User Manual

Diversify® PCR Random Mutagenesis Kit User Manual

Clontech

United States/Canada
800.662.2566
Asia Pacific
+1.650.919.7300
Europe
+33.(0)1.3904.6880
Japan
+81.(0)77.543.6116

Clontech Laboratories, Inc.
A Takara Bio Company
1290 Terra Bella Ave.
Mountain View, CA 94043
Technical Support (US)
E-mail: tech@clontech.com
www.clontech.com

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I. Introduction

PCR-based random mutagenesis is widely used for analyzing wild-type protein function and creating proteins with new or improved functions (e.g., directed protein evolution). The procedure generally involves performing a PCR reaction under conditions that reduce the fidelity of nucleotide incorporation, cloning the resulting PCR fragments, and then screening the resulting library for novel mutations which affect protein activity (You *et al.*, 1994; Wan *et al.*, 1998; Melnikov *et al.*, 1999). PCR-based random mutagenesis has gained popularity over chemical methods (e.g., nitrous acid, hydroxylamine, etc.) since it produces higher levels and a larger variety of mutations (Fromant *et al.*, 1995). Additionally, PCR-based random mutagenesis has advantages over the use of nucleotide analogs because analogs have an increased bias for certain point substitutions and must be removed from PCR products prior to cloning.

The Diversify PCR Random Mutagenesis Kit offers a variety of buffer conditions for performing random mutagenesis, allowing you to adjust the reaction for a desired error rate. The kit is based on the methods of Leung *et al.* (1989) and Cadwell and Joyce (1992) and has been optimized for use with the Clontech TITANIUM *Taq* PCR system. The primary advantages of the kit are:

- *Controlled* random mutagenesis: the buffer conditions can be varied to achieve the desired level of random mutagenesis.
- Amplification of large PCR fragments: we have confirmed amplification of fragments up to 4 kb in length. Theoretically, longer fragments can be mutated and amplified as well.
- High DNA yields using the TITANIUM *Taq* PCR system, which outperforms other PCR systems under the stress of error-prone PCR conditions. TITANIUM *Taq* includes TaqStart Antibody for automatic hot-start PCR.
- Wide mutational diversity: produce transition and transversion mutations.
- A rapid *in vitro* control reaction (patent pending) that allows you to confirm random mutagenesis of a control template just 2 hrs after your PCR reaction.

Diversify PCR mutagenesis provides control over the level of random mutation by independently varying the amounts of manganese and dGTP in the PCR reaction (Figure 1). The mutagenesis rate is first raised by increasing the amount of manganese in the reaction (up to 640 µM). Further increases in mutation rate are obtained by increasing the level of dGTP in the reaction, while keeping the concentration of manganese constant. The kit has been designed to provide mutation rates from 2 to 8 mutations per 1,000 bp, making it applicable to large and small PCR products. Higher mutation rates can be produced by performing a second round of PCR using a diluted aliquot of the primary mutagenesis reaction (Shafikhani *et al.*, 1997).

I. Introduction *continued*

Three of the mutation rates shown in Figure 1 (buffer conditions 1, 5, and 9) were determined directly by DNA sequencing (see Appendix). The remaining, intermediate points were determined by correlating mutagenesis levels from a reproducible, quantitative *in vivo* fidelity assay (Mo *et al.*, 1991) with results from DNA sequencing.

