Published in final edited form as:
J Virol Methods. 2010 August ; 167(2): 146–151. doi:10.1016/j.jviromet.2010.03.025.

Generation of random mutant libraries with multiple primers in a single reaction

Xiaozhi Lu, **Bhavna Hora**, **Fangping Cai**, and **Feng Gao***
Human Vaccine Institute, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Characterization of multiple sites in a single gene that are important in biological phenotypes is challenging due to the difficulty to generate many mutants representing all or a majority of combinations of mutations in the gene. Using the HIV-1 *env* and *pol* genes as templates, four random libraries were generated representing different combinations of mutations introduced by up to 36 mutagenesis primers in a single assay. Over 86% of the clones contained mutations and the mutants tended to have single or fewer mutations in the libraries. When protein size was used as a screening marker, all identified clones contained at least 2 mutations and up to 12 mutations were detected in a single clone. Nearly all mutant clones in each library contained unique mutations, indicating that mutants in the library were generated at random. Closely related mutations which were overlapped by neighboring mutagenesis primers were often introduced in this system. Analysis of the *env* library showed that some potential N-linked glycosylation sites did not increase the Env molecular mass significantly, suggesting they were not used for glycosylation or only limited carbohydrate moieties were added at these sites. This novel method can serve as a powerful tool to study the biological phenotypes of genes whose functions are determined by multiple sites.

Keywords

Random library; Mutant; HIV-1; Multiple site-directed Mutagenesis

1. INTRODUCTION

There is an increasing demand to develop more efficient and effective methods for engineering protein variants to study biological functions, structural biology and drug development (Wang et al., 2007; Wong et al., 2007). Site-specific mutagenesis at one or multiple sites has become an invaluable method for such studies (Follo and Isidoro, 2008; Ishii et al., 1998; Ling and Robinson, 1997; Varga-Orvos et al., 2007; Wong et al., 2007). Several commercial assays are currently available for introducing single mutations into a desired gene (Bi and Stambrook, 1998; Ling and Robinson, 1997; Varga-Orvos et al., 2007). Various PCR-based methods have also been used to generate genes with multiple mutations (An et al., 2005; Follo and Isidoro, 2008; Peng et al., 2006; Seyfang and Jin, 2004; Wang et al., 2007; Young and Dong, 2003). In one study, 9 and 11 mutation sites were introduced

*Corresponding author: Feng Gao, M.D., 3072B MSRB II, DUMC 103020, 106 Research Drive, Duke Human Vaccine Institute, Duke University Medical Center, Durham, NC 27710, USA, Tel: 1-919-668-6433, Fax: 1-919-681-8992, fgao@duke.edu.

simultaneously into two genes by the PCR-based mutagenesis method (Seyfang and Jin, 2004). However, these methods usually require multiple rounds of PCRs and purification of intermediate PCR products to remove parent templates, mispairing fragments and unused primers. A subcloning step is also required to express genes of interest. In addition, these methods are not suitable for generating random mutant libraries to study how various combinations of multiple mutations affect the phenotypes of the genes.

The QuickChange Multi Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) is one of the simplest and most applicable protocols for introducing multiple mutations into plasmid DNA in a single reaction (Hogrefe et al., 2002). Since mutagenesis can be performed with most backbone plasmid vectors, the final mutants can be used directly for downstream expression and functional studies without the subcloning step. The kit has been used widely for introducing five or fewer mutations in a single reaction (Bian et al., 2007; Hogrefe et al., 2002; Islam et al., 2007; Jinno et al., 2003; Sjostrand et al., 2007). Recently, it was also used to construct a library of mutants with a pool of 29 mutagenesis primers in a single reaction (Amin et al., 2004).

Human immunodeficiency virus type-1 (HIV-1) envelope glycoproteins are highly glycosylated. Each HIV-1 envelope glycoprotein has about 18–30 potential N-linked glycosylation sites (PNLGs) and some of them have been well characterized (Blay et al., 2006; Go et al., 2008; Irungu et al., 2008; Zhang et al., 2004). A few PNLGs were found to modify the ability of the Env proteins to induce neutralizing antibodies (Li et al., 2008; Quinones-Kochs et al., 2002; Reitter et al., 1998). To investigate how various combinations of PNLGs affect envelope glycoprotein expression and whether they could elicit stronger immune responses, random libraries of HIV-1 *env* mutants carrying up to 12 PNLGs mutations on individual genes were generated in this study. The effectiveness of the method was confirmed further by generating a similar *pol* gene library using 36 mutagenesis primers in a single reaction.

