Supporting Information

Solid phase click ligation for the synthesis of very long oligonucleotides

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S1. Oligonucleotide Synthesis and Purification

Standard DNA phosphoramidites, solid supports (controlled pore glass and macroporous polystyrene) and additional reagents were purchased from Link Technologies, Jena Bioscience, Berry Associates and Applied Biosystems. NAP gel-filtration columns were purchased from GE Healthcare and used according to the manufacturer’s instructions. All oligonucleotides were synthesized on an Applied Biosystems 394 automated DNA/RNA synthesizer using a standard 1.0 μmole phosphoramidite cycle of acid-catalyzed detritylation, coupling, capping, and iodine oxidation. Stepwise coupling efficiencies and overall yields were determined by automated trityl cation conductivity monitoring 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. In the case of oligonucleotides containing 5'-iodo dT fast deprotecting monomers were used (UltraMILD monomers, Pac-dA, Ac-dC and iPr-Pac-dG, Link Technologies). The coupling time for normal A, G, C, and T monomers was 35 s, and the coupling time for the modified phosphoramidite monomers (5'-iodo-dT, 5'-BCN and 5'-DMT-(N4-Fmoc)-2'-deoxycytidine was extended to 360 s. Aminolink C7 DNA was used for the introduction of the 3'-amino-moiety into oligonucleotides. Unless stated elsewhere, 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. In the case of oligonucleotides containing 5'-iodo dT deprotection was carried out for 5h at room temperature. The oligonucleotides were purified by reversed-phase HPLC on a Gilson system using a Luna 10μ C8 100Å pore Phenomenex column (10x250 mm) with a gradient of acetonitrile in triethylammonium acetate or ammonium acetate (0% to 50% over 20 min, flow rate 4 mL/min), (buffer A: 0.1 M triethylammonium acetate, pH 7.0, buffer B: 0.1 M triethylammonium acetate, pH 7.0, with 50% acetonitrile). Elution was monitored by UV absorption at 295 nm. After HPLC purification, oligonucleotides were desalted using NAP-25 then NAP-10 columns (GE Healthcare) when using triethylammonium acetate, or NAP-10 columns only when using ammonium acetate buffer. Electrospray mass spectrometry of oligonucleotides were recorded in water using a Bruker micrOTOF™ II focus ESI TOF MS instrument in ES⁻ mode. Data were processed using MaxEnt.
S2. Labelling of amino-modified oligonucleotides with azidohexyl

6-Azidohexanoic acid NHS ester (compound 3 in main paper, 2 mg) in DMSO (80 µL) was added post-synthetically to the freeze dried amino-modified oligonucleotide (1.0 µmol) in 0.5 M Na₂CO₃/NaHCO₃ buffer (80 µL) at pH 8.75. After 4 h at room temperature the fully-labelled oligonucleotide was desalted using a NAP-25 sephadex column then purified by reversed-phase HPLC as explained above in the oligonucleotide synthesis and purification section (S1).

S3. Labelling of oligonucleotides with DIBO alkyne

![DIBO alkyne](image)

DIBO active p-nitrophenyl carbonate (2 mg, shown above) was dissolved in DMF (80 µL) then added post-synthetically to the freeze dried amino-modified oligonucleotide (1.0 µmol) in 0.5 M Na₂CO₃/NaHCO₃ buffer (80 µL) at pH 8.75. The reaction mixture was left for 4 h at 55 °C then desalted using a NAP-25 sephadex column. The labelled oligonucleotide was purified by reversed-phase HPLC, then desalted using a NAP-25 sephadex column.

S4. Labelling of oligonucleotides with click-easy BCN

The click-easy BCN N-hydroxysuccinimide carbonate (compound 1 in main paper) was purchased from Berry Associates. 2 mg of this compound was dissolved in DMF (80 µL), then added post-synthetically to the freeze dried amino-modified oligonucleotide (0.5 µmol) in 0.5 M Na₂CO₃/NaHCO₃ buffer (80 µL) at pH 8.75. After 4 h at 55 °C, the fully-labelled oligonucleotide was desalted using a NAP-25 sephadex column, then purified by reversed-phase HPLC and desalted using a NAP-25 sephadex column.
S5. Conversion of 5'-iodo oligonucleotides to 5'-azide oligonucleotides

5'-Azide oligonucleotides ON1, ON2 (17-mers) and ON5 (61-mer) were assembled on the 1.0 μmole scale (trityl-off) as described in the general method, and were modified at the 5'-end by addition of 5'-iodo-dT phosphoramidite. To convert the 5'-iodo-dT to 5'-azido-dT, sodium azide (20 mg) was suspended in dry DMF (1 mL), heated for 15 min at 70 °C then cooled down. The supernatant was taken up into a 1 mL syringe, passed back and forth through the column periodically, then left at room temperature overnight or at 55 °C for 5 h. The column was then washed with DMF and acetonitrile 3 times and dried by the passage of a stream of argon gas. The Fmoc protecting groups* of ON1 and ON2 were cleaved using 20% piperidine in DMF as follows: two 1 mL syringes were attached to the column and the reagent was passed back and forth periodically (every 2 min) for 20 min at room temperature. The column was then thoroughly washed with DMF and acetonitrile 3 times and dried by the passage of a stream of argon gas.

* ON1 and ON2, which contain only T and C bases, were made with N4-Fmoc protected deoxycytidine phosphoramidite monomer which enabled deprotection of the oligonucleotides on the solid support. This was done in order to study templated click ligation reactions on the solid support. The templated click ligation method did not give high yields of ligated DNA and optimisation is required.