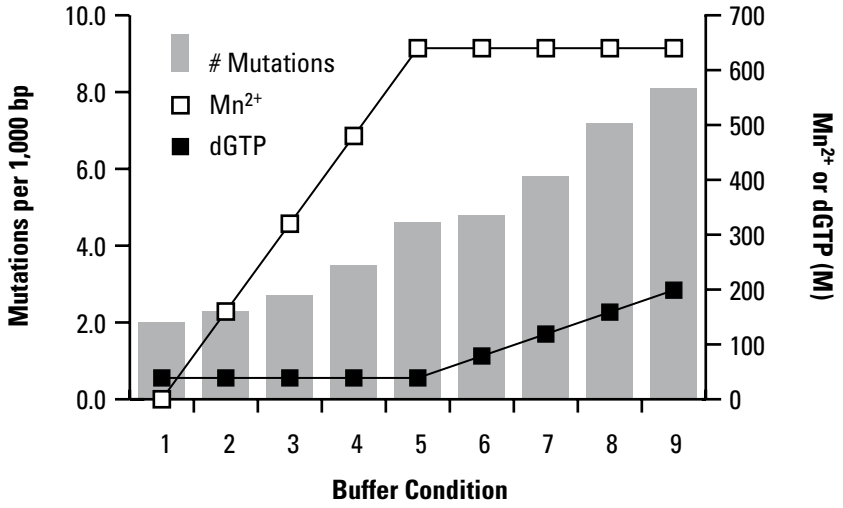

Figure 1. The level of Diversify® mutagenesis is controlled by varying the concentrations of manganese and dGTP. The concentrations of other dNTPs are held constant.

II. List of Components

Store all components at –20°C.

The following reagents are sufficient for 30 mutagenesis reactions of 50 µl each.

- **30 µl** **50X TITANIUM™ *Taq* DNA Polymerase** (includes TaqStart Antibody)

Concentration in 50X mix	Component	Final rxn concentration
50 %	Glycerol	1.0 %
20 mM	Tris-HCl (pH 8.0)	0.4 mM
100 mM	KCl	2 mM
0.1 mM	EDTA (pH 8.0)	2.0 µM
0.25 %	Tween-20	0.005 %
0.25 %	Nonidet P-40	0.005 %

- **200 µl** **10X TITANIUM™ *Taq* PCR Buffer**

Concentration in 10X mix	Component	Final rxn concentration
400 mM	Tricine-KOH (pH 8.0 at 25°C)	40 mM
160 mM	KCl	16 mM
35 mM	$MgCl_2$	3.5 mM
37.5 µg/ml	BSA	3.75 µg/ml

- **30 µl** **50X Diversify® dNTP Mix**
- **40 µl** **50X dNTP Mix** (standard dNTP mix; 10 mM each of dATP, dCTP, dGTP, and dTTP)
- **150 µl** **dGTP** (2 mM; final concentration variable from 40 to 200 µM)
- **120 µl** **Manganese Sulfate** ($MnSO_4$; 8 mM; final concentration variable from 0 to 640 µM)
- **10 µl** **Control PCR Template** (~1ng/µl)
- **10 µl** **Control Primer Mix** (10 µM each)

  Forward Primer: 5'–GAGCCTATGGAAAAACGCCAGCAAC–3'
  Reverse Primer: 5'–GCAAAAAAGGGAATAAGGGCGACAC-3'

- **10 µl** ***Taq* I Restriction Enzyme** (20 units/µl)
- **1.25 ml** **PCR Grade Water**

III. Additional Materials Required

The following reagents are required but not supplied.

- [optional] **Mineral oil** (We recommend Sigma Cat. No. M-3516.)
- **PCR reaction tubes**
- **Thermal cycler** (Hot-lid or non-hot-lid thermal cycler)
- **Dedicated pipettors**
- **PCR pipette tips** suitable to the above pipettors and preferably equipped with hydrophobic filters.
- **DNA size markers** (1 kb ladder or equivalent)
- **10X gel loading buffer** (Sambrook & Russell [2001] provides several recipes.)

IV. General Considerations

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. Template and Primer Design

Although the PCR reaction has been optimized for mutagenizing a 1 kb sequence, longer templates can be amplified by increasing the extension time as described in Section V.A. Dilute template to ~1 ng/µl before use.

Primer design is the single largest variable in PCR applications and the single most important factor in determining the success or failure of PCR reactions. Always check and recheck your primer design before constructing or ordering primers. For the Diversify protocol, we recommend that primers have the following characteristics:

- T_m around 70°C
- Length greater than 22 nucleotides; 25 to 30-mers are optimal
- 45–60% GC content
- 10 µM in concentration (each)

Additionally, ensure that the 3'-terminal ends of the primer pair are not complementary and have a low G-C content. Furthermore, primers should not contain sequences that create stable internal hairpin loops. If desired, you may incorporate restriction sites into your primers.