2. MATERIALS AND METHODS

2.1 Plasmid DNA and mutagenesis primers

Plasmid MCon3 (6.1 kb) contained a 1.7 kb HIV-1 group M consensus *env* gene (MCon3), in which all 24 PNLGs were eliminated. Individual mutagenesis primers were synthesized to restore one of the 24 PNLGs. Plasmid WEAU.WT6 (5.0 kb) contained a 1.2 kb HIV-1 *pol* gene fragment. Thirty-six primers were synthesized to introduce mutations conferring drug resistance to antiretroviral drugs (Johnson et al., 2007). All primers were synthesized and purified by standard desalting (IDT Inc., Coralville, IA). The 5′ ends of the primers were not phosphorylated. Mutagenic primers were 25–45 bases long and the desired point mutations were positioned in the middle of the primers.

2.2 Multiple site-directed mutagenesis

Mutagenesis reactions were carried out using the QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Each mutagenesis reaction contained 1x QuickChange Multi Buffer, 1x QuickChange Multi dNTPs, 100 ng of plasmid DNA, 50 ng (for 7- and 10-primer reactions) or 10 ng (for 24- and 36-primer reaction) of each primer, 0.75 μl QuickSolution, 1 μl QuickChange Multi enzyme blend, and double-distilled water to a final volume of 25 μl. The mutagenesis reaction was performed under the following conditions: denaturation at 95 °C for 1 min; 30 cycles of 95 °C for 1 min, 55 °C for 1 min; and 65 °C for 14 min (MCon3) or 12 min (WEAU.WT6). The dsDNA molecules had one strand bearing multiple mutations and containing nicks, which were repaired by the enzyme blend in the kit. The strands of the parent template DNA were digested subsequently with

Dpn I endonuclease in a 25 μl of reaction. The final reaction solution (1.5 μl) was used to transform 50 μl of Stratagene's XL10-Gold ultracompetent cells and cultured in 500 μl NZY + broth. The transformed bacteria were then plated on LB agar plates.

2.3 Library screening

Individual colonies were picked and cultured in 96-well plates in 1.25 ml of LB medium. Plasmid DNA was extracted using Perfectprep® Plasmid 96 Vac Direct Bind (Eppendorff, Westbury, NY, USA). Library screening was performed either by direct DNA sequencing of all selected colonies or by identifying clones which expressed HIV-1 Env proteins with increased molecular mass due to added carbohydrate molecules at PNLG positions in transfected 293T cells. All mutations were confirmed by sequencing.

2.4 Western Blot assay

Forty-eight hours after transfection in 293T cells in 24-well plates, the cell culture supernatant (12 μl) was mixed with 2.4 μl of 6 x sample buffer (300 mM Tris-HCl pH 6.8, 12 mM EDTA, 12% SDS, 864 mM 2-mercaptoethanol, 60% glycerol, 0.05% bromophenol blue). Samples were boiled for 5 minutes and then loaded into a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen; Carlesbad, CA). Following electrophoresis, the proteins were transferred onto a nitrocellulose membrane. The membrane was blocked in PBS containing 1% casein and 0.01% NaN_3 for 1 hour and then probed with a mouse mAb 13D5 (1ug/ml) to the HIV-1 Env protein (Gao et al., 2009). Finally, the Env proteins were detected with Alexa-Fluor 680 conjugated goat anti-mouse antibody (Invitrogen; Carlesbad, CA) using an Odyssey Infrared Imaging system (LiCor Biosciences; Lincoln, NE).

