# Single tube gene synthesis by phosphoramidate chemical ligation

# Supplementary Information

Afaf H. El-Sagheer[a,b] and Tom Brown[a*]

[a]Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford, OX1 3TA, UK

[b]Chemistry Branch, Dept. of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez University, Suez, 43721, Egypt.

*Tel: +44(0)1865 275413, e-mail: tom.brown@chem.ox.ac.uk

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## Experimental

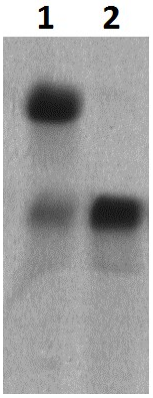
### General method for oligonucleotide synthesis and purification

Standard DNA phosphoramidites, solid supports, and additional reagents were purchased from Link Technologies Ltd and Applied Biosystems Ltd. 5'-Monomethoxytritylamino-2'-deoxythymidine,3'-[(2-cyanoethyl)-(*N,N*-diisopropyl)]-phosphoramidite was purchased from Glen Research (Catalog Number: 10-1932-90). All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/ RNA synthesizer using a standard 0.2 or 1.0 $\mu$mole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by the automated trityl cation conductivity monitoring facility and in all cases were >98.0%. All β-cyanoethyl phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for normal A, G, C, and T monomers was 60 s, and the coupling time for the 5′-amino dT phosphoramidite monomer was extended to 600 s. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C. Purification of oligonucleotides was carried out by reversed-phase HPLC on a Gilson system using a Brownlee Aquapore column (C8, 8 mm x 250 mm, 300Å pore) with a gradient of acetonitrile in triethylammonium bicarbonate (TEAB) increasing from 0% to 50% buffer B over 30 min with a flow rate of 4 mL/min (buffer A: 0.1 M triethylammonium bicarbonate, pH 7.0, buffer B: 0.1 M triethylammonium bicarbonate, pH 7.0 with 50% acetonitrile). Elution of oligonucleotides was monitored by ultraviolet absorption at 295 or 300 nm. After HPLC purification, oligonucleotides were freeze dried then dissolved in water without the need for desalting. For long oligonucleotides, polyacrylamide gel electrophoresis was used for purification. Oligonucleotide bands were then visualized using a UV lamp and the desired bands excised, crushed and soaked in water overnight at 37 °C. After evaporation, samples were desalted using NAP-25 followed by NAP-10 columns (G.E. Healthcare Life Sciences). All oligonucleotides were characterised by electrospray mass spectrometry using a Bruker micrOTOF II focus ESI-TOF MS instrument in $ESI^-$ mode. Data were processed using MaxEnt.

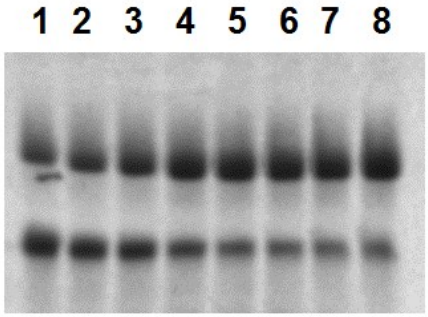
### Synthesis of 81-mer (ODN 1) template with one phosphoramidate ligation point

Oligonucleotides ODN 2 (1.0 nmol), ODN 3 (1.1 nmol) and splint ODN 4 (1.0 nmol) in 0.25 M HEPES (pH=7.2) with 1.0 M NaCl (80 μL) were annealed by heating at 90 °C for 5 min then cooling slowly to room temperature. A solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 10 μL) (0.1 M final concentration) and EDC.HCl (6.0 M, 10 μL) (0.6 M final concentration) was added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h. Reagents were removed using NAP-25 gel-filtration column and the ligated DNA was analysed and purified by denaturing 12% polyacrylamide gel electrophoresis (See figure S1). The reaction was scaled up 10-fold and aliquots were taken at different time points (figure S2).

The gel showed that the reaction was complete within 45 min and there was no difference in intensity of the product after 2 h.



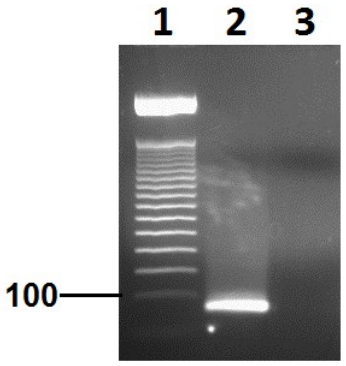

**Supplementary Figure S1.** 12% Denaturing PAGE analysis of 3 -phosphate/5 -amine oligonucleotides ligation to give the phosphoramidate-containing product. Lane 1; phosphoramidate reaction mixture (ODN 1, 81-mer), lane 2; reference starting material ODN 3. An excess of the amine oligonucleotide (ODN 3) was used, resulting in a residual lower band and complete consumption of the phosphate oligonucleotide.




