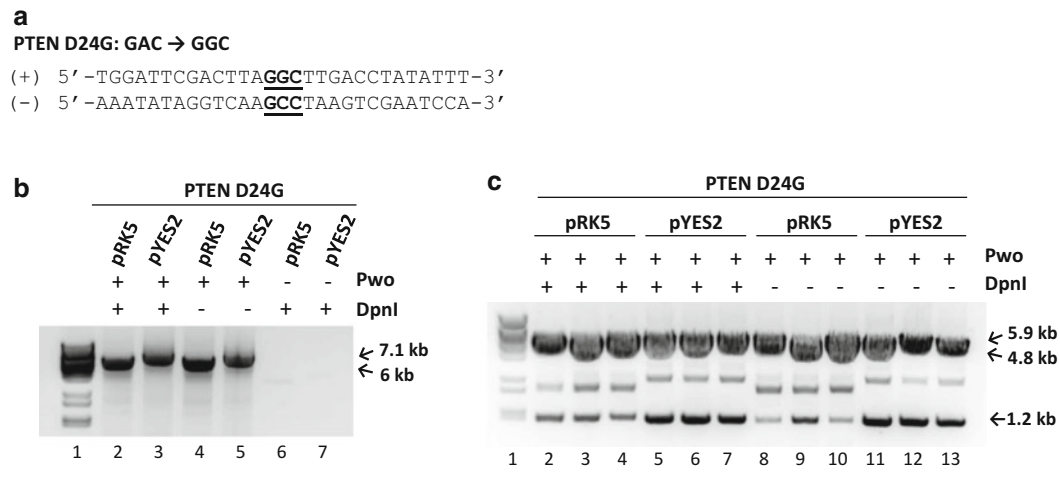
2.1 Site-Directed Mutagenesis of PTPs

1. Plasmid containing the PTP cDNA to be mutagenized (*see* **Note 1**).
2. Mutagenic oligonucleotide primers (*see* **Note 2**).
3. High-fidelity DNA polymerase and dNTPs (*see* **Note 3**).
4. DpnI restriction enzyme (target sequence 5′-Gm^{6}ATC-3′) (*see* **Note 4**).
5. *E. coli* competent cells (*see* **Note 5**).
6. TNE buffer: 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA.
7. 10× TCM buffer: 100 mM Tris–HCl pH 7.5, 100 mM $CaCl_2$, 100 mM $MgCl_2$.
8. LB medium: 1% Tryptone, 0.5% yeast extract, and 1% NaCl.
9. LB-Ampicillin (LB-Amp) plates: 1% Tryptone, 0.5% yeast extract, 1% NaCl, 0.01% NaOH, 1.5% agar, and 100 μg/ml ampicillin.
10. Plasmid DNA purification kit.
11. Agarose gel electrophoresis and DNA visualization reagents.
12. Thermocycler.
13. Shaking incubator.
14. Ultraviolet light detection system.

3 Methods

This mutagenesis method is based on the QuikChange™ Site-Directed Mutagenesis procedure, which consists of one-step inverse PCR from a methylated double-stranded DNA (dsDNA) plasmid followed by DpnI restriction enzyme treatment, eliminating the need for subcloning and single-stranded DNA (ssDNA) rescue. Our procedure utilizes a supercoiled dsDNA vector with the insert of interest, and synthetic oligonucleotide mutagenic primers with a standardized predesigned length (see below). The oligonucleotide primers are extended during temperature cycling by a high-fidelity DNA polymerase, which generates a non-methylated mutated nicked plasmid. The PCR product is directly treated with DpnI endonuclease to digest the parental methylated DNA template, followed by transformation into competent bacteria. Examples are provided for single-amino acid substitutions and