B. Choosing Buffer Conditions

The kit is intended for studying wild-type protein function and for directed protein evolution. For studying wild-type protein function, we suggest creating a single amino acid substitution per protein, which corresponds to approximately 1.5 mutations per gene (Vartanian *et al.*, 1996). This level of mutagenesis allows you to independently characterize the effect of each amino acid substitution on protein function. For directed protein evolution, mutagenesis rates that average 2 to 6 mutations per gene are regarded as most effective for creating mutant libraries to find proteins with enhanced activity (Shafikhani *et al.*, 1997). Mutational levels beyond 6 mutations per gene usually result in the complete loss of protein activity (Suzuki *et al.*, 1996); however, there have been exceptional cases where proteins tolerate extremely high levels of mutation (Vartanian *et al.*, 1996).

To choose the appropriate buffer conditions, you must consider the size of your target gene and the level of mutagenesis required. Table I shows the mutations created per 1,000 bp for given PCR conditions. First determine how many mutations you require per 1,000 bp, then use Table I to find PCR conditions which approximate your requirements. For example, if your PCR fragment is 500 bp long, and you wish to have an average of 2 to 3 mutations in each fragment, you need a mutagenesis rate between 4 and 6 mutations per 1000 bp. Condition 6 in Table I is the best choice to approximate this level of mutagenesis. The buffer composition of a standard PCR reaction is also included in Table I if you need to optimize other PCR parameters prior to performing random PCR.

IV. General Considerations *continued*

TABLE I: MUTAGENESIS PCR REACTION BUFFER CONDITIONS

	Buffer Condition									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Std.***
Mutations per 1,000 bp	2.0	2.3	2.7	3.5	4.6	4.8	5.8	7.2	8.1	0.4
$MnSO_4$ (µM; final rxn)	0	160	320	480	640	640	640	640	640	0
dGTP (µM; final rxn)	40	40	40	40	40	80	120	160	200	200

* Standard PCR reaction using TITANIUM *Taq* DNA Polymerase.

C. Mutational Bias

The mutational bias, or tendency of mutagenesis reactions to favor one type of mutation over another, varies among the PCR conditions shown in Table I. A common method for evaluating mutational bias is to consider the ratio of transitions to transversions (Ts/Tv). Transition mutations comprise purine ↔ purine and pyrimidine ↔ pyrimidine changes; transversions are purine ↔ pyrimidine type mutations. Another method is to consider the extent to which A or T bases are converted to G or C bases and vice versa (AT → GC/GC → AT). The mutational bias for buffer conditions 1, 5, and 9 has been determined by sequencing and is shown below in Table II, along with the ideal values characteristic of a truly random mutagenesis. Detailed information about the types of mutations is provided in the Appendix.

TABLE II: MUTATIONAL BIAS FOR DIVERSIFY® MUTAGENESIS

Buffer Condition (from Table I)	Mutations per 1,000 bp	Ts / Tv	AT → GC / GC → AT
1	2.0	0.9	7.3
5	4.6	1.3	2.9
9	8.1	3.9	13.7
Truly Random	-	0.5	1.0

As Table II shows, a high mutagenesis rate (condition 9) and low bias (condition 5) are mutually exclusive for a given number of cycles. Buffer condition 5 has minimal mutational bias and is the best choice for most directed evolution applications. Please note, however, that despite its mutational bias, buffer condition 9 has given useful results in a number of applications (Nishiya and Imanka, 1994; You and Arnold, 1994; Melnikov and Youngman, 1999). If you wish to have a higher mutagenesis rate with minimal mutational bias, you may perform additional rounds of PCR at buffer condition 5. For example, Shafikhani *et al.* (1997) obtained a rate of 29.2 mutations per 1,000 bp using a reaction similar to buffer condition 5. They achieved this high rate by performing six sequential rounds of PCR, diluting the product after each round. This rate corresponds to 4.9 mutations per 1,000 bp per round—equivalent to the rate of buffer condition 5.