**Supplementary Figure S2.** 12% Denaturing PAGE analysis for optimisation of 3 -phosphate/5 -amine oligonucleotides ligation to give the phosphoramidate template (ODN 1, 81-mer) top bands. Lanes 1-8; reaction mixture after 5, 10, 15, 30, 45, 60 120 and 360 min. An excess of the amine oligonucleotide was used resulting in a residual lower band and consumption of the phosphate oligonucleotide.

## PCR amplification of the 81-mer phosphoramidate-containing template (ODN 1)

GoTaq DNA polymerase was used to generate a PCR product from the 81-mer template (ODN 1) which includes one phosphoramidate linkage. Reagents and conditions: 4 μL of 5x buffer (Promega green PCR buffer) was used in a total reaction volume of 20 μL with 5 ng of the DNA template, 0.5 mM of each primer, 0.2 mM dNTP and 1.0 unit of GoTaq polymerase. The reaction mixture was loaded onto a 2% agarose gel in 1xTBE buffer. PCR cycling conditions: 95 °C (initial denaturation) for 2 min then 25 cycles of 95 °C (denaturation) for 15 s, 54 °C (annealing) for 20 s and 72 °C (extension) for 30 s. The reaction was then left at 72 °C for 5 min then loaded onto a 2% agarose gel in 1 X Tris/Borate/EDTA buffer (TBE) (Figure S3).
5 X Promega green PCR buffer was provided with the enzyme (Promega GoTaq DNA polymerase), pH 8.5 containing 7.5 mM $MgCl_2$ to give a final $Mg^{2+}$ concentration of 1.5 mM. The buffer contains Tris.HCl, KCl and two dyes (blue and yellow) that separate during electrophoresis to monitor the migration process.




**Supplementary Figure S3.** PCR amplification of the 81-mer phosphoramidate DNA template (ODN 1). Lane 1; 50 bp DNA ladder, lane 2; PCR using the phosphoramidate-containing template ODN 1, lane 3; control PCR without a phosphoramidate template.

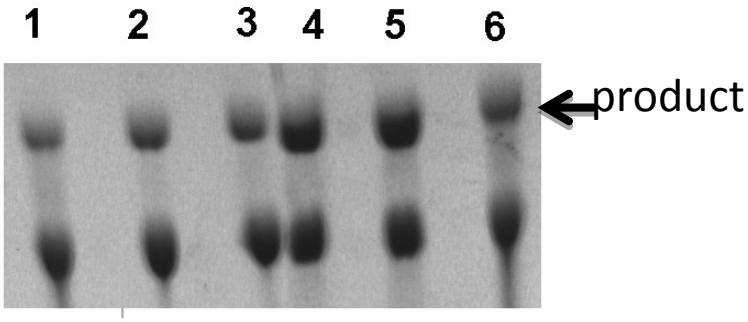
**Synthesis of 303-mer (ODN 5) template with two phosphoramidate ligation points**

Oligonucleotides ODN 6, ODN 7, ODN 8 with splints ODN 9 and ODN 10 (0.5 nmol of each) in 0.25 M HEPES (pH=7.2) with 1.0 M NaCl (80 μL) were annealed by heating at 90 °C for 5 min then cooling slowly to room temperature. A solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 10 μL) (0.1 M final concentration) and EDC.HCl (6.0 M, 10 μL) (0.6 M final concentration) was added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h. Reagents were removed using NAP-25 gel-filtration column and the ligated DNA was analysed by denaturing 6% polyacrylamide gel electrophoresis.

A mixture of 3 nmoles of each oligonucleotide and splints were dissolved in water and then divided into 6 samples and each mixed with 2x buffer. A solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 10 μL) (0.1 M final concentration) and EDC.HCl (6.0 M, 10 μL) (0.6 M final concentration) was added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 1 h then analysed by denaturing 6% polyacrylamide gel electrophoresis (Figure S4).

The following buffer systems were used:

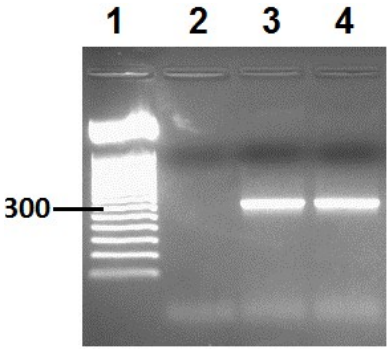
50 mM Tris (pH=8.5), 25 mM $MgCl_2$, 0.2 M NaCl; 10 mM phosphate (pH=7.0), 25 mM $MgCl_2$, 0.2 M NaCl; 0.25 M HEPES (pH=7.2), 1.0 M NaCl, 25 mM $MgCl_2$ and 0.25 M HEPES (pH=7.2), 1.0 M NaCl.