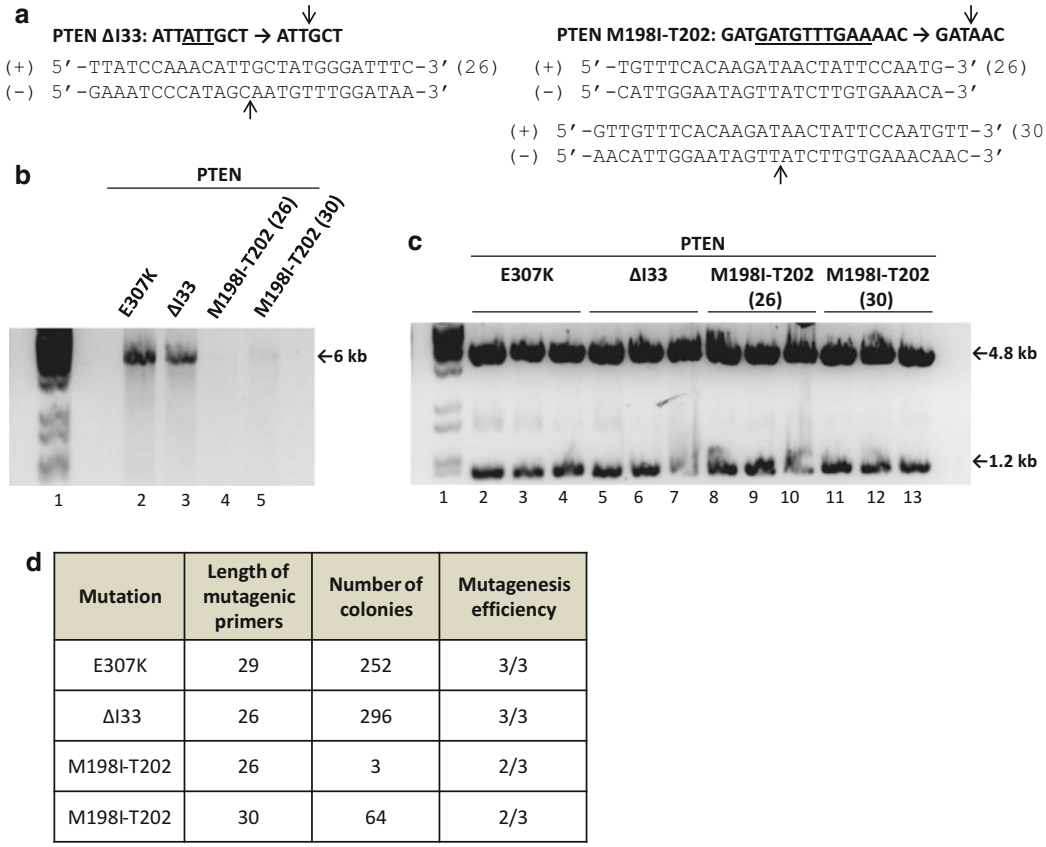
deletions on human PTEN (1.2 kb cDNA; NM_000314, NP_000305) (Figs. 1 and 2, Table 2) and for single and multiple-amino acid substitutions on human PTPRZ-B (4.3 kb cDNA; NM_001206838, NP_001193767) (Fig. 3). Note that for both single and multiple mutations we run a single PCR.



d

Mutagenesis conditions	Template plasmid	Number of colonies	Mutagenesis efficiency
+ Pwo + DpnI	pRK5-PTEN	198	3/3
	pYES2-PTEN	115	3/3
+ Pwo - DpnI	pRK5-PTEN	231	0/3
	pYES2-PTEN	172	0/3
-Pwo + DpnI	pRK5-PTEN	0	--
	pYES2-PTEN	0	--

Fig. 1 Monitoring of standardized site-directed mutagenesis using a PTEN amino acid substitution as an example. (**a**) Mutagenic primers of 29-mer predefined length (13 + 3 + 13, codon substituted *underlined*). The amino acid substitution (D24G) is indicated using the amino acid one-letter code. +, forward primer; –, reverse primer. (**b**) Linear DNA obtained on the mutagenic PCR, as resolved by 1 % agarose gel electrophoresis. pRK5 PTEN is 6 kb size, whereas pYES2 PTEN is 7.1 kb size. The parental circular DNA is in low amount and not visualized. Note the lack of PCR product in the absence of DNA polymerase (*lanes 6* and *7*). (**c**) Control restriction enzyme digestions (XbaI + SalI, cutting out the 1.2 kb PTEN insert) of plasmid DNA obtained from bacteria transformed with the mutagenic PCR product. Three colonies were analyzed from each condition. In *lanes 1* from (**b**) and (**c**), molecular markers (BstEII digestion of λ phage) are shown. (**d**) Efficiency of bacteria transformation with the mutagenic PCR product (number of bacteria colonies after transformation with the DpnI-treated PCR product) and mutagenesis efficiency (number of mutated samples with respect to the number of samples sequenced) achieved from each condition are indicated. Note that in the absence of DpnI, the parental template plasmid gives a background of non-mutated samples. In the absence of DNA polymerase (but presence of DpnI) no colonies should be obtained, as an indication of the efficiency of DpnI digestion



d

Mutation	Length of mutagenic primers	Number of colonies	Mutagenesis efficiency
E307K	29	252	3/3
ΔI33	26	296	3/3
M198I-T202	26	3	2/3
M198I-T202	30	64	2/3