3. RESULTS

Generation of a library of mutants with random combinations of PNLGs in the HIV-1 *env* gene is important to understand which PNLGs are necessary for glycosylation of envelope proteins, intracellular transportation mechanisms, T cell epitope presentation, and enhancement of immune responses to HIV-1. It has not been fully understood which PNLGs sites and combinations will affect Env biological functions. Obtaining and testing representative combinations of PNLGs in the Env proteins will be a critical step to study Env functionality. Among the 24 PNLGs in the MCon3 *env* gene, some are so close to each other that mutagenesis primers overlap. It was suspected initially that these overlapping primers might interfere with their ability to anneal to the same region in the template. Thus, two libraries with 7 and 10 primers each without any overlapping were made to test if these primers could be used simultaneously to generate clones with multiple random mutations. In the 10-primer library, 96 colonies were selected. Sequence analysis showed that 82 of 96 (85.4%) clones contained various numbers of mutations (Table 1). Among those mutants, the clones with 3–6 mutations were most common (76.8%). Although clones with ≤2 or ≥7 were few, clones with 8 or 9 mutations were generated.

In the second library with 7 non-overlapping primers, mutant clones were screened by Western blot analysis. Because the mutants with added PNLGs would express Env proteins with increased molecular mass, the clones with introduced mutations were expected to show bigger Env protein bands than the parental Env clone. Equal amounts of plasmid DNA from 92 clones were transfected into 293T cells, and equal amounts of culture supernatants were subjected to Western blot analysis. Based on Env protein band patterns, 68 of the 92 clones (73.9%) showed higher molecular mass than parental MCon3. This percentage was slightly lower than that identified by sequencing, possibly due to some mutations that did not result in glycosylation to increase the size of the Env proteins. Thirty-three clones were selected for sequencing analysis. The results showed that each clone contained at least two mutations

and a higher percentage (90.9%) of the clones had 3–6 mutations than the library screened by directly sequencing (Table 1).

It was demonstrated that more than five mutations could be generated simultaneously in individual clones and that screening for mutants by Western blot was an effective method to identify mutants carrying higher numbers of mutations based on increased molecular mass. Additional mutagenesis was performed using 24 primers in one reaction to generate a library with as many different combinations of added PNLGs as possible in order to identify PNLGs that may be important for glycosylation and elicitation of strong immune responses. Among 192 colonies selected, 152 were found to express Env proteins with increased molecular weights. Thirty-two clones that expressed bigger Env proteins at higher levels were selected for sequence analysis (Fig. 1). Sequence analysis showed that all 32 clones contained at least three mutations (Table 1). One clone (3F9) contained 12 mutations and two other clones (3B2 and 3F4) had 10 mutations each (Table 1 and Fig. 1). Clones with eight or more mutations had much larger molecular masses than others. Although clones with more than five mutations were frequently identified, more than half of them (59.4%) had three or four added PNLGs.

Examination of the mutation locations in the *env* gene showed that mutations 48, 87 and 187 at the beginning of the gene were detected more frequently than others (Fig. 1). These primers have similar GC contents (55–65%) as others used in the mutagenesis analysis and examining sequences in and near the mutagenesis primers in the template did not reveal any unique features. However, the distances among those primers at this region are bigger than those in other regions and there are no overlaps between the first three primers. The larger distance between primers might allow the primers to anneal more easily to the template and could result in higher frequencies of detected mutations in this region than other primers. Analysis of the library also showed that some added PNLGs did not increase the Env molecular mass as significantly as others with the same number of PNLGs, for example, 3C12 vs. 3A6 and 3B3 vs. 3B8 (Fig. 1). This finding suggests that some potential sites may not be used for glycosylation or only limited carbohydrate moieties were added at these sites. All mutations were detected at least once among 32 clones selected, suggesting all primers in the reaction were utilized randomly.

To test if more mutagenesis primers could be used to generate comprehensive libraries in a single reaction, another random library was made using 36 mutagenesis primers. The wild type HIV-1 *pol* gene was used as the template and the drug-resistant mutations in both the protease and reverse transcriptase genes were used according to the recommendation by the International AIDS Society–USA Drug Resistance Mutations Group (Johnson et al., 2007). Ninety-six colonies were selected after transformation. Since no biological markers could be available for screening clones with mutations, direct sequencing was used to determine mutations in selected clones. Sequence analysis showed that 90 clones had various numbers of mutations (1 through 9) and 57.3% of them had at least three mutations (Table 1). All mutants contained unique single or combinations of mutations, except two clones that had the same K219Q mutation. The result again suggested that the library was generated at random. With as many as 36 primers in one mutagenesis reaction, only six selected clones had no mutations among 96 clones. Although a higher percentage of the clones (70.8%) had four or fewer mutations than in the 10-primer library (52.1%), 8 or 9 mutations were also identified in two clones (Table 1).