**Supplementary Figure S4.** 6% Denaturing PAGE analysis of 3 -phosphate/5 -amine oligonucleotide ligation to give the product containing two phosphoramidate linkages (ODN 5, 303-mer). Lane 1; 0.2 M NaCl, 25 mM $MgCl_2$, lane 2; 50 mM Tris (pH=8.5), 25 mM $MgCl_2$, 0.2 M NaCl, lane 3; 10 mM phosphate (pH=7.0), 25 mM $MgCl_2$, 0.2 M NaCl, lane 4; 0.25 M HEPES (pH=7.2), 1.0 M NaCl, 25 mM $MgCl_2$, lane 5; 0.25 M HEPES (pH=7.2), 1.0 M NaCl, lane 6; 0.4 M NaCl.

**PCR amplification of the 303-mer double phosphoramidate template ODN 5 using GoTaq DNA polymerase, cloning and sequencing**

Following the above method for PCR amplification of 81-mer ODN 1, the PCR product was purified by extraction from a 2% agarose gel (Figure S5) using a QIAquick Gel Extraction kit. It was then inserted into vector pCR2.1. TOPO for subcloning. Cloning was carried out using a standard TOPO cloning protocol. Standard automated Sanger DNA sequencing was performed and the data shown in Figure S6.




**Supplementary Figure S5.** PCR amplification of the 303-mer DNA template ODN 5. Lane 1; 50 bp DNA ladder, lane 2; control PCR without phosphoramidate linkage, lane 3 and 4; PCR using the phosphoramidate-containing template ODN 5 (303-mer).

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Res3954(303) Reference                AAGCTTTATTAAAATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCAATTTTGGTTGAATTAGATGGTGATG-TTAATGGTCACAAATTTTCTGTCTCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAA
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**Supplementary Figure S6.** Sequence alignment of 20 Clones from PCR of ODN 5 (2x phosphoramidate linkages in 303-mer section of EGFP gene, in red with the ligation points in blue). All the sequences are identical indicating the biocompatibility of the phosphoramidate linkage. Only a few mutations were observed and these are far from the ligation points (see Table S1). The mutations could have occurred during sequencing or during oligonucleotide synthesis and purification.

**Table S1**. Mutations found in sequence data in Figure S6

| TC | NMC | MC | IM | DM | SM | LPM | TB | TM |
|---|---|---|---|---|---|---|---|---|
| 20 | 13 | 7 | 1 | 10 | 1 | 0 | 6060 | 12 |

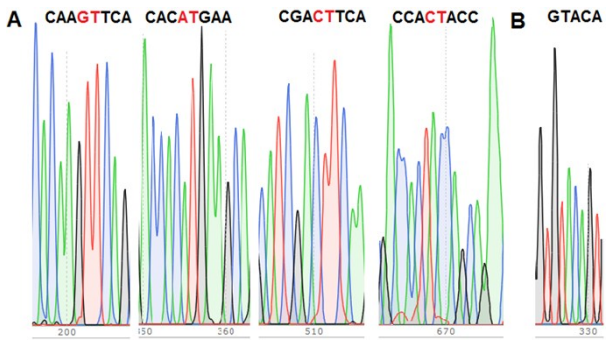
TC: total clones, NMC: non mutant clones, MC: mutant clones, IM: insertion mutation, DM: deletion mutation, SM: substituted mutation, LPM: ligation point mutation, TB: total number of bases, TM: total number of mutation.