IV. General Considerations *continued*

D. Control Reaction

To ensure that the mutagenesis reaction is working correctly, you should perform the *Taq* I PCR Fidelity Assay (Figure 2) in parallel with your experimental reactions. In this assay, a DNA sequence containing a series of pseudo-*Taq* I restriction enzyme sites is amplified under highly mutagenic (buffer 9;Table I) or weakly mutagenic (buffer 1) conditions. These sites contain only three of the four bases (TCGA) required for *Taq* I cleavage. Under mutagenic conditions, some of these pseudo-sites are converted to *Taq* I sites. Digestion of the resulting PCR product with *Taq* I enzyme, followed by electrophoretic analysis, will verify that mutations have been successfully incorporated. We recommend that you perform this control alongside your first mutagenesis reaction to verify that the system is working. Subsequently, the control can be used for troubleshooting.

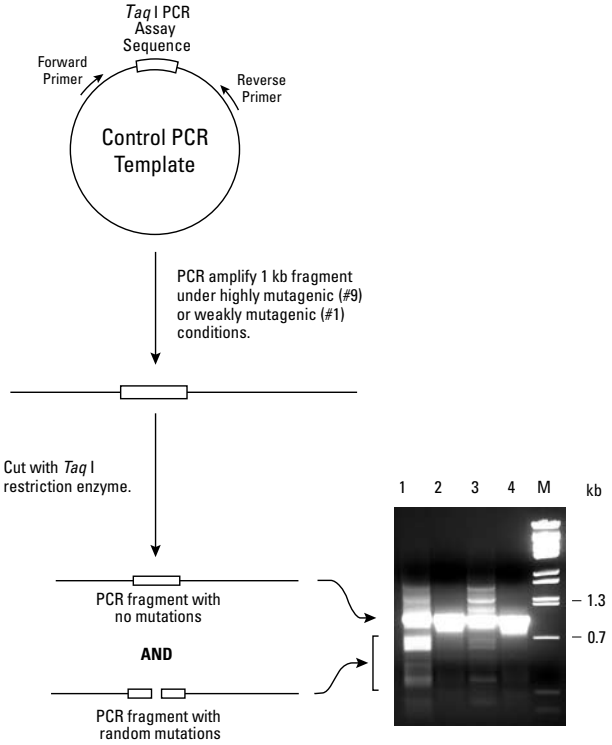

Figure 2. *Taq* I PCR Fidelity Assay. Following amplification of the Control PCR template and *Taq* I restriction digest, a broad band from 550–750 bp should be apparent in the highly mutagenic sample (buffer condition 9; lane 1), but not in the weakly mutagenic sample (buffer 1; lane 2). Additionally, a second broad band from 250–450 bp, representing the small arm of the amplified fragment, can sometimes be seen (lane 1). Both samples will show a bright band at 1 kb. For comparison, lanes 3 and 4 show the PCR product of buffer conditions 9 and 1, respectively, after mock-digestion without *Taq* I enzyme. Lane M: λ/*Bst*E II DNA size markers.

V. Random Mutagenesis Procedure

A. Setting Up and Running Reactions

After deciding which buffer condition(s) suit your desired level of mutagenesis, set up reactions as shown in Table III. At a minimum, you should run three reactions: one each with buffer conditions 1 and 9 for performing the control *Taq* I PCR Fidelity Assay, as well as one for your experimental reaction(s).

1. Prepare reactions on ice by combining the following reagents in the order shown:

TABLE III: MUTAGENESIS REACTIONS

	Volumes by Buffer Condition (µl)									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**Std.**[a]
Mutations per 1,000 bp	2.0	2.3	2.7	3.5	4.6	4.8	5.8	7.2	8.1	–
PCR Grade Water	40	39	38	37	36	35	34	33	32	41
10X TITANIUM *Taq* Buffer	5	5	5	5	5	5	5	5	5	5
$MnSO_4$ (8 mM)	0	1	2	3	4	4	4	4	4	0
dGTP (2 mM)	1	1	1	1	1	2	3	4	5	0
50X Diversify dNTP Mix	1	1	1	1	1	1	1	1	1	0
50X dNTP Mix	0	0	0	0	0	0	0	0	0	1
Primer mix[b]	1	1	1	1	1	1	1	1	1	1
Template DNA[c]	1	1	1	1	1	1	1	1	1	1
TITANIUM *Taq* Polym.	1	1	1	1	1	1	1	1	1	1
Total volume	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**	**50**

