Combination of DMT-mononucleotide and Fmoc-trinucleotide phosphoramidites in oligonucleotide synthesis affords an automatable codon-level mutagenesis method

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Background: Synthetic DNA has been used to introduce variability into protein-coding regions. In protocols that produce a few mutations per gene, the sampling of amino-acid sequence space is limited by the bias imposed by the genetic code. It has long been apparent that the incorporation of trinucleotides in the synthetic regime would circumvent this problem and significantly enhance the usefulness of the technique.

Results: A new method is described for the creation of codon-level degenerate oligodeoxyribonucleotides that combines conventional dimethoxytrityl (DMT) mononucleoside phosphoramidite chemistry with 9-fluorenylmethoxycarbonyl (Fmoc) trinucleotide phosphoramidites (whose synthesis is reported in the paper). The substoichiometric use of these Fmoc-trinucleotides in an automatable, solid-phase synthesis procedure afforded DNA fragments comprising the wild-type sequence and a controllable distribution of mutants within two- and three-codon stretches of DNA, within the multiple cloning site of the conventional cloning vector pUC19.

Conclusions: DMT and Fmoc are compatible protecting groups in conventional oligonucleotide synthesis methods, resulting in controllable levels of codon-based mutagenesis.

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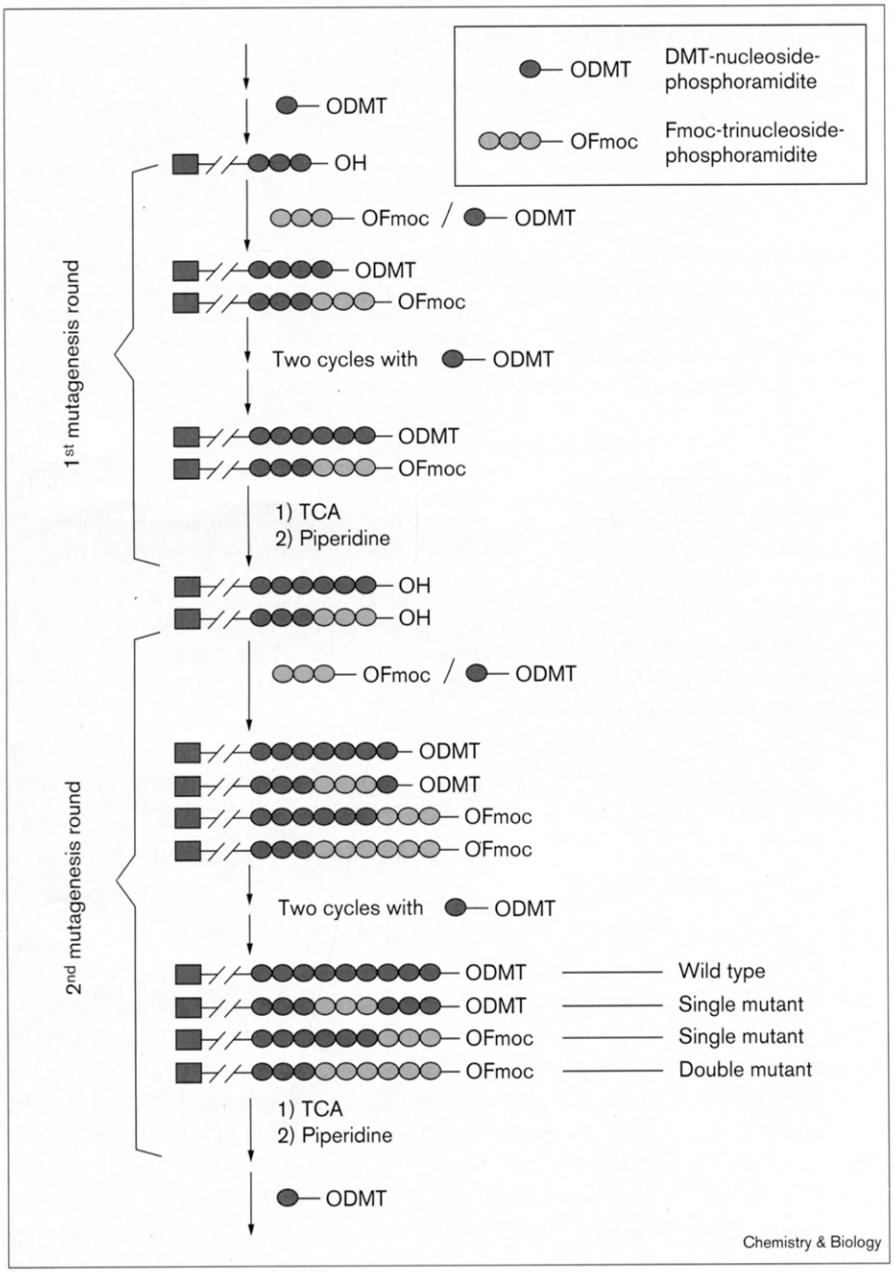
Introduction

It has been recognized for a number of years that mutagenesis methods aimed at creating diversity in gene fragments of various sizes suffer from important drawbacks: a bias introduced by the genetic code and inherent limitations in the distribution of replacements that result from the substitution of bases one by one, as opposed to three at a time [1]. These limitations are particularly relevant when the goal is to achieve one or a few mutations per gene (nonsaturating conditions) as opposed to saturating conditions concentrated in one or a few contiguous codons [2,3]. For example, directed evolution methods frequently require uniform generation of diversity over whole genes [4,5], but a mutant collection averaging one amino-acid replacement per gene will only sample about 25% of all possible replacements with currently available methods [3]. Alanine-scanning mutagenesis, which is performed by introducing alanine replacements one by one [6], would similarly benefit from the creation of libraries containing a high representation of one alanine replacement per gene.