**Single tube synthesis of the entire EGFP gene**

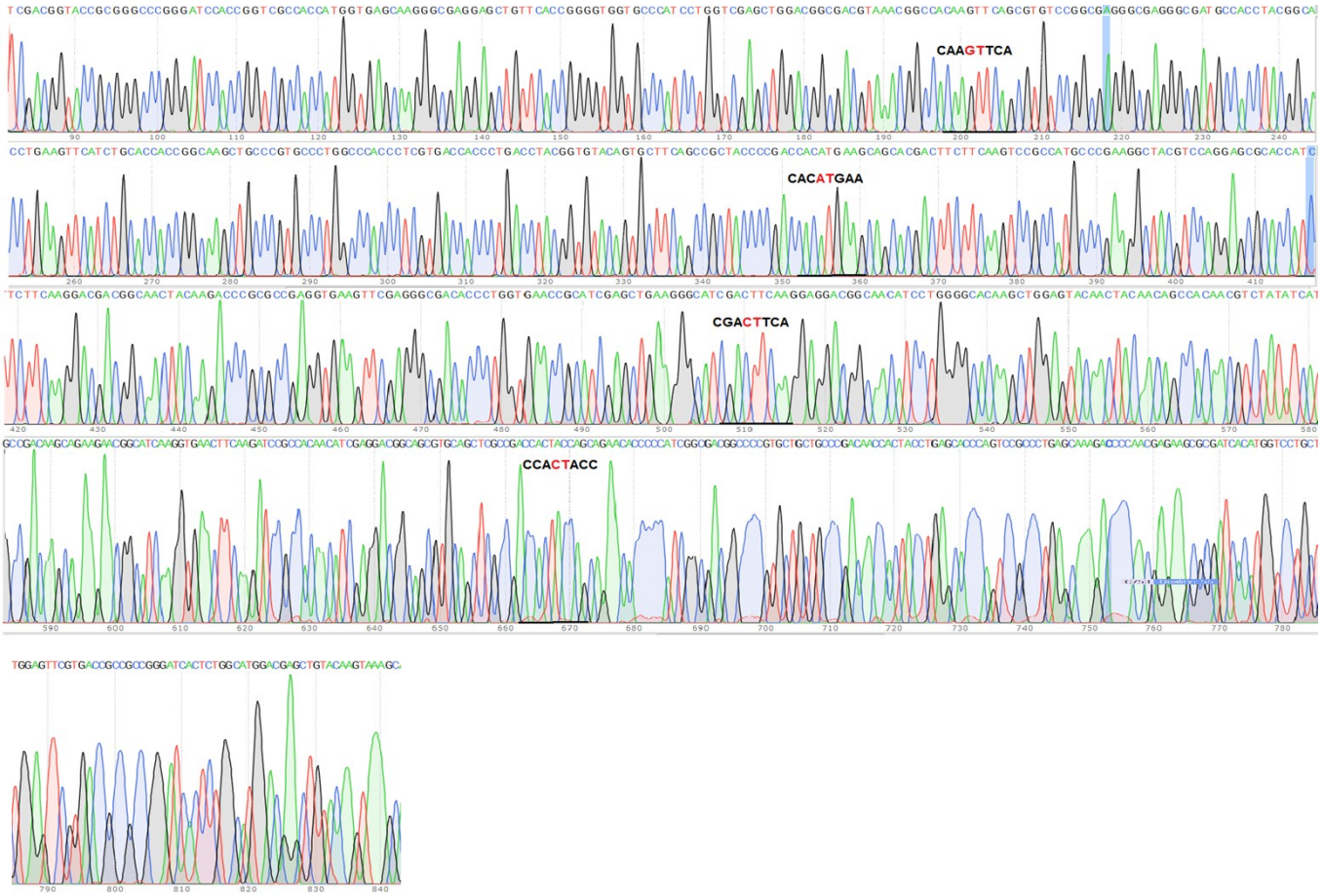
The synthesis of the entire EGFP gene in one tube was achieved by mixing 0.1 nmole of each of the 10 oligonucleotides (ODN 15-ODN 24 for both forward and reverse strands), freeze drying them together then re-dissolving them in 100 µL HEPES Buffer (0.25 M, pH=7.2) with 1.0 M NaCl. The oligonucleotides mixture was annealed by heating at 90 °C for 5 min then cooled slowly to room temperature. EDC.HCl (30 mg) and a solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 30 µL) were added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h. Reagents were removed using NAP-25 gel-filtration column and the ligated DNA was analysed by denaturing 4% polyacrylamide gel electrophoresis. The band was cut and DNA was extracted then used in PCR.

**PCR amplification of the double stranded phosphoramidate EGFP gene by phosphoramidate ligation using GoTaq DNA polymerase**

A PCR product from the whole EGFP gene duplex was generated using GoTaq DNA polymerase under the same conditions explained above for PCR of 81-mer ODN 1. The PCR product was purified by extraction from a 2% agarose gel (Figure 2A) using a QIAquick Gel Extraction kit. It was then inserted into the vector pCR2.1. Cloning into the TOPO vector was done with a standard TOPO cloning protocol. Automated Sanger DNA sequencing was performed; and the data is shown in Figure S7 and Figure S8. This procedure was carried out by Eurofins GmbH.




**Supplementary Figure S7**. Data from cloning and sequencing of the PCR product from the phosphoramidate EGFP gene (762-mer) showing the faithful copying at the ligation points (shown in red in the sequence text) (A) and the water mark GTACA (B). All clones show the water mark which was inserted into the sequence of the synthesised EGFP gene as a unique signature to differentiate it from potential contaminant DNA.




**Supplementary Figure S8**. Data from cloning and sequencing of the PCR product of the phosphoramidate EGFP gene (762-mer). The data show that the polymerase copied the gene faithfully including the bases around the phosphoramidate ligation points (shown in red in the inserted sequence text). Only one deletion mutation was found in this clone.

**Expected EGFP sequence with the ligation points in red and a watermark in blue**

TCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTG
GTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTG
AAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGTGTACAGTGCTTCAGCCGC
TACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGAC
GACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAG
GAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGC
ATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGC
GACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATG
GTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGC

**Found EGFP sequence watermark in blue and only one deletion mutation in red (Figure S8)**

CGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCG
AGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGT
TCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGTGTACAGTGCTTCAGCCGCTACC
CCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACG
GCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGG
ACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCA
AGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACG
GCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCC
TGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGC

Two more clones were sequenced and similar results were obtained with a small number of mutations which were far from the ligation points. Two deletion mutations were found in one clone whereas in the other clone, 4 deletion mutation and 2 substitution mutation were found. The mutation could have occurred during sequencing or oligonucleotide synthesis and purification. The mutation rate is consistent with that expected