Sequence analysis showed that unintended mutations were detected in some clones. Most of these clones had only one such mutation and only a few were found to contain a maximum of two unintended mutations. The frequency of clones with unintended mutations (8.9%; 23/257) was higher than that generated with the mutation kit designed for introduction of a

single mutation into the interested gene (0.9%; 2/235; unpublished data). However, the mutagenesis reaction with ≥24 primers did not increase the frequency of clones with unintended mutations than that with ≤10 primers (9.3% vs. 8.5%).

Since some primers were overlapping each other in the two libraries with larger numbers of primers (24 or 36), it was determined whether mutations could be introduced simultaneously into the genes by overlapping primers. In the 24-primer library, four clones were found carrying adjacent mutations introduced by overlapping primers (Table 2). Examination of the primer locations showed two different scenarios. In some cases, the primers did not overlap the mutation bases (3F4, 3F9 and 3B2) and the overlapping portion from one primer was able to completely anneal to the template while the overlapping region from the other primer was left free as shown in clone 3F9, in which three mutations were induced by overlapping mutagenesis primers (Fig. 2A). In other cases, the mutations were so close that mutagenesis primers overlapped each other and did not leave space for any primers to completely anneal to the template (Fig. 2B). For both mutations to be introduced into the same molecule, the portions of the overlapping regions from both primers must be free to allow the parts of the primers containing mutations to anneal to the template (Fig. 2B). When more primers (36) were used to generate a random library, more mutations that were close to each other were identified. Eleven out of 96 clones were found to contain two or more mutations that were overlapped by neighboring primers. In one clone (1F2), up to four mutations were introduced by two sets of overlapping primers (L76V/V82A and L210W/K219Q) in a singe reaction (Table 2). The L210W and K219Q mutations were most frequent and were found in six mutant clones. Other combinations were less frequent and were found in only two or three cases (Table 2).

4. DISCUSSION

Random libraries containing various combinations of mutations using multiple mutagenesis primers can be generated in a single reaction using the QuickChange Multi Site-Directed Mutagenesis Kit. In four independent random mutant libraries with multiple mutagenesis primers, percentages of clones carrying mutations were high (73.9%–93.8%). When 24 or 36 primers are used in a single reaction, the number of clones required to cover all possible random combinations is large. In the current study, only 33 or 96 clones were analyzed in one library, which represented a small portion of all possible combinations or mutations. However, all possible mutants can theoretically be identified when a large number of clones are screened.

After analyzing over 200 mutants, the clones were rarely found to contain identical combinations of mutations. In addition, all primers in the mutagenesis reaction were utilized to introduce mutations and up to 12 mutations could be identified in a single clone. This finding is very similar to what was reported in a previous study (Amin et al., 2004). Closely related mutations that were overlapped by neighboring mutagenesis primers were also generated in this system. Successful identification of clones with closely positioned mutations suggests that the free ends of the primers were repaired by the enzyme blend in the kit. This can significantly increase the utility of the method to generate random libraries by including overlapping primers. The results of this study also show that the length of mutagenesis oligos can be shorter and the mutation bases are not required to be at the center of the primer. This could significantly simplify the primer design.

Many HIV-1 Env mutants were generated in the random libraries. Analysis of the sizes of expressed HIV-1 Env proteins showed that some of the PNLGs were utilized for the addition of carbohydrates as previously reported (Go et al., 2009; Go et al., 2008). Characterization of more mutants from the library will allow us to further determine which

PNLGs are involved in adding carbohydrates to the Env protein. Such mutants are being studied to determine if modifying the number and positions of PNLGs can increase their ability to elicit stronger immune responses. The mutant clones carrying various combinations of drug-resistant mutations can be used to determine how the various combinations of the mutations affect their susceptibility to drugs and viral fitness.

When coupled with biological markers (e.g., protein size) for screening, the desired mutants can be quickly identified for further studies. For genes with multiple critical sites that are important for biological functions, this new method can serve as a useful tool to generate a library of clones in order to determine how each individual site and/or combinations of various sites affect the biological phenotypes of genes of interest.

Acknowledgments

We thank Barton Haynes for critical review of the manuscript. This work was supported by grants from the National Institutes of Health: AI061734 and GM065057.