Fig. 2 Monitoring of standardized site-directed mutagenesis using PTEN amino acid substitution and nucleotide deletions as examples. (**a**) Mutagenic primers of 26-mer or 30-mer predefined length (13 + 0 + 13 or 15 + 0 + 15, respectively, where 0 indicates the *underlined* deleted nucleotides). The *arrows* indicate the nucleotide junction after the deletion. The amino acid changes after the nucleotide deletion are indicated as follows: ΔI33, deletion of I33; M198I-T202, M198I substitution plus deletion of residues 199–201. +, forward primer; –, reverse primer. (**b**) Linear DNA obtained on the mutagenic PCR (pRK5 PTEN as template plasmid), as resolved by 1 % agarose gel electrophoresis. The E307K amino acid substitution (29-mer mutagenic primers) was included for comparison. (**c**) Control restriction enzyme digestions (XbaI + SalI, cutting out the 1.2 kb PTEN insert) of plasmid DNA obtained from bacteria transformed with the mutagenic PCR product. Three colonies were analyzed from each condition. In *lanes 1* from (**b**) and (**c**), molecular markers (BstEII digestion of λ phage) are shown. (**d**) Efficiency of bacteria transformation with the DpnI-treated PCR product, and mutagenesis efficiency are indicated. Note that two different set of primers (26-mer or 30-mer) were used for the M198I-T202 mutagenesis. Note that sometimes the amplified PCR product is barely detected [see *lanes 4* and *5* from panel (**b**)], but it is enough to obtain colonies with the desired mutation

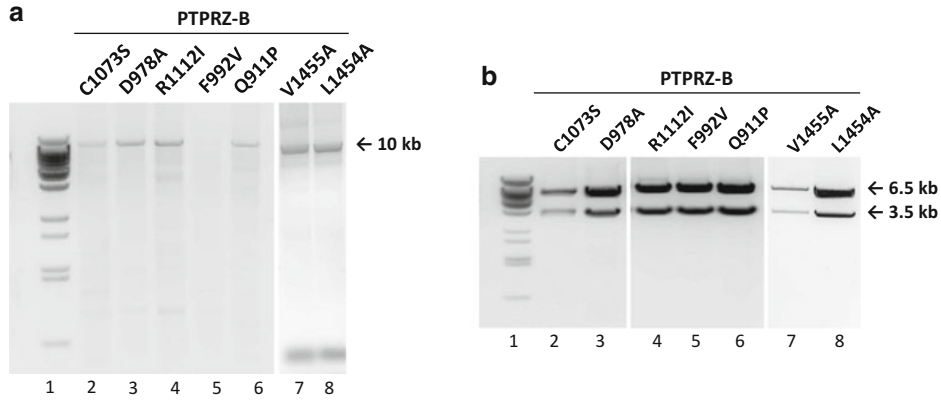
3.1 Site-Directed Mutagenesis of PTPs

1. Design the mutagenic oligonucleotide primers. In our protocol for amino acid substitutions, the mutagenic primers are two fully complementary primers of standardized predefined length (usually 29-mer), with the mutated codon in the center (Fig. 1a) (*see* **Note 2**) (Mingo et al., submitted). For deletions of short sequences of nucleotides, the design of mutagenic primers follows similar rules, with the junction of the deleted sequence in the center (Fig. 2a).