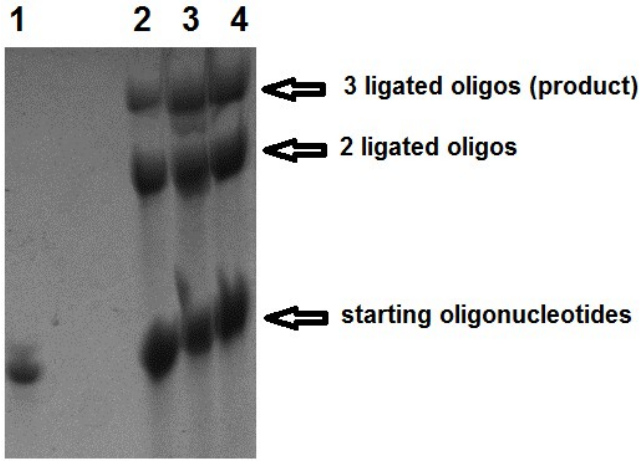
from oligonucleotide syntheses. All clones show the water mark which was inserted in the sequence of the synthesised EGFP as a unique signature to differentiate it from potential contaminant DNA. (Figure S7).

**Orthogonality of phosphoramidate and CuAAC reactions to form 303-mer and 331-mer DNA strands**

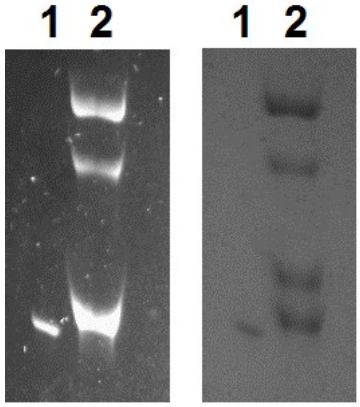
For CuAAC reactions, 0.5 nmole of ODN 35 and ODN 36 and ODN 37 with 3 -alkyne and 5 -azide and splints ODN 9 and ODN 10 in 0.2 NaCl (40 μL) were annealed by heating at 90 °C for 5 min then cooling slowly to room temperature. A solution of $Cu^{I}$ click catalyst was prepared from *tris*-hydroxypropyltriazole (0.7 μmol in 0.2 M NaCl, 17.0 μL), and sodium ascorbate (1.0 μmol in 0.2 M NaCl, 2.0 μL) and $CuSO_4.5H_2O$ (0.1 μmol in 0.2 M NaCl, 1.0 μL) was added to the above annealed oligonucleotides. The mixture was kept at room temperature for 2 h before analysis by denaturing 8% polyacrylamide gel electrophoresis.

For phosphoramidate reactions, 0.5 nmole of ODN 6 and ODN 7 and ODN 8 with 3 -phosphate and 5 -amine and splints ODN 9 and ODN 10 in 40 uL buffer (0.25 M HEPES, 1.0 M NaCl) were annealed by heating at 90 °C for 5 min then cooling slowly to room temperature. EDC.HCl (10 mg) and a solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 10 μL) were added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h before it was analysed by denaturing 8% polyacrylamide gel electrophoresis.

For orthogonal CuAAC and phosphoramidate reactions, 0.5 nmole of ODN 6 (3 -phosphate), ODN 38 (3 -propargyl and 5 -amine), ODN 37 (5 -azide) and splints ODN 9 and ODN 10 in 40 uL buffer (0.25 M HEPES, 1.0 M NaCl) were annealed by heating at 90 °C for 5 min then cooling slowly to room temperature. A solution of $Cu^{I}$ click catalyst was prepared from *tris*-hydroxypropyltriazole (0.35 μmol in 0.2 M NaCl, 17.0 μL), sodium ascorbate (1.0 μmol in 0.2 M NaCl, 1.0 μL) and $CuSO_4.5H_2O$ (0.1 μmol in 0.2 M NaCl, 1.0 μL) followed by EDC.HCl (10 mg) and a solution of 1-(2-hydroxyethyl) imidazole (1.0 M, 10 μL) were added to the annealed oligonucleotides and the reaction mixture was kept at room temperature for 2 h before being analysed by denaturing 8% polyacrylamide gel electrophoresis. Figure S9 shows similar results for all three reactions indicating the orthogonality of CuAAC click and phosphoramidate ligations. The orthogonal CuAAC and phosphoramidate reactions were repeated under the same conditions using fluorescently labelled oligonucleotide ODN 39 (3 -phosphate, 5'-Cy3), ODN 38 (3 -propargyl and 5 -amine), ODN 40 (5 -azide) and splints ODN 41 and ODN 42. Similar result was obtained as indicated by denaturing 8% polyacrylamide gel-electrophoresis (Figure S10). In all above reactions, salt was removed using NAP-25 or ethanol precipitation.