[a] Standard PCR reaction using TITANIUM *Taq* DNA Polymerase
[b] Experimental or Control Primer Mix (10 µM each primer)
[c] Experimental or Control PCR Template (~1 ng/µl)

2. Mix well and spin briefly to collect all liquid at the bottom of the tubes.
   Note: If you are not using a hot-lid thermal cycler, overlay contents with mineral oil.
3. Commence thermal cycling using the following parameters for either hot-lid or non-hot-lid thermal cyclers.
   - 94°C for 30 sec
   - 25 cycles:
     94°C 30 sec
     68°C 1 min*
   - 68°C for 1 min
   - 4°C soak

   * For experimental mutagenesis reactions with templates longer than 1 kb, add 1 min of extension time per additional kb.

V. Random Mutagenesis Procedure *continued*

B. *Taq* I PCR Fidelity Assay

When the PCR reaction is complete, examine the results of the control mutagenesis reactions by performing a *Taq* I restriction digest and agarose gel electrophoresis.

1. Prepare a 20 µl digest for each of the control reactions (buffer conditions 1 and 9) as follows:

15 µl	PCR mutagenesis reaction (#1 or #9)
4 µl	distilled H_2O
1 µl	*Taq* I Restriction Enzyme (2 units)
20 µl	**Total volume**

2. Incubate at 65°C for 1 hr.
3. Add 3 µl of 10X gel loading buffer to each digest. Electrophorese 15 µl of each reaction on a 1% agarose/EtBr gel along with 1 kb ladder DNA size markers or equivalent. Stop electrophoresis when dye front has migrated three-quarters of the length of the gel.
4. Photograph the gel and compare its band pattern to the pattern in Figure 2. Both reaction mutagenesis conditions should show a bright band at 1 kb. The 1 kb band will typically be brighter in condition 1. In condition 9, a broad band from 550 to 750 bp indicates that random mutations were successfully introduced.

After verifying that the control reaction was successful, you may proceed to construct a mutagenized library by cloning the PCR products of your experimental mutagenesis into the vector of your choice. For TA-cloning, we recommend the AdvanTAge PCR Cloning Kit (Cat. No. 639507).

VI. Troubleshooting

A. No product observed

PCR component missing or degraded	Use a checklist when assembling reactions. Always perform the *Taq* I PCR Fidelity Assay to ensure that each component is functional. If this positive control does not work, repeat the positive control only. If the positive control still does not work, repeat again replacing individual components to identify the faulty reagent.
Too few cycles	Increase the number of cycles (3–5 additional cycles at a time).
Annealing temp. too high	Decrease the annealing temperature in increments of 2–4°C.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt long, try using a longer primer. If the original primer(s) had a GC content of less than 45%, try to design a primer with a GC content of 45–60%.
Not enough template	Repeat PCR using a higher concentration of DNA (after trying more cycles).
Poor template quality	Check template integrity by electrophoresis on a standard TBE-agarose gel. If necessary, repurify your template using methods that minimize shearing and nicking.
Denaturation temp. too high or low	Optimize denaturation temperature by decreasing or increasing it in 1°C increments. (A denaturation temperature that is too high can lead to degradation of the template, especially for long target sequences.)
Denaturation time too long or too short	Optimize denaturation time by decreasing or increasing it in 10 sec increments. (A denaturation time that is too long can lead to degradation of the template, especially for long target sequences.)
Extension time too short	(Especially with longer templates) Increase the extension time in 1 min increments.
Too little enzyme	TITANIUM *Taq* is supplied at an optimized 50X concentration. Therefore, try to optimize the cycle parameters as described above before increasing the enzyme concentration. In rare cases, the yields can be improved by increasing the concentration of the enzyme mix. However, increasing the concentration >2X is likely to lead to higher background levels.

VI. Troubleshooting *continued*

[Mg^{2+}] is too low	TITANIUM *Taq* performs well at a broad range of Mg^{2+} concentration. Therefore, as long as you use the included buffer, it is unlikely that a lack of product is due to problems with the Mg^{2+} concentration. However, high concentrations of EDTA or other metal chelators in the template stock solution can reduce the effective concentration of Mg to below a minimum level.