A number of strategies have been used to improve the performance of nonsaturating mutagenesis methods [3,7,8], with synthetic DNA offering, in principle, the best starting point to achieve the most sophisticated mutagenesis schemes. Degenerate oligonucleotides (collections of synthetic DNA fragments that vary in one or more positions) have been used as mutagenic agents for more than a decade [9], and synthetic DNA offers an obvious approach to drastically enhance the diversity generated: whole mutagenic oligonucleotides (oligos) could be synthesized using trinucleotides, thereby easily controlling the proportion of degenerate codons by varying the molar ratio of wild-type codon trinucleotides with respect to a mixture of up to 20 other trinucleotides, coding for the rest of the amino acids [2,10–12]. It should be noted, however, that most of the work described so far has been performed using DMT-trinucleotides at saturating conditions, with Shortle and Sondek [10] reporting substoichiometric coupling, for insertions only, combining DMT-mononucleotides and DMT-trinucleotides. A major disadvantage of using exclusively trinucleotides is the difficulty of maintaining the stock of trinucleotides and the correspondingly high increased cost. Employing only monomers, the introduction of a controlled proportion of wild-type versus degenerate codons has also been achieved using the splitting-column method [13,14,8], which requires the interruption of the oligonucleotide synthesis and the separation of the solid support into two

Figure 1

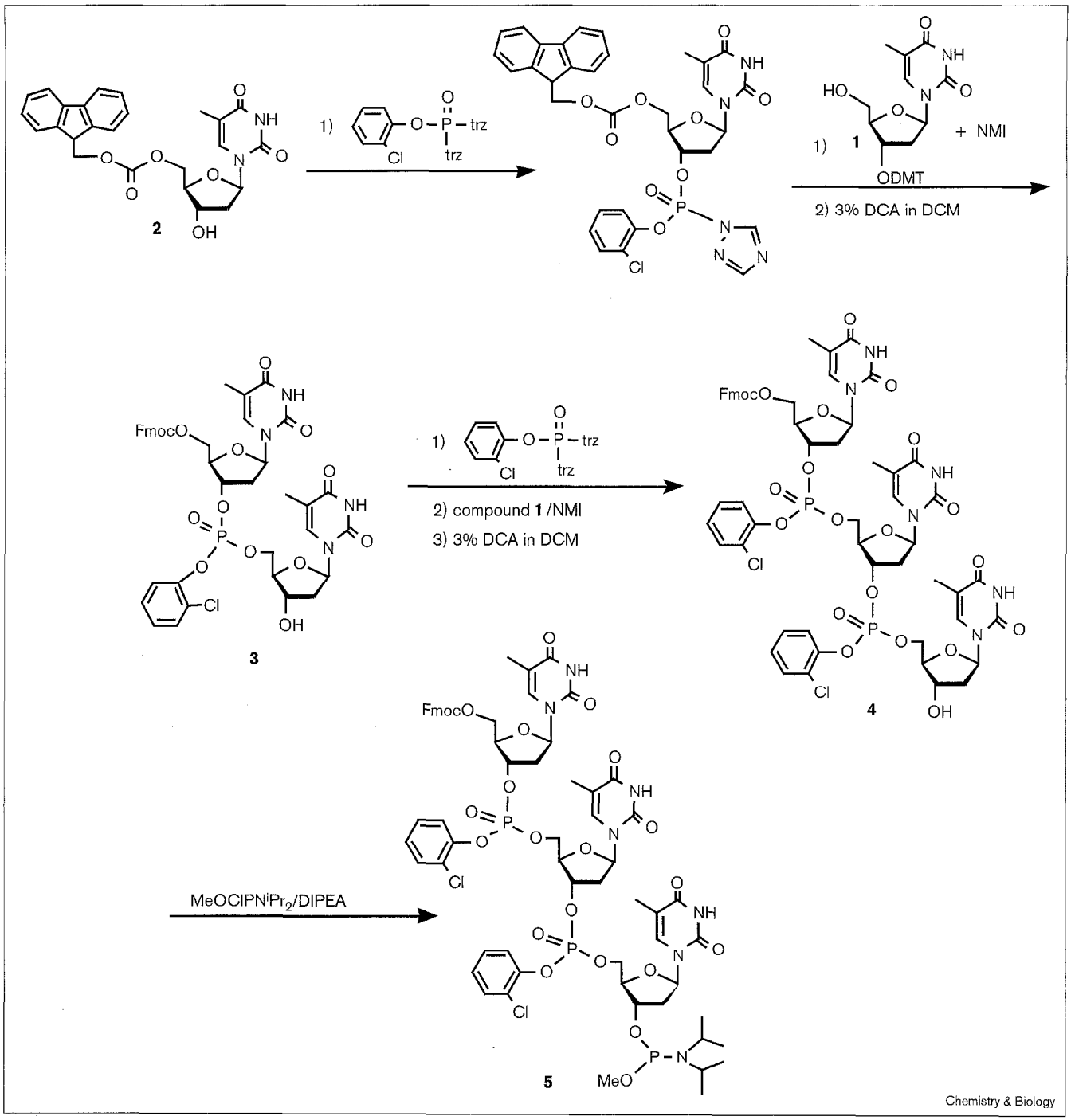


The Fmoc/DMT combining method for synthesis of oligonucleotide libraries. Synthesis of the wild-type sequence is performed using DMT-nucleoside-phosphoramidites until the point where a mutation must be introduced, where coupling with an appropriate mixture of the following DMT-nucleoside-phosphoramidite and the Fmoc-trinucleoside-phosphoramidite is performed to produce the first mutagenesis cycle. The next two cycles are performed using DMT-nucleoside-phosphoramidites to reach a uniform length. At this point the Fmoc group is removed with piperidine and the DMT is removed in the usual manner. A mutagenesis cycle has been completed and can be repeated as necessary.

parts (in different proportions, if wanted); one part is subjected to three mononucleotide couplings with the wild-type sequence, whereas the other is subjected to three consecutive nucleotide couplings with appropriate mixtures of mononucleotides. The two portions of the resin are re-mixed and the cycle repeated as needed. Here, the main drawback is that frequent separation and re-mixing of the resin is tedious, hard to automate and deleterious to the overall yield.

Shortle and Sondek proposed in 1992 [10] a scheme that could potentially achieve the controlled doping of oligos in a codon-oriented manner via substoichiometric coupling of trinucleotide phosphoramidites (Figure 1). No experimental results, however, have appeared so far using the proposed approach, which involved the use of alternative chemistries at the 5′ protecting group. We report here the experimental implementation of such a method, and demonstrate that 9-fluorenylmethoxycarbonyl (Fmoc) and dimethoxytrityl (DMT) are protecting groups compatible with each other and with the conventional phosphoramidite-synthesis protocols, thereby permitting fully automated synthesis of mutagenic oligos with codon-based variability.