**Supplementary Figure S9**. Orthogonal phosphoramidate and CuAAC reactions for ligation of three oligonucleotides to make a 303-mer product. Lane 1; ODN 6, lane2; 2 x CuAAC reactions, lane 3; 2 x phosphoramidate reactions, lane 4; orthogonal phosphoramidate and CuAAC reactions. Denaturing 8% polyacrylamide gel-electrophoresis.




**Supplementary Figure S10**. Orthogonal phosphoramidate and CuAAC reactions for ligation of three oligonucleotides to make a fluorescent 331-mer product. Lane 1; starting material ODN 39, lane2; orthogonal phosphoramidate and CuAAC reactions using ODN 39 (3 -phosphate, 5'-Cy3), ODN 38 (3 -propargyl and 5 -amine), and ODN 40 (5 -azide). Denaturing 8% polyacrylamide gel-electrophoresis. Left (gel was visualised by Cy3 emission), right (gel was visualised by UV shadowing)

## Transcription of phosphoramidate template ODN 27 and control ODN 31

MegaScript T7 Transcription Kit (ThermoFislher Scientific, cat. no. AM1333) was used according to the manufacturer's recommended protocol. Reaction mixtures were prepared in the following order at room temperature: phosphoramidate template ODN 27 (5 µM, 2.5 µl), long coding strand ODN 32 (5 µM, 2.75 µl), water (14.75 µl), reaction buffer (10x, 5 µl), ATP (5 µl), CTP (5 µl), GTP (5 µl), UTP (5 µl) and enzyme mix (5 µl). The reaction mixture was then incubated at 37 °C and 10 µl aliquots removed at the specified times and mixed with an equal volume of formamide before storing at -80 °C. Samples were then loaded on 12% denaturing polyacrylamide gel (1x TBE, 7 M urea, W x D x H = 18 x 0.2 x 24.4 cm) at 20 W for 2 h.

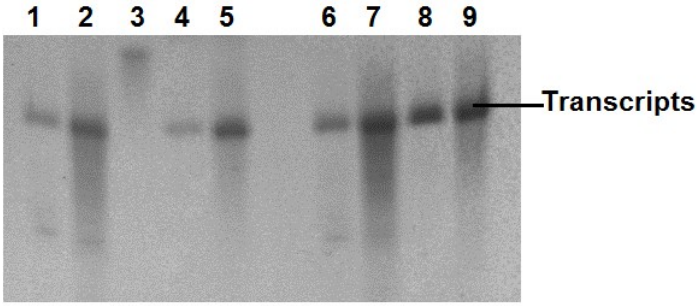
The same reaction was repeated using short coding strand ODN 33 and gave similar results.

For comparison of efficiency, the experiments were also performed using the control unmodified template ODN 31 using long coding strand ODN 32 and short coding strand ODN 33 (Figure S11).

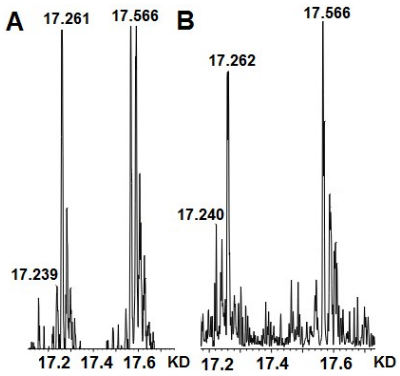
Oligonucleotide bands were then visualized using a UV lamp and the desired bands excised, crushed and soaked in buffer (50 mM Tris-HCl, pH 7.5, 25 mM NaCl) overnight at 37 °C. After evaporation of the solvent, samples were desalted using two NAP-25 columns (G.E. Healthcare Life Sciences, cat. no. 17-

0852-01). The expected product was confirmed by mass spectrometry of transcripts formed from phosphoramidate-containing and control strands using the long coding strand.

Transcription reaction (70 µl total volume) was performed as above using phosphoramidate (ODN 27) or control (ODN 31) template and long coding strand (ODN 32). The reaction mixture was left for 16 h before mixing with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1,v/v) (from Invitrogen) to remove excess reagents. The mixture was mixed vigorously and the top layer (transcripts) was removed. The RNA transcripts were precipitated by adding sodium acetate (3 M, 50 µl) followed by isopropanol (150 µl). The mixture was left at -80 °C for 3 h then centrifuged at 4 °C and 13 RPM for 10 min. The RNA was dried then dissolved in 20 µl water where 0.5 µl was analysed by mass spectrometry. The crude transcripts gave the same (expected) mass for phosphoramidate and control templates.




**Supplementary Figure S11.** Transcription of 79-mer unmodified and phosphoramidate-containing DNA templates. Lane 1 and 2, reaction using phosphoramidate template (ODN 27) and short coding strand (ODN 33) for 2 and 4 h respectively; lane 3; template ODN 31 lane 4 and 5, reaction using control template (ODN 31) and short coding strand (ODN 33) for 2 and 4 h respectively; Lane 6 and 7, reaction using phosphoramidate template (ODN 27) and long coding strand (ODN 32) for 2 and 4 h respectively; lane 8 and 9, reaction using control template (ODN 31) and long coding strand (ODN 32) for 2 and 4 h respectively. 15% polyacrylamide gel.