References

Amin N, Liu AD, Ramer S, Aehle W, Meijer D, Metin M, Wong S, Gualfetti P, Schellenberger V. Construction of stabilized proteins by combinatorial consensus mutagenesis. Protein Eng Des Sel 2004;17:787–93. [PubMed: 15574484]

An Y, Ji J, Wu W, Lv A, Huang R, Wei Y. A rapid and efficient method for multiple-site mutagenesis with a modified overlap extension PCR. Appl Microbiol Biotechnol 2005;68:774–8. [PubMed: 16249879]

Bi W, Stambrook PJ. Site-directed mutagenesis by combined chain reaction. Anal Biochem 1998;256:137–40. [PubMed: 9466810]

Bian XL, Rosas-Acosta G, Wu YC, Wilson VG. Nuclear import of bovine papillomavirus type 1 E1 protein is mediated by multiple alpha importins and is negatively regulated by phosphorylation near a nuclear localization signal. J Virol 2007;81:2899–908. [PubMed: 17192311]

Blay WM, Gnanakaran S, Foley B, Doria-Rose NA, Korber BT, Haigwood NL. Consistent patterns of change during the divergence of human immunodeficiency virus type 1 envelope from that of the inoculated virus in simian/human immunodeficiency virus-infected macaques. J Virol 2006;80:999–1014. [PubMed: 16379001]

Follo C, Isidoro C. A fast and simple method for simultaneous mixed site-specific mutagenesis of a wide coding sequence. Biotechnol Appl Biochem 2008;49:175–83. [PubMed: 17640176]

Gao F, Scearce RM, Alam SM, Hora B, Xia S, Hohm JE, Parks RJ, Ogburn DF, Tomaras GD, Park E, Lomas WE, Maino VC, Fiscus SA, Cohen MS, Moody MA, Hahn BH, Korber BT, Liao HX, Haynes BF. Cross-reactive monoclonal antibodies to multiple HIV-1 subtype and SIVcpz envelope glycoproteins. Virology 2009;394:91–8. [PubMed: 19744690]

Go EP, Chang Q, Liao HX, Sutherland LL, Alam SM, Haynes BF, Desaire H. Glycosylation site-specific analysis of clade C HIV-1 envelope proteins. J Proteome Res 2009;8:4231–42. [PubMed: 19610667]

Go EP, Irungu J, Zhang Y, Dalpathado DS, Liao HX, Sutherland LL, Alam SM, Haynes BF, Desaire H. Glycosylation site-specific analysis of HIV envelope proteins (JR-FL and CON-S) reveals major differences in glycosylation site occupancy, glycoform profiles, and antigenic epitopes' accessibility. J Proteome Res 2008;7:1660–74. [PubMed: 18330979]

Hogrefe HH, Cline J, Youngblood GL, Allen RM. Creating randomized amino acid libraries with the QuikChange Multi Site-Directed Mutagenesis Kit. Biotechniques 2002;33:1158–60. 1162, 1164–5. [PubMed: 12449398]

Irungu J, Go EP, Zhang Y, Dalpathado DS, Liao HX, Haynes BF, Desaire H. Comparison of HPLC/ESI-FTICR MS versus MALDI-TOF/TOF MS for glycopeptide analysis of a highly glycosylated HIV envelope glycoprotein. J Am Soc Mass Spectrom 2008;19:1209–20. [PubMed: 18565761]

Ishii TM, Zerr P, Xia XM, Bond CT, Maylie J, Adelman JP. Site-directed mutagenesis. Methods Enzymol 1998;293:53–71. [PubMed: 9711602]

Islam A, Shen X, Hiroi T, Moss J, Vaughan M, Levine SJ. The brefeldin A-inhibited guanine nucleotide-exchange protein, BIG2, regulates the constitutive release of TNFR1 exosome-like vesicles. J Biol Chem 2007;282:9591–9. [PubMed: 17276987]

Jinno H, Tanaka-Kagawa T, Ohno A, Makino Y, Matsushima E, Hanioka N, Ando M. Functional characterization of cytochrome P450 2B6 allelic variants. Drug Metab Dispos 2003;31:398–403. [PubMed: 12642465]