Figure 2



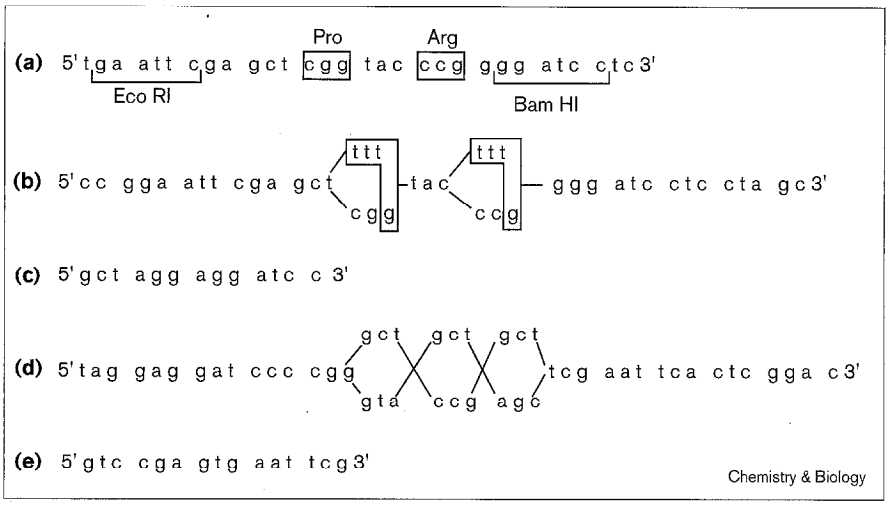
Chemical synthesis of Fmoc-TTT-phosphoramidite **5**. Synthon **4** was assembled using the phosphotriester method as described in the Materials and methods section. DCA, dichloroacetic acid; DCM, dichloromethane; NMI, N-methylimidazole; DIPEA, N,N-diisopropylethylamine.

Results and discussion

Selection of protecting groups and synthesis of Fmoc-trinucleotide-phosphoramidites

Out of several alternative 5′ hydroxyl protecting groups (e.g., levulinyl (Lev) [15], 2-dansylethoxycarbonyl (Dnseoc) [16], 2-(2,4-dinitrobenzenesulphenyloxymethyl) benzoyl (DNBSB) [17], 9-fluorenylmethoxycarbonyl (Fmoc) [18–20], t-butyldimethylsilyl (TBDMS) [21], t-butyldiphenylsilyl (TBDPS) [22]), we chose Fmoc for use in further experiments, because of its commercial

Figure 3



The oligonucleotides synthesized in this study. **(a)** Wild-type sequence coding for the β-galactosidase α-fragment. A change in the proline codon destroys the *Sac*I restriction site, whereas a change in the arginine codon destroys the *Sma*I restriction site. **(b)** Oligonucleotide libraries (LMRL or HMRL) were created using different proportions of DMT-dG^{ib}-phosphoramidite to Fmoc-TTT-phosphoramidite. **(c)** The first extension primer (EP1). **(d)** Oligonucleotide libraries (LM–AS or HM–AS) for alanine scanning were created using dilute solutions of Fmoc-GCT-phosphoramidite. **(e)** The second extension primer (EP2).

availability, proven performance in biopolymer synthesis [18,19], stability, regio-selectivity [20] and rapid removal in mild basic conditions. Synthon **4**, precursor of the Fmoc-TTT-phosphoramidite **5**, was synthesized as shown in Figure 2 using the phosphotriester method in solution phase, through a fully protected scheme that made use of 3′-O-DMT-thymidine **1** and 5′-O-Fmoc-thymidine **2**. Intermediate compounds were purified by flash column chromatography and their structure and purity was spectroscopically verified (see the Materials and methods section). Phosphitylation of **4** with the monophosphitylating reagent N,N-diisopropyl-methyl-phosphonamidic chloride afforded the target compound **5**, which was only semi-purified by rapid flash chromatography in the presence of 10% pyridine in dichloromethane. Upon *n*-hexane precipitation, we obtained a white powder that was stable, when stored dry at –20°C, for at least 2 years. In acetonitrile and standard temperature, the compound had a half-life of 6.7 days (as measured by high performance liquid chromatography (HPLC) [23]), which is ample time to conduct automated oligonucleotide synthesis.

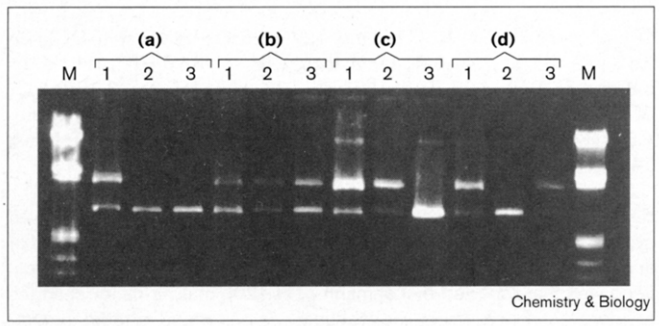
To carry out a preliminary assessment of the suitability of levulinyl as protecting group, we synthesized 5′-O-Fmoc-2-N-isobutyryldeoxyguanosine-3′-O-methyl-N,N-diisopropyl-phosphoramidite and 5′-O-Lev-2-N-isobutyryldeoxy-guanosine-3′-O-methyl-N,N-diisopropylphosphoramidite. Preliminary results indicate that levulinyl derivatives are indeed more stable in solution than those containing Fmoc. At 10 mM concentration in acetonitrile, Fmoc- and Lev-containing compounds had half-lives of 6.5 and 20.1 days, respectively. These results suggest that the levulinyl group could have advantages for the protection of the trimers. We confirmed, however, that one problem associated with levulinyl nucleosides is the low regioselectivity of the levulinyl group towards the 5′ hydroxyl group at the moment of nucleoside protection, yielding a high quantity of the 3′ byproduct [24], whose elimination by column chromatography was difficult.

In order to demonstrate the feasibility of the synthetic route shown in Figure 2 for the preparation of other Fmoc-trinucleotides, we also synthesized the Fmoc-GCT-phosphoramidite, which corresponds to an alanine codon, useful in the alanine-scanning mutagenesis procedure [6]. In this case, during the preparation of the Fmoc-GC dimer, significant amounts of byproduct were generated (evident by thin layer chromatography (TLC) analysis) after acidic hydrolysis of the 3′-DMT protecting group, as a direct consequence of the depurination of N-isobutyryl-deoxyguanosine to acid. After column purification, the dimer could be obtained in a pure form, but only at a 17% overall yield. To solve the problem, we decided to use N-benzoyl-deoxycytidine instead of 3′-O-DMT-N-benzoyl-deoxycytidine for the internucleotidic coupling, even at the expense of possible 3′-3′ coupling. After column purification, the 5′-3′ Fmoc-GC dimer was obtained in a pure form with a 62% overall yield. The authenticity of this compound was confirmed using HPLC, after removal of all protecting groups and direct comparison with a GC dimer prepared by solid-phase automated synthesis (see the Supplementary material). The Fmoc-GC dimer was then similarly reacted with unprotected thymidine to obtain the Fmoc-GCT trimer, precursor of the Fmoc-GCT-phosphoramidite, which was subsequently column-purified.

Design and synthesis of mutagenic oligonucleotides

We used the Fmoc-TTT-phosphoramidite to test the compatibility with DMT-based chemistry, employing the oligonucleotide synthesis protocol shown in Figure 1

Figure 4



Restriction analysis of representative library clones. **(a)** Wild-type sequence, **(b)** double mutant, **(c)** single mutant in *Sac*I site, **(d)** single mutant in *Sma*I site. Lanes 1, plasmid without digestion, lanes 2, *Sma*I digestion; lanes 3, *Sac*I digestion; M, molecular weight marker.