**Supplementary Figure S12.** ES- Mass spectra of A), the RNA transcripts from the phosphoramidate-containing template (ODN 27) and B), the normal template (ODN 31). The transcripts have the expected 5'-triphosphate and an additional 3'-cytidine. Required mass = 17.236 KD. Found mass, 17.239 (transcript with 5'-triphosphate), 17.261 (transcript with 5'-triphosphate + $Na^+$) and 17.566 (transcript with 5'-triphosphate and 3'-cytidine).

## Table S2: Oligonucleotides used in this study

**$^{N}$T= 5′-amino dT, p=3′-phosphate, X=3′-propargyl-5-Me-dC[1], $^{z}$T= 5′-azido dT[1], Y=5′-Cy3**

| Code | Oligonucleotide sequences (5′-3′) |
|---|---|
| **ODN 1** | GCATTCGAGCAACGTAAGATCGCTAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGAC ACACATAACC |
| **ODN 2** | GCATTCGAGCAACGTAAGATCGCp |
| **ODN 3** | $^{N}$TAGCACACAATCTCACACTCTGGAATTCACACTGACAATACTGCCGACACACATAACC |
| **ODN 4** | TGTGTGCTAGCGATCTTA splint |
| **ODN 5, 303-mer** | AAGCTTTATTAAAATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCAATTTTGGTTGAATTAGATG GTGATGTTAATGGTCACAAATTTTCTGTCTCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACC TTAAAATTTATTTGTACTACTGGTAAATTGCCAGTTCCATGGCCAACCTTAGTCACTACTTTCGGTTATGG TGTTCAATGTTTTGCTAGATACCCAGATCATATGAAACAACATGACTTTTTCAAGTCTGCCATGCCAGAAG GTTATGTTCAAGAAAGAAC |
| **ODN 6** | AAGCTTTATTAAAATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCAATTTTGGTTGAATTAGATG GTGATGTTAATGGTCACAAATTTTCTGTCp |
| **ODN 7** | $^{N}$TCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAATTTATTTGTACTACTGGTAAAT TGCCAGTTCCATGGCCAACCTTAGTCACTACp |
| **ODN 8** | $^{N}$TTTCGGTTATGGTGTTCAATGTTTTGCTAGATACCCAGATCATATGAAACAACATGACTTTTTCAAGTCT GCCATGCCAGAAGGTTATGTTCAAGAAAGAAC |
| **ODN 9** | CCATAACCGAAAGTAGTGACTAAG Splint for 303-mer template ligation |
| **ODN 10** | ACCTTCACCGGAGACAGAAAATTT Splint for 303-mer template ligation |
| **ODN 11** | GTTCTTTCTTGAACATAA PCR Primer 1 for 303-mer template |
| **ODN 12** | AAGCTTTATTAAAATGTCTA PCR Primer 2 for 303-mer template |
| **ODN 13, EGFP forward strand** | pTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCG GGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGC GAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTG GCCCACCCTCGTGACCACCCTGACCTACGGTGTACAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGC ACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGC AACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCAT CGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATA TCATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGC GTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCA CTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGT TCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGC |
| **ODN 14 EGFP reverse strand** | pGGCCGCTTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGAC CATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCA GCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCG ATGTTGTGGCGGATCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTG GCTGTTGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCT CGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTG AAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTG GTCGGGGTAGCGGCTGAAGCACTGTACACCGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCA GCTTGCCGGTGGTGCAGATGAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGAC ACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTC CTCGCCCTTGCTCACCATGGTGGCGACCGGTGGATCCCGGGCCCGCGGTACCG |
| **ODN 15** | pTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCG GGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGp 120-mer |
| **ODN 16** | $^{N}$TTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCA CCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGTGTACAGTGCTTCAGCCGC TACCCCGACCACAp 154-mer |
| **ODN 17** | $^{N}$TGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAA GGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGC TGAAGGGCATCGACp 155-mer |
| **ODN 18** | $^{N}$TTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCA TGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTG CAGCTCGCCGACCACp 156-mer |
| **ODN 19** | $^{N}$TACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGT CCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGG |

| | |
|---|---|
| | ATCACTCTCGGCATGGACGAGCTGTACAAGTAAAGC 177-mer |
| **ODN 20** | pGGCCGCTTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGAC CATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCA GCAGCACGGGGCCGp 155-mer |
| **ODN 21** | $^{N}$TCGCCGATGGGGGTGTTCTGCTGGTAGTGGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGA TCTTGAAGTTCACCTTGATGCCGTTCTTCTGCTTGTCGGCCATGATATAGACGTTGTGGCTGTTGTAGTTG TACTCCAGCTTGp 153-mer |
| **ODN 22** | $^{N}$TGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATGCCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGC CCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTTGAAGAAGATGGTGCGCTCCTGGACG TAGCCTp 147-mer |
| **ODN 23** | $^{N}$TCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGTAGCGGCTGAAGCACTGTACAC CGTAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGCAGATGAACTTCAGG GTCAGCTTGCCGp 153-mer |
| **ODN 24** | $^{N}$TAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACGTCGCCGTCCAGCTCGA CCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACCGGTGGATCCCGG GCCCGCGGTACCG 154-mer |
| **ODN 25** | TCGACGGTACCGCGGGCC PCR primer for EGFP forward strand |
| **ODN 26** | GCTTTACTTGTACAGCTCGTCC PCR primer for EGFP reverse strand |
| **ODN 27** | CACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACTTCTCCCTATAGTGAGTCGTATTAGG ACCAGCGT transcription template |
| **ODN 28** | $^{N}$TCGCCCTTGCTCACCATGGTGGCGACTTCTCCCTATAGTGAGTCGTATTAGGACCAGCGT |
| **ODN 29** | CACCCCGGTGAACAGCTCCp |
| **ODN 30** | GCAAGGGCGAGGAGCTGTTC splint |
| **ODN 31** | CACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACTTCTCCCTATAGTGAGTCGTATTAGG ACCAGCGT control for transcription |
| **ODN 32** | ACGCTGGTCCTAATACGACTCACTATAGGGAGAAGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCA CCGGGGTG long coding strand |
| **ODN 33** | ACGCTGGTCCTAATACGACTCACTATAGGGAGAAGTCGCC short coding strand |
| **ODN 34** | pppGGGAGAAGUCGCCACCAUGGUGAGCAAGGGCGAGGAGCUGUUCACCGGGGUGc transcript |
| **ODN 35** | AAGCTTTATTAAAATGTCTAAAGGTGAAGAATTATTCACTGGTGTTGTCCCAATTTTGGTTGAATTAGATG GTGATGTTAATGGTCACAAATTTTCTGTX |
| **ODN 36** | $^{Z}$TCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAATTTATTTGTACTACTGGTAAAT TGCCAGTTCCATGGCCAACCTTAGTCACTAX |
| **ODN 37** | $^{Z}$TTTCGGTTATGGTGTTCAATGTTTTGCTAGATACCCAGATCATATGAAACAACATGACTTTTTCAAGTCT GCCATGCCAGAAGGTTATGTTCAAGAAAGAAC |
| **ODN 38** | $^{N}$TCCGGTGAAGGTGAAGGTGATGCTACTTACGGTAAATTGACCTTAAAATTTATTTGTACTACTGGTAAAT TGCCAGTTCCATGGCCAACCTTAGTCACTAX |
| **ODN 39** | YGGCCGCTTTACTTGTACAGCTCGTCCATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGAC CATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCAGGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCA GCAGCACGGGGCCGp |
| **ODN 40** | $^{Z}$TTCAGCCGGTGCGTGATCAGAAAGGCGAGCTGCAATACTTTATTGGGGTTCAGTTAGATGGATCCGATCA TGTGG |
| **ODN 41** | TCACCTTCACCTTCACCGGACGGCCCCGTGCTGCTGCCCG splint 1 for 331 click amidate ligation |
| **ODN 42** | CTGATCACGCACCGGCTGAAGTAGTGACTAAGGTTGGCCA splint 1 for 331 click amidate ligation |

**Table S3: Oligonucleotide mass spectra**

| Code | Expected | Found |
|---|---|---|

| | | |
|---|---|---|
| ODN 1 | 24736 | 24739 |
| ODN 2 | 7135 | 7137 |
| ODN 3 | 17619 | 17619 |
| ODN 6 | 31056 | 31060 |
| ODN 7 | 31215 | 31216 |
| ODN 8 | 31474 | 31473 |
| ODN 15 | 37365 | 37367 |
| ODN 16 | 47237 | 47241 |
| ODN 17 | 47881 | 47886 |
| ODN 18 | 48229 | 48233 |
| ODN 19 | 54332 | 54330 |
| ODN 20 | 48197 | 48197 |
| ODN 21 | 47443 | 47447 |
| ODN 22 | 45296 | 45302 |
| ODN 23 | 47831 | 47835 |
| ODN 24 | 47261 | 47260 |
| ODN 27 | 24163 | 24162 |
| ODN 28 | 18384 | 18382 |
| ODN 29 | 5797 | 5797 |
| ODN 35 | 31031 | 31031 |
| ODN 36 | 31216 | 31217 |
| ODN 37 | 31500 | 31499 |
| ODN 38 | 31190 | 31193 |
| ODN 39 | 48625 | 48626 |
| ODN 40 | 23336 | 23336 |

**Reference**

1- A. H. El-Sagheer, A. P. Sanzone, R. Gao, A. Tavassoli and T. Brown, Proc. Natl. Acad. Sci. USA., 2011, 108, 11338-11343.