Table 2
Efficiency of amino acid substitution-standardized mutagenesis of PTEN[a]

Mutation[b]	GC content (%)	Tm[c]	Number of colonies	Mutagenesis efficiency
M1I (ATG → ATA)	48	75	22	1/1
K6E[d] (AAA → GAA)	48	75	12	1/1
K6I[d] (AAA → ATA)	45	74	8	1/1
N12T[e] (AAC → ACC)	41	72	12	1/1
R14G[d] (AGG → GGG)	41	72	20	1/2
D24G (GAC → GGC)	38	71	58	1/1
E43G (GAA → GGA)	48	75	11	1/1
I50T (ATT → ACT)	31	68	19	1/1
Y65C[e] (TAC → TGC)	34	69.5	8	1/1
Y68N[e] (TAC → AAC)	34	69.5	9	2/3
Y68H[e] (TAC → CAC)	38	71	15	1/1
Y68C[e] (TAC → TGC)	38	71	32	1/1
L70P[e] (CTT → CCT)	38	71	14	1/1
K80E[d] (AAA → GAA)	41	72	5	1/1
I101T[d] (ATC → ACC)	41	72	20	1/1
D107G[d] (GAT → GGT)	48	75	10	1/1
L112P (CTA → CCA)	45	74	10	1/1
H123R (CAC → CGC)	45	74	25	1/1
R130L (CGA → CTA)	45	74	38	1/1
G132D[d] (GGT → GAT)	38	71	32	1/2
R142P (CGG → CCG)	24	65	44	1/1
R159G[d] (AGG → GGG)	48	75	39	1/1
Q171E (CAG → GAG)	48	75	15	1/2
M205V (ATG → GTG)	45	74	19	1/1
D252V (GAT → GTT)	41	72	55	1/1
K254T[e] (AAA → ACA)	45	74	26	1/1
V255A (GTA → GCA)	41	72	100	1/1

[a]Mutagenesis was performed using the plasmid pYES2 PTEN as the template, and 29-mer-length mutagenic primers, as shown in Fig. 1a
[b]The nucleotide substitutions and the resulting amino acid changes (one-letter amino acid code) are indicated
[c]Tm was calculated according to the QuikChange™ manual (Agilent Technologies)
[d]One of the primers from the mutagenic primer pair has G or C in 3′ position
[e]Both primers from the mutagenic primer pair have G or C in 3′ position
Data are shown as in Fig. 1d



c

Mutation	Number of colonies	Mutagenesis efficiency
C1073S	13	2/2
D978A	50	1/2
R1112I	37	2/2
F992V	8	2/2
Q911P	15	2/2
V1455A	16	1/2
L1454A	37	2/2

Mutation	Number of colonies	Mutagenesis efficiency		
		D978A	C1073S	D978A/ C1073S
D978A/ C1073S	10	4/6	0/6	2/6

Fig. 3 Monitoring of standardized site-directed mutagenesis using PTPRZ-B amino acid substitutions as examples. (**a**) Linear DNA obtained on the mutagenic PCR (pCDNA3 PTPRZ-B as template plasmid, 10 kb size), as resolved by 1 % agarose gel electrophoresis. The amino acid substitutions are indicated using the amino acid one-letter code. (**b**) Control restriction enzyme digestions (XbaI, cutting out a 3.5 kb fragment of PTPRZ-B insert) of plasmid DNA obtained from bacteria transformed with the mutagenic PCR product. Two colonies were analyzed from each condition (see *left panel* c; digestion is only shown for one colony each). In *lanes 1* from (**a**) and (**b**), molecular markers (BstEII digestion of λ phage) are shown. (**c**) In the *left panel*, the efficiency of bacteria transformation with the DpnI-treated PCR product and mutagenesis efficiency are indicated, corresponding to the mutagenesis shown in (**a**). Note that sometimes the amplified PCR product is barely detected [see *lane 5* from panel (**a**)], but it is enough to obtain colonies with the desired mutation. In the *right panel*, data are shown for the simultaneous obtaining of two mutations at two distinct target sites, running a single PCR reaction with a mix of two pairs of mutagenic primers

2. Prepare the mutagenic PCR mix (25 μl): 14.25 μl H_2O, 5 μl template plasmid (at 5 ng/μl) (*see* **Note 1**), 1.25 μl dNTP mix (at 4×2.5 mM), 2.5 μl buffer Pwo 10× (Roche), 0.1 μl DNA polymerase Pwo (at 5 U/μl; Roche), and, in the case of a single mutation (one primer pair), 1 μl of each mutagenic primer (at 10 μM). In the case of multiple mutations (one primer pair/mutation), we keep constant the final concentration of primers preparing a mix of primer pairs from the 10 μM primer stocks (same volume each primer) and adding 2 μl of the mix to the mutagenic PCR mix.