Johnson VA, Brun-Vezinet F, Clotet B, Gunthard HF, Kuritzkes DR, Pillay D, Schapiro JM, Richman DD. Update of the drug resistance mutations in HIV-1:2007. Top HIV Med 2007;15:119–25. [PubMed: 17720996]

Li Y, Cleveland B, Klots I, Travis B, Richardson BA, Anderson D, Montefiori D, Polacino P, Hu SL. Removal of a single N-linked glycan in human immunodeficiency virus type 1 gp120 results in an enhanced ability to induce neutralizing antibody responses. J Virol 2008;82:638–51. [PubMed: 17959660]

Ling MM, Robinson BH. Approaches to DNA mutagenesis: an overview. Anal Biochem 1997;254:157–78. [PubMed: 9417773]

Peng RH, Xiong AS, Yao QH. A direct and efficient PAGE-mediated overlap extension PCR method for gene multiple-site mutagenesis. Appl Microbiol Biotechnol 2006;73:234–40. [PubMed: 17021880]

Quinones-Kochs MI, Buonocore L, Rose JK. Role of N-linked glycans in a human immunodeficiency virus envelope glycoprotein: effects on protein function and the neutralizing antibody response. J Virol 2002;76:4199–211. [PubMed: 11932385]

Reitter JN, Means RE, Desrosiers RC. A role for carbohydrates in immune evasion in AIDS. Nat Med 1998;4:679–84. [PubMed: 9623976]

Seyfang A, Jin JH. Multiple site-directed mutagenesis of more than 10 sites simultaneously and in a single round. Anal Biochem 2004;324:285–91. [PubMed: 14690693]

Sjostrand D, Carlsson J, Paratcha G, Persson B, Ibanez CF. Disruption of the GDNF binding site in NCAM dissociates ligand binding and homophilic cell adhesion. J Biol Chem 2007;282:12734–40. [PubMed: 17322291]

Varga-Orvos Z, Nagy ZB, Meszaros A, Kokeny S, Gergely P Jr, Tamas L, Poor G. Multiplex site-directed mutagenesis strategy including high-efficiency selection of the mutant PCR products. Biotechnol Lett 2007;29:1921–5. [PubMed: 17687622]

Wang J, Zhang S, Tan H, Zhao ZK. PCR-based strategy for construction of multi-site-saturation mutagenic expression library. J Microbiol Methods 2007;71:225–30. [PubMed: 17936385]

Wong TS, Roccatano D, Schwaneberg U. Steering directed protein evolution: strategies to manage combinatorial complexity of mutant libraries. Environ Microbiol 2007;9:2645–59. [PubMed: 17922750]

Young L, Dong Q. TAMS technology for simple and efficient in vitro site-directed mutagenesis and mutant screening. Nucleic Acids Res 2003;31:e11. [PubMed: 12560514]

Zhang M, Gaschen B, Blay W, Foley B, Haigwood N, Kuiken C, Korber B. Tracking global patterns of N-linked glycosylation site variation in highly variable viral glycoproteins: HIV, SIV, and HCV envelopes and influenza hemagglutinin. Glycobiology 2004;14:1229–46. [PubMed: 15175256]