(detailed in the Materials and methods section). As a model study, we decided to introduce a certain proportion of the TTT codon at two positions within the wild-type sequence of the poly-linker segment of the gene encoding the α-peptide of β-galactosidase in a conventional cloning vector (see Figure 3). A DNA cassette derived from these oligos could be cloned as an *Eco*RI–*Bam*HI fragment and the substitution of the arginine and proline codons by TTT would destroy a *Sma*I or a *Sac*I restriction site, respectively. In order to demonstrate the controllability of the mutagenesis rate and the distribution of mutants, we synthesized two oligonucleotide libraries, at high mutagenic rates (HMRL) and low mutagenic rates (LMRL) (1:1.36 and 1:7.9 trimer to monomer molar ratio, respectively). As both codons have guanine at the third position, the procedure required the preparation of only two mixtures of the DMT-G monomer with the Fmoc-TTT phosphoramidites, one for each of the two different molar ratios. The incorporation of monomers during the synthesis was monitored spectrophotometrically; the incorporation of Fmoc-containing synthons was inferred by subtraction, with an average yield of 28.6% for HMRL and 8.1% for LMRL.

The Fmoc-GCT-phosphoramidite was employed to scan three contiguous amino acids (valine, proline and serine) located between the *Eco*RI and *Bam*HI sites of the poly-linker region described above, as shown in Figure 3d (coding strand complementary to that shown in Figure 3a). Again, we synthesized two alanine-scanning libraries at different mutagenesis rates, employing the protocol described by Shortle and Sondek [10] instead of preparing mixtures of monomers and trimers. In this case, we used only diluted solutions of the trimer, coupling them at the beginning of the codons to be scanned, and skipping the capping step. The library at high mutagenesis rate (HM–AS) was prepared with the Fmoc-trimer at 31.1 mM concentration in acetonitrile, whereas the library at low mutagenesis rate (LM–AS) was prepared at an Fmoc-trimer concentration of 16.7 mM.

Cloning of duplex DNA fragments and distribution analysis

Purified DNA cassettes were obtained with DNA polymerase and extension primer 1 (EP1) using the HMRL or LMRL oligos as template. The cassettes were cloned into plasmid pUC19 as *Eco*RI–*Bam*HI restriction fragments. For each mutagenesis rate we analyzed 40 clones by restriction enzyme digesting with *Sma*I and *Sac*I; the observed rate of disappearance of the restriction site was taken as indication of replacement by the mutagenic trinucleotide TTT (Figure 4 and Table 1). Sequencing of selected clones (one for each category and mutagenesis rate) confirmed that all of them contained only the expected changes. The small sample analyzed is reasonably consistent with the distribution of replacements expected theoretically.

Similarly, the DNA cassettes for alanine scanning were created using the HM–AS and LM–AS oligonucleotides with extension primer 2 (EP2). In this case, we analyzed 50 clones for each mutagenesis rate by sequencing the DNA directly. With LM–AS, we obtained 42 wild-type clones, seven single mutants and one double mutant, whereas in the case of HM–AS we obtained 36 wild-type, 11 single

Table 1

***Sac*I/*Sma*I restriction analysis of individual clones of the oligonucleotide libraries at low (LMRL) and high (HMRL) mutagenesis rate.**

Clone phenotype	LMRL			HMRL		
	Number of clones	Experimental fraction (%)	Theoretical fraction (%)	Number of clones	Experimental fraction (%)	Theoretical fraction (%)
Wild type	36	90.0	84.4	20	50.0	50.9
Double mutants	1	2.5	0.6	6	15.0	8.2
*Sac*I single mutant	1	2.5	7.4	4	10.0	20.4
*Sma*I single mutant	2	5.0	7.4	10	25.0	20.4
Total single mutants	3	7.5	14.8	14	35.0	40.8

The theoretical fraction was calculated using the binomial distribution equation. Forty clones were analyzed in total in each case (LMRL and HMRL).

mutants and three double mutants. Again, the results broadly correlate with the expected mutagenesis rate.

Significance

Our results demonstrate that it is possible to combine two different 5′ protecting groups in an automated oligonucleotide synthesis protocol and obtain enzymatically and biologically active molecules. The combination of 9-fluorenylmethoxycarbonyl (Fmoc)-trinucleotides and dimethoxytrityl (DMT)-mononucleotides allowed us to manipulate the introduction of sequence substitutions at the codon level, resulting in a controlled distribution of single and multiple amino-acid replacements in the coded protein. The general application of this, or related protocols, should provide for a new mutagenesis method, with significant advantages over pre-existing ones.

Materials and methods

^1H and ^{31}P nuclear magnetic resonance (NMR) analyses were obtained at 300 and 121 MHz on a Varian VXR. The samples were tested on a mixture of $CDCl_3$-DMSO containing tetramethylsilane as internal reference or 85% H_3PO_4 as external reference, respectively. Fast atom bombardment-mass spectrometry (FAB–MS) analyses were obtained on a Jeol JSM-SX 102A with double beam using *m*-nitrobenzylalcohol as matrix. Melting points (mp) were determined on a Fisher-Johns melting point apparatus and are reported uncorrected. All reactions were followed using TLC on aluminum-backed silica gel 60 F_{254} sheets (Merck) using $CHCl_3$/MeOH (9:1 v/v) as the elution system. The products were purified by flash column chromatography using silica gel 60H (Merck, 5–40 µm) as the stationary phase and methanol gradients in CH_2Cl_2 for the elution process. The reagents employed for synthesis of the Fmoc-trinucleotide-phosphoramidites were all purchased from Aldrich and used without additional treatments. The mutagenic oligonucleotides were assembled on an Applied Biosystems DNA Synthesizer 381A using a 0.2 mmol standard protocol as recommended by the manufacturer. Oligonucleotide libraries were created through a combination of DMT-nucleoside-Me-phosphoramidites (Glen Research) and the Fmoc-trinucleotide-phosphoramidites synthesized in this work. All additional reagents for oligonucleotide synthesis were prepared from Aldrich. Reagents for electrophoresis including acrylamide, agarose and buffers were all purchased from Sigma or Bio-Rad. Restriction endonucleases, T4 DNA ligase, Klenow polymerase, pUC19 plasmid and dNTP's were bought from Boheringer Mannheim and used according to standard protocols.