3. Run the mutagenic PCR, using the following cycling conditions (*see* **Note 6**):
   - For PTEN mutagenesis: 1 min at 95 °C, followed by 18 cycles of 50 s at 95 °C (denaturation), 50 s at 60 °C (annealing), and 5 min at 68 °C (extension), and followed by a final 7-min extension at 68 °C and cooling at 4 °C.
   - For PTPRZ-B mutagenesis: 1 min at 95 °C, followed by 18 cycles of 50 s at 95 °C (denaturation), 50 s at 60 °C (annealing), and 7 min at 68 °C (extension), and followed by a final 7-min extension at 68 °C and cooling at 4 °C.
4. Transfer 10 μl of the PCR product to a new Eppendorf tube and add 0.15 μl of the DpnI restriction enzyme (20 U/μl) (*see* **Note 4**). Mix carefully the solution and incubate at 37 °C for 2–3 h (*see* **Note 7**). For long-term storage, keep the undigested PCR product at 4 °C, and the DpnI-digested PCR product at −20 °C (*see* **Note 8**).
5. *Optional*: Run 5–10 μl of the undigested PCR product on 1% agarose gel to monitor the efficiency of the PCR amplification. A linear DNA migrating as the size of the template should be detected (*see* **Note 9**).
6. Transform bacteria: Mix, in a 14 ml polypropylene round-bottom tube, 10 μl of TCM 10×, 10 μl of TNE, and 2 μl of the DpnI-treated PCR product (*see* **Note 10**). Keep on ice for 5 min; then, add 20 μl *E. coli* competent cells (*see* **Note 5**) and incubate on ice for 15–30 min. Heat-shock the mix by incubating the tube in a 42 °C water bath for 90 s, followed by incubation at 20 °C for 10 min. Add 300 μl of LB medium, and incubate under shaking at 37 °C for 80 min.
7. Plate the transformation mix on LB-ampicillin agar plates, air-dry, and incubate inverted at 37 °C for 16 h (*see* **Note 11**).
8. Pick individual colonies to inoculate 3 ml of LB-ampicillin and grow for 16 h at 37 °C under constant shaking.
9. Purify the plasmid DNA according to standard procedures and check the resulting plasmid for the presence of the desired mutation by restriction enzyme and DNA sequencing analysis (*see* **Notes 12** and **13**).

An example of an amino acid substitution mutagenesis on PTEN, using different template plasmids and a pair of 29-mer predefined length mutagenic primers, is provided in Fig. 1. These experiments illustrate the monitoring of the mutagenesis by analyzing the efficiency of the PCR (amount of amplicon and number of bacteria colonies after transformation) and of the mutagenesis (number of sequencing-confirmed mutated samples per number of samples sequenced). Examples of mutagenesis results obtained using different template plasmids (different vectors with PTEN

and PTPRZ-B cDNA inserts) and mutagenic primers are shown in Figs. 2 and 3. In all cases, the PCR product resolved by agarose gel electrophoresis is shown. Note that the amount of this amplified linear DNA correlates with the number of bacteria colonies obtained after transformation with the DpnI-digested PCR product. Data on the mutagenesis efficiency are also provided. In addition, an example of the simultaneous incorporation of two separate mutations in the PTPRZ-B cDNA (resulting in two different amino acid substitutions) is provided in Fig. 3c. Finally, examples from an extensive mutagenesis of PTEN are also given in Table 2.