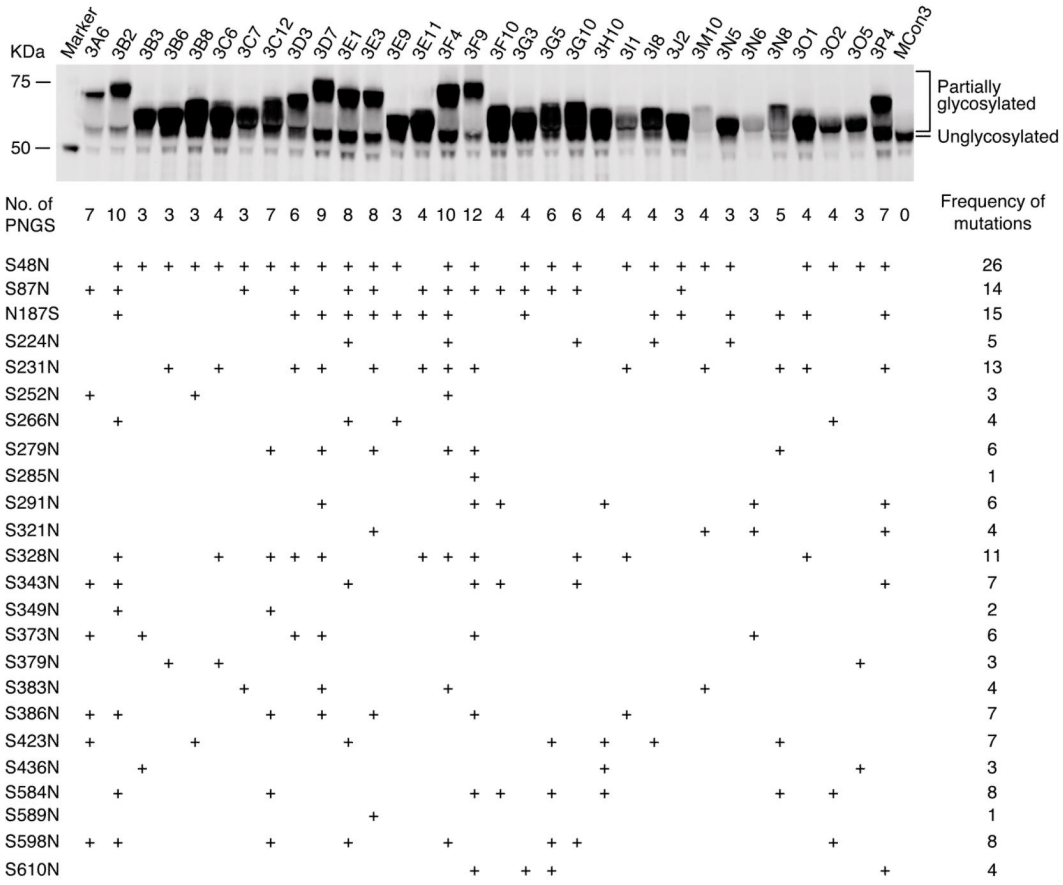

Figure 1.

Identification of clones containing introduced mutations by Western blot and sequencing analysis. The same amount (0.5 μg) of each plasmid DNA was transfected into 293T cells. Equal volume (12 μl) of culture supernatant was loaded into NuPAGE® Novex 4–12% Bis-Tris gels. Anti-HIV-1 Env mAb 13D5 and Alexa Fluor® 680 conjugated goat anti-mouse IgG were used to detect Env proteins. The image was acquired using the Odyssey Infrared Imaging System (Li Cor Bioscience, Lincoln, NE). The clones with added PNLGs showed increased molecular weight of the expressed Env Proteins. The mutation sites introduced in individual clones were confirmed by sequence analysis.