B. Multiple products

Too many cycles	Reducing the cycle number may eliminate non-specific bands.
Annealing temp. too low	Increase the annealing/extension temperature in increments of 2–3°C.
Suboptimal primer design	Redesign your primer(s) after confirming the accuracy of the sequence information. If the original primer(s) was less than 22 nt long, try using a longer primer. If the original primer(s) had a GC content of less than 45%, try to design a primer with a GC content of 45–60%.
Touchdown PCR needed	"Touchdown" PCR significantly improves the specificity of many PCR reactions in various applications (Don *et al.*, 1991; Roux, 1995). Touchdown PCR involves using an annealing/extension temperature that is several degrees *higher* than the T_m of the primers during the initial PCR cycles. The annealing/extension temperature is then reduced to the primer T_m for the remaining PCR cycles. The change can be performed either in a single step or in increments over several cycles.
Contamination	See Section D.

C. Products are smeared on gel

Too many cycles	Reduce the cycle number by 3–5 cycles to see if non-specific bands go away.
Denaturation temp. too low	Try increasing the denaturation temperature in increments of 1°C.
Extension time too long	Decrease the extension time in 1–2 min increments.
Poor template quality	Check template integrity by electrophoresis on a denaturing agarose gel. Repurify your template if necessary.
Touchdown PCR needed	See "Touchdown PCR needed" under previous section.

VI. Troubleshooting *continued*

Too much enzyme	TITANIUM *Taq* is supplied at an optimized 50X concentration; however, a 1X final concentration of the enzyme mix may be too high for some applications. If smearing is observed, first try optimizing the cycle parameters as described above, then try reducing the enzyme concentration to 0.5–0.2X.
Too much template	Try a lower concentration of DNA template in the PCR reaction.
Contamination	See Section D.

D. Dealing with contamination

Contamination most often results in extra bands or smearing. It is important to include a negative control (a control that replaces the DNA template with PCR-grade H_2O but still includes the primers) in every PCR experiment to determine if the PCR reagents, pipettors, or PCR reaction tubes are contaminated with previously amplified targets.

If possible, set up the PCR reaction and perform the post-PCR analysis in separate laboratory areas with separate sets of pipettors.

Laboratory benches and pipettor shafts can be decontaminated by depurination. Wipe surfaces with 1 N HCl followed by 1 N NaOH. Then neutralize with a neutral buffer (e.g., Tris or PBS) and rinse with ddH_2O.

We advise using commercially available aerosol-free pipette tips.

E. Mutation rate too high or too low

If clones resulting from your mutagenic PCR reaction are mostly inactive, the mutation rate may be too high. Try using a buffer condition with a lower rate of mutagenesis. If the mutation rate is still too high using buffer condition 1 (Table III), the error rate of a standard PCR reaction may be sufficient to randomly mutate your gene of interest. This technique might be useful for very large proteins that are also hypersensitive to mutation.

If the error rate is too low for your application (at your preferred level of mutational bias; see Section IV.C), you can perform multiple rounds of PCR, diluting the product 1,000-fold for each subsequent reaction. The optimal dilution factor may vary depending on your specific template and the yield of the PCR reaction.

VII. References

Cadwell, R. C. & Joyce, G. F. (1992) Randomization of genes by PCR mutagenesis. *PCR Methods Appl.* **2**(1):28–33.

Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K. & Mattick, J. S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**:4008.

Fromant, M., Blanquet, S. & Plateau, P. (1995) Direct random mutagenesis of gene-sized DNA fragments using polymerase chain reaction. *Anal. Biochem.* **224**(1):347–353.

Leung, D. W., Chen, E. & Goeddel, D. V. (1989) A method for random mutagenesis of a defined DNA segment using a modified polymerase chain reaction. *Technique* **1**(1): 11–15.

Melnikov, A. & Youngman, P. J. (1999) Random mutagenesis by recombinational capture of PCR products in *Bacillus subtilis* and *Acinetobacter calcoaceticus*. *Nucleic Acids Res.* **27**(4):1056–1062.