4 Notes

1. Mutagenesis protocols coupled to digestion with DpnI restriction enzyme (target sequence 5′-Gm^{6}ATC-3′) require a Dam-methylated DNA plasmid template, which is produced by most of laboratory cloning *E. coli* strains (*dam*+).
2. In our standardized protocol, we use mutagenic primers of predefined length, irrespective of their Tm, GC content, and GC localization (Table 2) (Mingo et al., submitted). As a general rule, for single-amino acid substitutions, we use 29-mer mutagenic primers (13 + 3 + 13; 13 nucleotides flanking each side of the codon to be changed [underlined]). For deletion of one amino acid, we use 26-mer primers (13 + 0 + 13; where 0 indicates the deleted nucleotides). For larger nucleotide deletions we increase the length of the mutagenic primers (15 + 0 + 15) (see an example in Fig. 2). It is possible that for templates difficult to amplify, the design of the mutagenic primers needs further optimization. For simultaneous mutagenesis of several target sequences (multiple mutagenesis), we prepare a mix of all the mutagenic primer pairs and use the same final amount of total primers than for a single mutagenesis. In our experience, double and triple mutations are easily obtained by this procedure in a single PCR reaction.
3. High-fidelity thermostable DNA polymerases are required to avoid incorporation of undesired mutations. In addition, some thermostable DNA polymerases, such as Taq polymerase, add undesired "A-tails" in the PCR product. The examples shown here have been performed using Pwo DNA polymerase (Roche), but many other high-fidelity thermostable DNA polymerases are suitable.
4. The efficiency of the digestion of the PCR product with the DpnI restriction enzyme determines the amount of undesired parental plasmid that is transformed into bacteria, which produces wild-type (parental plasmid) colonies. This constitutes one of the key parameters of the mutagenesis efficiency.

Examples of control experiments to test DpnI efficiency are shown in Fig. 1. Note that in the absence of DNA polymerase no colonies should be obtained if the DpnI enzyme is fully efficient. The examples shown have been performed using DpnI from New England Biolabs, but many other DpnI enzymes are suitable.

5. We routinely use $CaCl_2$-competent DH5α *E. coli* cells [60] for direct transformation of the DpnI-digested PCR product. To make a stock of competent bacteria from 50 ml culture, we resuspend the final bacteria pellet in a volume of 2 ml $CaCl_2$ 0.1 M, and keep frozen at −80 °C in aliquots of 50 μl. Commercial ultra-competent bacteria, available from different vendors, are also suitable.
6. Optimization of PCR cycling conditions may be necessary if the template plasmid is of large size or difficult to amplify. As a general rule, 1 min of extension is recommended per kb of template size. Number of cycles can be increased, although this may raise the frequency of undesired mutations. In addition, the amount of parental template plasmid can be increased, although this may result (depending on the DpnI efficiency) in higher background from the parental plasmid (the transformation efficiency of the circular parental plasmid exceeds that of the linear PCR product) (*see* also **Note** 7).
7. It is advisable to test the efficiency of the DpnI digestion with different incubation times, taking into consideration the amount of template plasmid used (*see* Fig. 1). For convenience with time distribution, overnight digestion with DpnI can be performed, although nonspecific digestion will decrease the mutagenesis efficiency.
8. If required, the PCR product can be stored at 4 or −20 °C for some time before being treated with DpnI. Similarly, the DpnI-digested PCR product may be stored for longer periods at −20 °C before the transformation of bacteria is taken up.
9. In general, the number of colonies obtained from the mutagenesis is proportional to the amount of amplified DNA from the mutagenic PCR (monitored by agarose gel electrophoresis in **step 5**; the parental template plasmid is not detectable at these amounts). It is recommended to transform bacteria with the DpnI-digested PCR product even when the amplicon is not detectable on gel, since a small number of colonies is enough to obtain the desired mutation (*see* Figs. 2 and 3).
10. The amount of DpnI-digested PCR product to transform bacteria can be increased, although this may increase background due to the parental plasmid (depending on the DpnI efficiency; *see* also **Note** 7).

11. Our routine mutagenesis experiments yield 20–200 colonies after transformation with 2 μl of DpnI-digested PCR product. If no colonies are obtained, *see* **Notes 2**, **6**, and **10**.
12. Before sequencing, we routinely check by restriction analysis that the amplified DNA corresponds to the template plasmid.
13. The mutation efficiency under our standardized mutagenesis conditions is about 85 %. Sequencing of one to three colonies should be enough to obtain the desired mutation from a single mutagenesis experiment. In the case of multiple mutagenesis, take into consideration that a mix of individual and multiple mutations is likely to be obtained, necessitating the analysis of a larger number of colonies (*see* Fig. 3c, right panel).

Acknowledgements

This work was supported in part by grants SAF2013-48812-R from Ministerio de Economía y Competitividad (Spain), 2013111011 from Gobierno Vasco, Departamento de Salud (Basque Country, Spain), and BIO13/CI/001/BC from BIOEF/EITB maratoia (Basque Country, Spain) (to RP).

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