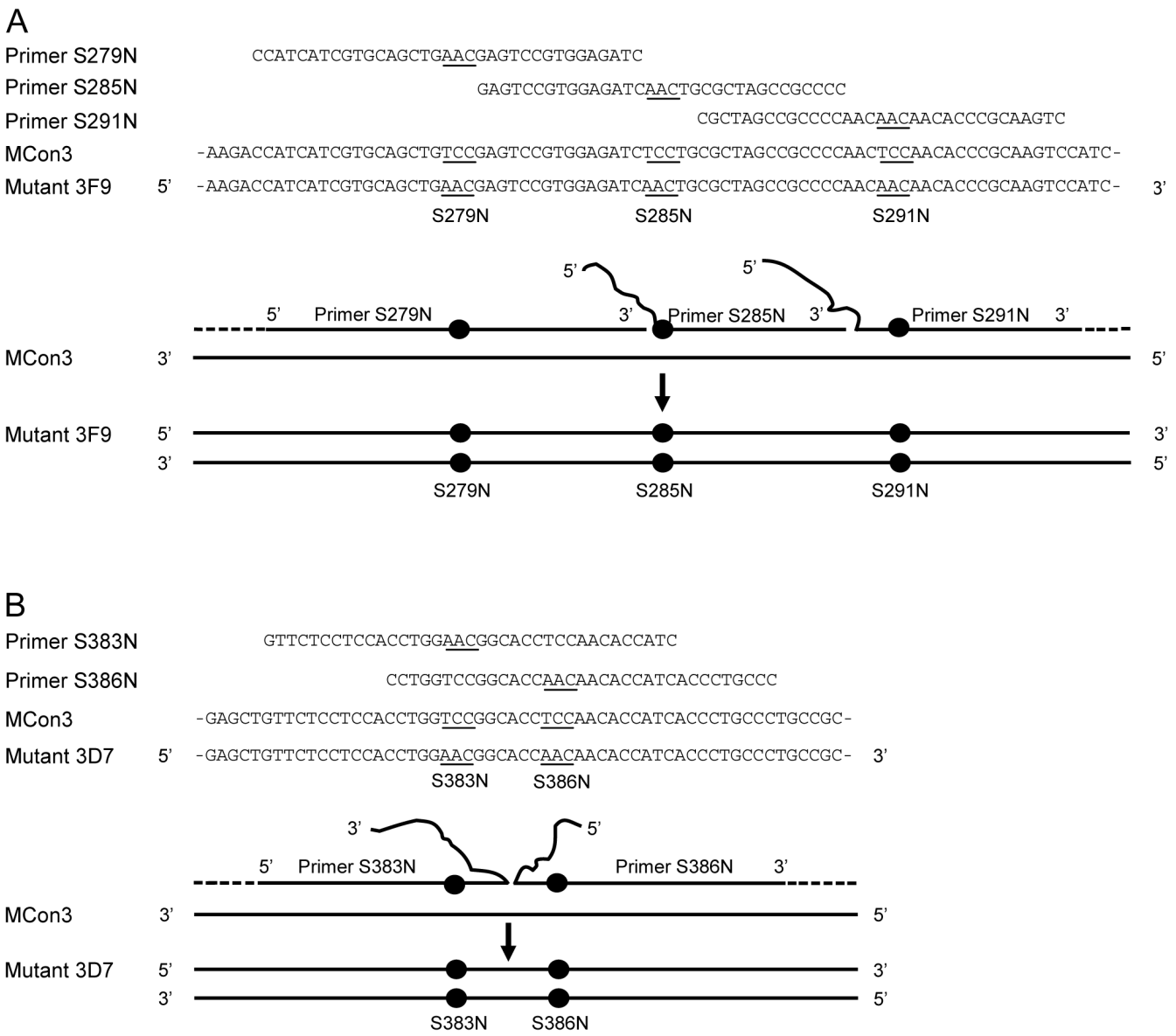

Figure 2.

Generation of clones with mutations close to each other by overlapping mutagenesis primers. Sequences of overlapping primers, parent clone MCon3 and resulting mutants are aligned. The codon in which the mutation was introduced is underlined. The mutation positions are indicated with black dots. (A) Mutagenesis primers overlap each other but do not overlap the actual mutation bases. (B) Mutagenesis primers overlap each other and the mutations.

Table 1

Random libraries containg various combinations of mutations

Template	No. of Primers	Colonies Screened	Mutation Frequency	No. of Clones Sequenced	No. of mutations												
					0	1	2	3	4	5	6	7	8	9	10	11	12	13 or more
MCon3	7	92	73.9%[a]	33	0	0	1	5	7	12	6	2						
MCon3	10	96	85.4%[b]	96	14	5	9	18	18	14	13	3	1	1	0			
MCon3	24	192	79.2%[a]	32	0	0	0	9	10	1	3	3	2	1	2	0	1	0
WEAU.WT6	36	96	93.8%[b]	96	6	14	21	17	16	9	9	2	1	1	0	0	0	0

[a] Percentage of clones showing Env protein band at increased size by Western blot analysis

[b] Percentage of clones with mutations by directly DNA sequencing screening.

Table 2

Multiple mutations introduced by overlapping primers in individual clones

Template	No. of primers in the mutagenesis reaction	Mutant ID	Mutations generated by overlapping primers
MCon3	24	3F4	S224N+S231N
		3F9	S279N+S285N+S291N
		3B2	S343N+S349N
		3D7	S383N+S386N
WEAU.WT6	36	1H2	M46I+I54M
		1G5	I47V+I54M
		1C8	L76V+V82A
		1B1	L76V+V84A
		1F2	L76V+V82A, L210W+K219Q
		1A7	L210W+K219Q
		1B11	L210W+K219Q
		1C4	L210W+K219Q
		1C5	L210W+K219Q
		1C7	L210W+K219Q
		1F1	T215Y+P225H