Syntheses

3′-O-(4,4′-dimethoxytrityl)thymidine (1). 5′-O-t-butyldimethylsilylthymidine (3.56 g, 10 mmol), prepared as described by Sproat *et al.* [22], was co-evaporated with pyridine (2 × 30 ml) and re-dissolved in 60 ml of the same solvent. 4,4′-O-dimethoxytrityl chloride (DMT-Cl, 4.61 g, 13.6 mmol) was added in powder under nitrogen and the reaction was kept overnight under magnetic stirring. On the following day, when the reaction was shown to be almost complete using TLC analysis, the excess of DMT-Cl was quenched with methanol for 30 min and the mixture was concentrated to dryness. The oily residue was taken up in dichloromethane and washed with saturated sodium bicarbonate and brine. After drying and filtering from anhydrous sodium sulfate, the organic phase was concentrated to an oil and subjected to flash column chromatography to quickly remove the 4,4′-O-dimethoxytritylmethyl ether byproduct. The semi-purified intermediate 5′-O-t-butyldimethylsilyl-3′-O-(4,4′-O-dimethoxytrityl)thymidine, obtained as a foam, was dissolved in 100 ml of THF and treated with 1.2 equivalents of tetrabutylammonium fluoride overnight to remove the TBDMS group. The reaction work-up was as before and the product was carefully purified by flash chromatography using a gradient of ethyl acetate (AcOEt) in *n*-hexane. The product was eluted with 90% of AcOEt and was finally obtained as a white powder (3.55 g) by precipitation from cold *n*-hexane.

Overall yield 65.3%; R_f 0.74; mp 127–128°C; ^1H NMR ($CDCl_3$): δ 9.13 (H3, 1H, s), 7.44-6.83 (aromatics, 14H, m), 6.15 (H1′, 1H, dd, J= 8.8 and 5.9 Hz), 4.36 (H3′, 1H, bs), 3.97 (H4′, 1H, bs), 3.78 (MeO-DMT, 6H, s), 3.65 (H5′, 1H, m), 3.30 (H5″, 1H, m), 1.9 (H2′α, 1H, m), 1.82 (H7, 3H, d, J= 1.2 Hz), 1.7 (H2′β, 1H, m). FAB-MS (m-NBA): m/z 545 ($M+H^+$).

5′-O-(9-Fluorenylmethoxycarbonyl)thymidine (**2**). This compound was synthesized from fluorenylmethoxycarbonyl chloride (13 mmol, 3.36 g) and thymidine (10 mmol, 2.42 g) in pyridine as solvent. The protocol used was that reported by Lehmann *et al.* [20], making minor changes to improve it. For example, the reaction was performed at room temperature instead of 0°C with no practical difference between product distribution. In both cases, the reactions seemed to proceed simultaneously, so we allowed 5 min for the compounds to react. Although the product was purified by column chromatography, those fractions (recovered with acetone) that were contaminated with its 3′ counterpart were treated with a small amount of *n*-hexane to selectively obtain the precipitation of compound **2** as a white powder (3.1 g).

Yield: 66.7%; R_f 0.6; mp 190–191°C; ^1H NMR ($CDCl_3$/DMSO): δ 10.24 (H3, 1H, s), 7.76–7.31 (Fmoc-aromatics, 8H), 7.34 (H6, 1H, q, J= 1.0 Hz), 6. 36 (H1′, 1H, t, J= 6.5 Hz), 5.03 (OH 3′, 1H, d, J= 4.5 Hz), 4.53 and 4.45 (CH_2 of Fmoc, 2H, 2dd, J= 10.5 and 7.0 Hz), 4.44 and 4.39 (H5′, 2H, 2dd, J= 12.0, 11.5, 5.0 and 3.0 Hz), 4.36 (H3′, 1H, m), 4.25 (CH of Fmoc, 1H, t, J= 7 Hz), 4.12 (H4′, 1H, dd, J= 4.0 and 3.0 Hz), 2.36 (H2′ß, 1H, ddd, J= 14.0, 6.5 and 4.0 Hz), 2.08 (H2′α, 1H, ddd, J= 13.5, 6.5 and 6.5 Hz), 1.80 (H7, 3H, d, J= 1.0 Hz). FAB-MS (m-NBA): m/z 465 ($M+H^+$).

5′-O-(9-Fluorenylmethoxycarbonyl)thymidine-3′-yl(o-chlorophenyl)phosphate-5′-ylthymidine (**3**). Compound **2** (5 mmol, 2.32 g, previously co-evaporated with anhydrous pyridine) was activated with 60 ml of a 0.1 M solution of the phosphorylating reagent *o*-chlorophenylphosphoroditriazolide. After 2 h of reaction, when the TLC analysis showed that activation was complete, the reaction mixture was transferred, using a plastic canule, to another flask containing compound **1** (4.62 mmol, 2.5 g, previously dried as above), and dry, double-distilled N-methylimidazole (NMI, 1.24 ml) was added to catalyze this coupling reaction. When the reaction was complete, 2 ml of a 10% aqueous solution in pyridine was added to quench the excess of activated nucleoside and was worked up as compound **1**. The fully protected dimer was then subjected to detritylation at 0°C with 80 ml of 5% dichloroacetic acid in dichloromethane for 5 min, after which the solution was quickly neutralized with saturated $NaHCO_3$, followed by brine. After drying over anhydrous Na_2SO_4, filtration and concentration of the organic phase, the remaining yellowish oil was purified by flash column chromatography, employing a methanol gradient in CH_2Cl_2. Compound **3** eluted with 3% methanol and was obtained as a white powder (2.76 g) by precipitation in cold *n*-hexane.

Yield: 63%; R_f 0.40; mp 120–121°C; ^1H NMR ($CDCl_3$/DMSO) of the diastereomeric mixture: δ 11.30-11.14 (H3, 2H, 4s), 7.87–7.22 (Fmoc-aromatics + *o*-chlorophenyl-aromatics + H6, 14H, m), 6.20 (H1′, 2H, q, J = 6.9 Hz), 5.43 (OH 3′, 1H, d, J = 4.5 Hz), 5.17 (H3′, 1H, bs), 4.42-4.26 (4H5′ + 1H4′ + 1H3′ + CH and CH_2 of Fmoc, m, 9H), 4.0 (H4′, 1H, bs), 2.45 (H2′, 2H, m), 2.13 (H2″, 2H, m), 1.71 (H7, 3H, s), 1.70 (H7, 3H, s); ^{31}P NMR ($CDCl_3$/DMSO): δ -11.13 (s); FAB-MS (m-NBA): m/z 879 ($M+H^+$).

5′-O-(9-Fluorenylmethoxycarbonyl)thymidine-3′-yl(o-chlorophenyl)phosphate-5′-ylthymidine-3′-yl(o-chlorophenyl)phosphate-5′-ylthymidine (**4**). This compound was synthesized using the protocol described for compound **3** but at a 3 mmol scale. Compound **3** was first activated with *o*-chlorophenyl-phosphoroditriazolide and then reacted with compound **1**. The fully protected trimer was subsequently detritylated. Compound **4** was obtained as a white powder after purification and precipitation.