Mo, J. Y., Maki, H. & Sekiguchi, M. (1991) Mutational specificity of the dnaE173 mutator associated with a defect in the catalytic subunit of DNA polymerase III of *Escherichia coli*. *J. Mol. Biol.* **222**(4):925–936.

Nishiya, Y. & Imanaka, T. (1994) Alteration of substrate specificity and optimum pH of sarcosine oxidase by random and site-directed mutagenesis. *Appl. Env. Microbiol.* **60**(11):4213–4215.

Roux, K. H. (1995) Optimization and troubleshooting in PCR. *PCR Methods Appl.* **4:**5185–5194.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press (Cold Spring Harbor, NY).

Shafikhani, S., Siegel, R. A., Ferrari, E. & Schellenberger, V. (1997) Generation of large libraries of random mutants in *Bacillus subtilis* by PCR-based plasmid multimerization. *BioTechniques* **23**(2): 304–310.

Suzuki, M., Christians, F. C., Kim, B., Skandalis, A., Black, M. E. & Loeb, L. A. (1996) Tolerance of different proteins for amino acid diversity. *Mol. Divers.* **2**(1–2):111–118.

Vartanian, J. P., Henry, M. & Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions. *Nucleic Acids Res.* **24**(14):2627–2631.

Wan, L., Twitchett, M. B., Eltis, L. D., Mauk, A. G. & Smith, M. (1998) *In vitro* evolution of horse heart myoglobin to increase peroxidase activity. *Proc. Natl. Acad. Sci. U.S.A.* **95**(22):12825–12831.

You, L. & Arnold, F. H. (1994) Directed evolution of subtilisin E in *Bacillus subtilis* to enhance total activity in aqueous dimethylformamide. *Protein Eng.* **9**(1):77–83.

VIII.Related Products

For the latest and most complete listing of all Clontech products, please visit **www.clontech.com**

Products	Cat. No.
• TITANIUM™ *Taq* DNA Polymerase	639208 639209
• TITANIUM™ *Taq* PCR Kit	639211 639210
• Matchmaker™ Yeast Two-Hybrid Systems	many

Appendix: Mutational Data from DNA Sequencing

A mutagenized library was constructed and sequenced to determine the frequency and type of mutations introduced. The results of this sequence analysis are shown in Table IV. Additionally, the distribution of mutations per clone is shown in Figure 3.

TABLE IV: DIVERSIFY® MUTAGENESIS SEQUENCING DATA

	Buffer Condition		
	1	**5**	**9**
$MnSO_4$ (µM)	0	640	640
dGTP (µM)	40	40	200
Total bp sequenced	18,414	20,705	15,148
Mutations per 1,000 bp	2.0	4.6	8.1
Total mutations found	36	96	123
Type of Mutation (%)			
Ts: A → G or T → C	33.3	42.7	74.0
Ts: G → A or C → T	8.3	11.5	4.9
Tv: A → T or T → A	16.7	26.0	13.8
Tv: A → C or T → G	27.8	8.3	4.1
Tv: G → C or C → G	0.0	0.0	1.6
Tv: G → T or C → A	0.0	6.3	0.8
Insertions	2.8	2.1	0.0
Deletions	11.1	3.1	0.8

Ts = Transition
Tv = Transversion

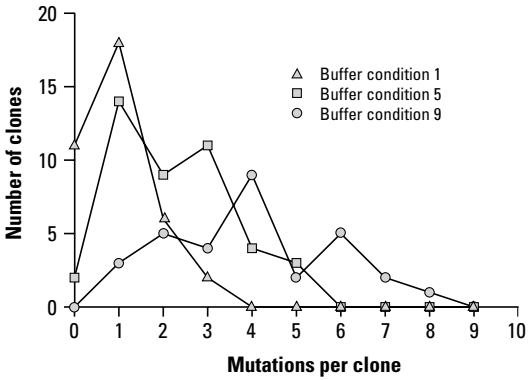

Figure 3. Distribution of mutations per clone for different Diversify® buffer conditions. Data represent sequencing results from a 490 bp region of the mutagenized DNA sequence.

Notes

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