Yield: 67.8%; R_f 0.36; mp 128–130°C; ^{1}H NMR ($CDCl_3$/DMSO) of the diastereomeric mixture: δ 11.29 (H3, 2H, bs), 11.22 (H3, 1H, bs), 7.86–7.21 (Fmoc-aromatics + *o*-chlorophenyl-aromatics + H6, 19H), 6.19 (H1′, 3H, 2d, J = 6.9 and 6.0 Hz), 5.42 (OH 3′, 1H, d, J = 4.2 Hz), 5.22 (H3′, 1H, bs), 5.16 (H3′, 1H, bs), 4.54-4.28 (CH and CH_2 of Fmoc + 6H5′ + 2H4′ + 1H3′, 12H, m), 3.99 (H4′, 1H, bs), 2.47 (H2′, 4H, m), 2.132 (H2′, 2H, m), 1.70 (H7, 9H, s). ^{31}P NMR ($CDCl_3$/DMSO): δ −11.089 (1P, s), −11.270 (1P, 2s). FAB–MS (m-NBA): m/z 1299 ($M+H^+$).

5′-O-(9-Fluorenylmethoxycarbonyl)thymidine-3′-yl(o-chlorophenyl)-phosphate-5′-ylthymidine-3′-yl(o-chlorophenyl)-phosphate-5′-ylthymidine-3′-O-methyl-N,N-diisopropylphosphoramidite (**5**). The Fmoc-trimer **4** (1 mmol, 1292 mg) was dried by coevaporation with anhydrous pyridine (2 × 20 ml), followed by coevaporation with anhydrous THF (20 ml) until formation of a white foam, which was redissolved in 20 ml of THF, followed by syringe addition of N,N-diisopropylethylamine (DIPEA, 4 mmol, 696 µl) and N,N-diisopropyl-methylphosphonamidic chloride (3 mmol, 603 µl). After 20 min magnetic stirring, the reaction was quenched with saturated $NaHCO_3$ (5 ml), diluted with CH_2Cl_2 and subjected to standard work up. The remaining oil was diluted with CH_2Cl_2 (5 ml) and purified by flash column chromatography with 10% of pyridine in dichloromethane. The fractions that contained the compound were joined, concentrated and precipitated at −40°C in *n*-hexane with strong stirring. After drying overnight, over phosphorus pentoxide under a high vacuum, the Fmoc-trimer-phosphoramidite **5** was obtained as a white powder, which was stable at −20°C for at least 2 years.

Yield: 50.4%; R_f 0.580; 1H NMR ($CDCl_3$/DMSO) of the diastereomeric mixture: δ (Fmoc-aromatics + *o*-chlorophenyl-aromatics + H6, 19H), 6.24 (H1′, 3H, m), 5.24 (2H3′, m), 4.60-4.18 (1H3′ + CH and CH_2 of Fmoc + 6H5′ + 3H4′, 13H, m), 3.62-3.45 (CH of isopropyl, 2H, m), 3.37 and 3.36 (CH_3OP, 3H, 2d, J = 13.2 Hz), 2.65-2.17 (H2′, 6H, m), 1.88–1.72 (H7, 9H, m), 1.241 (CH_3 of isopropyl, 12H, d, J = 3.3 Hz); ^{31}P NMR ($CDCl_3$/DMSO): δ 146.40 and 146.90 (1P), −11.10 (2P).

5′-O-(9-Fluorenylmethoxycarbonyl)-2N-isobutyryldeoxyguanosine (**Fmoc-dG**). This compound was synthesized as compound **2**, employing 2N-isobutyryldeoxyguanosine (10 mmol, 3.37 g) and Fmoc-Cl (13 mmol, 3.36 g) in pyridine as solvent. After normal work up and column chromatography purification, we obtained 7.83 g of a white powder corresponding to pure Fmoc-dG. This compound was eluted with 9% MeOH in CH_2Cl_2.

Yield: 70.0%; R_f 0.35; mp 118–120°C; ^{1}H NMR ($CDCl_3$): δ 12.41 (H1, 1H, s), 10.57 (NH of dG, 1H, s), 7.93 (H8, 1H, s), 7.68-7.13 (Fmoc-aromatics, 8H), 6.18 (H1′, 1H, t, J = 6.0 Hz), 5.47 (OH 3′, 1H, bs), 4.80 (H3′, 1H, s), 4.49 (CH_2 of Fmoc + 2H5′ + H4′, 5H, m), 4.14 (CH of Fmoc, 1H, t, J = 7.2 Hz), 2.85 (CH of isobutyryl, 1H, sept, J = 6.6 Hz), 2.64 (H2′β, 1H, bs), 2.46 (H2′β, 1H, bs), 1.20 (CH_3 of isobutyryl, 6H, d, J = 6.6 Hz).

5′-O-(9-Fluorenylmethoxycarbonyl)-2N-isobutyryldeoxyguanosine-3′-yl(o-chlorophenyl)-phosphate-5′-yl-4N-benzoyldeoxycytidine (**Fmoc-GC dimer**). This compound was prepared as dimer **3**, using Fmoc-dG (5 mmol, 2.80 g) and three molar equivalents of 4N-benzoyldeoxycytidine (15 mmol, 4.97 g) instead of compound **1** and **2**. The main difference with respect to the procedure of dimer **3**, was the absence of the acid step no longer necessary to remove a DMT group. After normal work-up, column chromatography purification and cold *n*-hexane precipitation, we obtained 2.8 g of a white powder corresponding to the Fmoc-GC dimer. The title compound was eluted with 7% MeOH in CH_2Cl_2.

Yield: 56%; R_f 0.38; mp 128–130°C; ^{1}H NMR ($CDCl_3$) of the diastereomeric mixture: δ 12.03 and 11.98 (H1 of dG, 1H, 2s), 10.78 and 10.34 (NH of dG, 1H, 2s), 8.92 (NH of dC, 1H, bs), 8.39 and 8.19 (H6 of dC, 1H, 2d, J = 7 Hz), 7.85-7.40 (Fmoc-aromatics + *o*-chlorophenyl-aromatics + benzoyl-aromatics + H8 of dG + H5 of dC, 19H, m), 6.24, 6.06 and 5.87 (H1′, 2H, 3m), 5.42 and 5.37 (H3′ of dG, 1H, 2m), 4.72 (H3′ of dC, 1H, m), 4.63 (H5′ of dC, 2H, m), 4.49-4.20 (CH_2 of Fmoc + H4′ of dG + H4′ of dC + 2H5′ of dG + H3′ of dC, 7H, m), 4.17 (CH of Fmoc, 1H, t, J = 7.5 Hz), 2.98-2.92 (CH of isobutyryl, 1H, m) 2.91-2.63 (2H2′ of dG + 2H2′ of dC, 4H, m), 1.26, 1.23 and 1.20 (CH_3 of isobutyryl, 6H, 3d, J = 7 Hz). ^{31}P NMR ($CDCl_3$): δ −8.39 and −8.20 (2s).

5′-O-(9-Fluorenylmethoxycarbonyl)-2N-isobutyryldeoxyguanosine-3′-yl(o-chlorophenyl)-phosphate-5′-yl-4N-benzoyldeoxycytidine-3′-yl(o-chlorophenyl)phosphate-5′-ylthymidine (**Fmoc-GCT trimer**). This compound was synthesized by the procedure described for the Fmoc-GC dimer, using as starting materials the Fmoc-GC dimer (3 mmol, 3.18 g) and thymidine (9 mmol, 2.18 g). After normal work-up, column chromatography purification and cold *n*-hexane precipitation, we obtained 2.21 g of a white powder corresponding to the Fmoc-GCT trimer. The title compound was eluted with 8% MeOH in CH_2Cl_2.

Yield: 53%; R_f 0.36; mp 133–135°C; ^{1}H NMR ($CDCl_3$) of the diastereomeric mixture: δ 12.14 (H1 of dG, 1H, s), 10.43, 10.30 and 10.23 (NH of dG, 1H, 3s), 9.81, 9.68 and 9.58 (H3 of dT, 1H, 3bs), 8.58 and 8.07 (H6 of dC, 1H, 2m), 7.98 (H8 of dG, 1H, m), 7.92-7.04 (aromatics, 24H, m), 6.20 (H1′, 3H, m), 5.41 (H3′ of dG, 1H, m), 5.33 (H3′ of dC, 1H, m), 4.63-4.25 (CH_2 of Fmoc + H4′ and H5′ of dG + H4′ and H5′ of dC + H3′,H4′ and H5′ of dT, 12H, m), 4.17 (CH of Fmoc, 1H, t, J = 7Hz), 3.00-2.15 (2H2′ of dG + 2H2′ of dC + 2H2′ of dT + CH of isobutyryl, 7H, m), 1.79 (H7 of dT, 3H, m), 1.17 (CH_3 of isobutyryl, 6H, m); ^{31}P NMR ($CDCl_3$): δ −6.90, −6.98, −7.07, −7.12, −7.16, −7.48, −7.58, −7.74 (8s).

5′-O-(9-Fluorenylmethoxycarbonyl)-2N-isobutyryldeoxyguanosine-3′-yl(o-chlorophenyl)-phosphate-5′-yl-4N-benzoyldeoxycytidine-3′-yl(o-chlorophenyl)phosphate-5′-ylthymidine-3′-O-methyl-N,N-diisopropylphosphoramidite (**Fmoc-GCT-phosphoramidite**). Phosphitylation of the Fmoc-GCT trimer (1 mmol, 1.47 g) was performed using the procedure described for preparation of compound **5**. In this case, however, the Fmoc-GCT-phosphoramidite was purified over silica gel 60 (40–63 µm) instead of silica gel 60H, obtaining a product in high purity. The compound was eluted with 20% pyridine in CH_2Cl_2 and precipitated over cold *n*-hexane to obtain 671 mg of a white powder that was ready dissolved in acetonitrile.

Yield: 41%; ^{1}H NMR ($CDCl_3$): δ 12.10 (H1 of dG, 1H, 1s), 10.68, 10.65, 10.62 and 10.29 (NH of dG, 1H, 3s), 8.89 (H3 of dT, 1H, bs), 8.22-8.06 (H6 of dC, 1H, m), 7.83-7.10 (aromatics, 20 H, m), 6.39-5.89 (H1′, 3H, m), 5.40-5.31 (H3′ of dG + H3′ of dC, 2H, m), 4.66-4.32 (CH_2 of Fmoc + H4′ of dG + H4′ and H5′ of dC + H3′, H4′ and H5′ of dT, 10H, m), 4.22 (CH of Fmoc + H5′ of dG, 3H, m), 3.56 (CH of isopropyl, 2H, m), 3.36 (MeOP, 3H, m), 3.10-2.10 (CH of isobutyryl + H2′ of dG + H2′ of dC + H2′ of dT, 7H, m), 1.83 (H7 of dT, 3H, m), 1.26 (CH3 of isopropyl, 12H, m), 1.16 (CH_3 of isobutyryl, 6H, m); ^{31}P NMR ($CDCl_3$): δ 150.60-149.97 (P of phosphoramidite, 1P, m), −6.62 to −7.44 (P of phosphates, 2P, m).

5′-O-(9-Fluorenylmethoxycarbonyl)-2N-isobutyryldeoxyguanosine-3′-O-methyl-N,N-diisopropylphosphoramidite (**Fmoc-dG-phosphoramidite**). This compound was synthesized and purified by the procedure described by Balgobin *et al.* [19]. After precipitation over cold *n*-hexane, a white powder corresponding to the title compound was obtained and dried overnight over phosphorus pentoxide under high vacuum.

^{1}H NMR ($CDCl_3$) of the diastereomeric mixture: δ 11.9 (H1, 1H, bs), 8.96 (NH of dG, 1H, bs), 7.79 and 7.78 (H8, 1H, 2s), 7.76-7.22 (Fmoc-aromatics, 8H, m), 6.25 (H1′, 1H, dd, J = 7.5 and 6.0 Hz), 4.66 (H3′+1H5′, 2H, m), 4.48 (CH_2 of Fmoc, 2H, m), 4.43-4.38 (1H5′ + H4′, 2H, m), 4.26 and 4.25 (CH of Fmoc, 1H, 2t, J = 7.5 Hz), 3.60 (CH of isopropyl, 2H, m), 3.4 (MeOP, 3H, 2d, J = 13.5 Hz), 2.8 (CH of isobutyryl, 1H, m), 2.59 (H2′, 2H, m), 1.22-1.16 (CH_3 of isopropyl + CH_3 of isobutyryl, 18H, m). ^{31}P NMR ($CDCl_3$): δ 150.04 and 149.77 (2s).

5′-O-Levulinyl-2N-isobutyryldeoxyguanosine-3′-O-methyl-N,N-diisopropylphosphoramidite (**Lev-dG-phosphoramidite**). 5′-O-Lev-2-isobutyryldeoxyguanosine (1 mmol, 435 mg, synthesized with the procedure

reported by van Boom *et al.* [24]) was phosphitylated under the conditions used for compound **5**, to afford a white foam after purification.

^{1}H NMR ($CDCl_3$) of the diastereomeric mixture: δ 11.98 (H1, 1H, bs), 9.1 (NH of dG, 1H, bs), 7.78 and 7.77, (H8, 1H, 2s), 6.21 and 6.20 (H1′, 1H, 2t, J = 7.0 Hz), 4.58 (H3′ + 1H5′, 2H, m), 4.36 (1H5′ + H4′, 2H, m), 3.59 (CH of isopropyl, 2H, m), 3.40 and 3.39 (CH_3OP, 3H, 2d, J = 13.5 Hz), 2.83 (CH of isobutyryl, 1H, m), 2.72 (CH_2-CO2 + H2′α, 3H, m), 2.57 (CH_2COMe + H2′ß, 3H, m), 2.15 and 2.14 (CH_3 of levulinyl, 3H, 2s), 1.49 and 1.48 (CH_3 of isobutyryl, 6H, 2d, J = 7.0 Hz), 1.18 (CH_3 of isopropyl, 12H, d, J =7.0 Hz). ^{31}P NMR ($CDCl_3$): δ 149.92 and 149.62 (2s).

Synthesis of oligonucleotide libraries

For the experiments with the Fmoc-TTT-phosphoramidite, two oligonucleotide libraries with the sequence: 5′ CC GGA ATT CGA GCT CGX TAC CCX GGG ATC CTC CTA GC 3′ were synthesized by the phosphite-triester method, employing a combination of DMT-nucleoside-Me-phosphoramidites (for assembling of the wild-type sequence) and the Fmoc-trimer-phosphoramidite **5**. Here **X** represents mixtures with different proportions of DMT-dG^{ib}-phosphoramidite and the Fmoc-trimer-phosphoramidite **5**. In the library at low mutagenesis rate (LMRL), **X** corresponded to an acetonitrile/THF (3:1 v/v) solution being 0.087M in DMT-dG^{ib}-phosphoramidite and 0.011 M in Fmoc-trimer-phosphoramidite **5** (rate 7.9:1.0). In the case of the library at high mutagenesis rate (HMRL), **X** was 0.075 M in DMT-dG^{ib}-phosphoramidite and 0.055 M in Fmoc-trimer-phosphoramidite **5** (rate 1.36:1.0). The coupling efficiency of the trimer was calculated by the difference between the DMT cation released in the 14th and 15th coupling and the 20th and 21st coupling. Measuring the absorbance at 304 nm of the Fmoc group released with 5% piperidine in acetonitrile gave a very similar result.

The synthesis of the mutagenic oligonucleotides required the use of the fifth vial of the synthesizer for the **X** mixture and to program the synthesis in three steps:

fragment **1**:	5′ CC**X** GGG ATC CTC CTA GC 3′
fragment **2**:	5′ CG**X** TAC 3′ fragment 1
fragment **3**:	5′ CC GGA ATT CGA GCT 3′ fragment 2

The synthesis cycles for the first two fragments were programmed Tr-on and that of the third fragment was Tr-off. As soon as the synthesis of fragment **1** concluded, it was subjected, in the same column, to a 1 min manual treatment with 3 ml of a 5% piperidine solution in acetonitrile to remove the Fmoc group. After washing with acetonitrile, the column was re-connected to the on-line tubing and fragment **2** was synthesized. The process for removal of the Fmoc group was repeated and the synthesis of the fragment **3** was finally performed to generate the fully protected oligonucleotide library. The oligonucleotides were de-methylated with thiophenol/TEA/dioxane, followed by a treatment with 0.4 M 2-pyridinealdoxime and 0.4 M 1,1,3,3-tetramethylguanidinium in dioxane/water (1:1 v/v) and 28% NH_4OH.The oligonucleotides were purified by electrophoresis in 15% polyacrilamide gels containing 8 M urea.

A third oligonucleotide (extension primer 1, EP1) with the sequence 5′GCT AGG AGG ATC C3′ was synthesized to prime the mutagenic oligonucleotides in order to construct the mutagenic cassettes with the aid of Klenow polymerase.

The alanine-scanning experiments were performed with the Fmoc-GCT-phosphoramidite. In this case, as above, we also synthesized two oligonucleotide libraries with different levels of substoichiometric coupling of the trinucleotide, now with the sequence:

5′ TAG GAG GAT CCC CGG GTA**X** CCG**X** AGC**X** TCG AAT TCA CTC GGA C 3′

where **X** represented the coupling of diluted solutions of the Fmoc-GCT-phosphoramidite (16.7 mM and 31.1 mM acetonitrile solutions for libraries LM-AS and HM-AS respectively). This trimer was placed in vial X of the synthesizer and coupled through a modified cycle of synthesis lacking the capping step to allow all hydroxyl groups that did not react with the trimer to react in the next cycle with the DMT-nucleoside-Me-phosphoramidite that comprised the first nucleotide of the wild-type codon. After three monomer couplings, the Fmoc was removed as above and the synthesis was completed.

Finally, a second extension primer (EP2) with the sequence 5′GCT CGA GTG AAT TCG 3′ was synthesized to generate the alanine-scanning cassettes.

Recombinant DNA methods

Following established recombinant DNA methods [25] we processed and cloned synthetic DNA to generate mutant libraries. 50 pmol of each gel-purified oligo (HMRL/LMRL with EP1 or LM–AS/HM–AS with EP2) were annealed by incubating for a few minutes at 95°C and allowing to cool down to 37°C. Full duplex molecules were obtained by the action of Klenow fragment DNA polymerase I. The duplex DNA products were gel-purified after digestion with *Eco*RI and *Bam*HI and ligated to plasmid pUC19 [26] previously digested with the same enzymes. The ligation mixture was electroporated into *E. coli* JM101 [26] and the transformed cells plated in selective media containing ampicillin and incubated overnight. 40 clones for each of the HMRL and LMRL libraries, and 50 clones for each of the LM–AS and HM–AS libraries were picked at random for each of the four mutagenic regimes, cultured in liquid medium and plasmid DNA isolated by mini-preps [25] to perform restriction analysis with enzymes *Sac*I and *Sma*I, in the case of HMRL and LMRL. The nucleotide sequence of the relevant DNA segment was obtained for 16 colonies (four for each restriction digestion outcome: no *Sac*I or *Sma*I site present, *Sac*I but no *Sma*I, *Sma*I but no *Sac*I, both sites present), utilizing Thermosequenase according to the manufacturer's procedure (Amersham). In the case of LM–AS and HM–AS, clones picked at random were analyzed directly by DNA sequencing ^{1}H and ^{31}P NMR spectrums of Fmoc-GCT-phosphoramidite.

Supplementary material

Supplementary material available with the online version of this paper includes the ^{1}H and ^{31}P NMR spectra of Fmoc-GCT-phosphoramidite.

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