S6. Synthesis of 5'-iodo-3'-azide oligonucleotides ON16 and ON19

5'-Iodo-3'-azide oligonucleotides ON16 and ON19 were synthesised on the 1.0 μmole scale, as explained in the oligonucleotide synthesis and purification section, using 5'-iodo-dT phosphoramidite monomer and aminolink C7 synthesis column. As the 5'-iodo group is labile under the normal conditions of oligonucleotide deprotection (5 h at 55 °C in aqueous ammonia), fast deprotecting monomers were used (UltraMILD monomers, Pac-dA, Ac-dC and iPr-Pac-dG, Link Technologies). Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 5 h at room temperature*. NAP-10 gel filtration was used to remove the cleaved protecting groups, after which the deprotected oligonucleotides were freeze-dried and labelled with 6-azidohexanoic acid NHS ester as explained in section (S2) - labelling oligonucleotides with azide.
*it was found that using 0.05M potassium carbonate in methanol for 4 hr at room temperature, as recommended by the supplier, gave a mixture of the correct product and a by-product which resulted from elimination of the 5'-iodine (loss of HI).

S7. CuAAC click ligation reactions
A solution of Cu\(^{1}\) click catalyst was prepared from tris-hydroxypropyltriazole ligand (THPTA)\(^{2}\) (3.2 μmol in H\(_2\)O, 5.2 μL), sodium ascorbate (1.6 μmol in H\(_2\)O, 3.2 μL) and CuSO\(_4\).5H\(_2\)O (0.16 μmol in H\(_2\)O, 1.6 μL). The Cu\(^{1}\) solution was mixed with the 3'-propargyl-oligonucleotide (ON3, 3eq or ON7, 10eq) in 10 μL of H\(_2\)O and 20 μL of dry DMF. The mixture was added to the azide oligonucleotide in the solid support (2 nmole). All CuAAC reactions were carried out in PCR tubes (250 μL, sealed with parafilm) and heated in a heating block at 40 °C for 21 h, 55 °C for 21 h or 70 °C for 7 h. After the click reaction, the solid support was washed 3 times with H\(_2\)O and 3 times with acetonitrile, then dried by the passage of a stream of argon gas. The resultant ligated oligonucleotide was cleaved from the solid support, deprotected by treating with concentrated aqueous ammonia solution for 5 h at 55 °C in a sealed tube. The cleaved protecting groups were removed using a NAP-10 gel-filtration column (GE Healthcare). All ligated oligonucleotide products were analysed by reversed-phase HPLC and mass spectrometry.

S8. SPAAC click ligation reactions
The 3'-BCN-oligonucleotide (ON9 or ON11) was dissolved in 20 μL of 10% pyridine/H\(_2\)O (pH 9.8) and added to the azide oligonucleotide on the solid support (2 nmole). Similarly, the azide-labelled oligonucleotide (ON14, ON16 or ON19) was dissolved in 10 μL of 10% pyridine/H\(_2\)O (pH 9.8) and added to the alkyne oligonucleotide in the solid support (2 nmole). All SPAAC reactions were carried out in PCR tubes (250 μL, sealed with parafilm) and put in a heating block at 40 °C for 21 h. After the click reaction, the solid support was washed 3 times with phosphate buffer (10 mM phosphate, 0.2 M NaCl, pH 7.0), H\(_2\)O and acetonitrile, and dried by the passage of a stream of argon gas. The resultant ligated oligonucleotide was cleaved from the solid support, then deprotected by treating with concentrated aqueous ammonia solution for 5 h at 55 °C in a sealed tube. The cleaved protecting groups were removed using a NAP-10 gel-filtration column (GE Healthcare). All the
ligated oligonucleotide products were analysed by reversed-phase HPLC and mass spectrometry.

**S9. SPAAC reactions for the synthesis of ON18 and ON21 (ligating three oligos on solid support)**

5'-BCN oligo (ON13) was ligated to 3'-azide-5'-iodo oligo (ON16 or ON19) for 21 h at 40 °C, as explained above in the SPAAC reaction section (S8). Next, to convert 5'-iodo to 5'-azido on solid support, sodium azide (10 mg) was suspended in dry DMF (0.5 mL), heated for 15 min at 70 °C then cooled down and transferred to 1.5 ml eppendorf tube containing the washed 5'-iodo oligonucleotide on solid support (ON17a or ON20a). The mixture was heated for 5 h at 55 °C then washed with DMF, water and acetonitrile. This was followed by reacting of the 5'-azido oligonucleotides (ON17b and ON20b) with 3'-BCN oligonucleotide (ON11) for 21 h at 40 °C followed by washing and deprotection as explained above in the SPAAC reaction section (S8).

**S10. DIBO click ligation reaction**

![HPLC chromatogram and mass spectrum (ESI) of the ligated product of 5´N₅-TTTTTTTTTTTT-500 Å resin with 3´-DIBO-TTTTTTTTTTTTTTTT](image1.png)

**Figure S1. Use of Dibenzocyclooctyne (DIBO) in solid-phase SPAAC ligation reactions**

HPLC chromatogram and mass spectrum (ESI) of the ligated product of 5´N₅-TTTTTTTTTTTT-500 Å resin with 3´-DIBO-TTTTTTTTTTTTTTTT. (calculated: 7351, found: 7352). The reversed-phase HPLC chromatogram of the product (blue) shows the expected mixture of diastereomers/regioisomers.
S11. PAGE gels, HPLC chromatograms and mass spectra

**Figure S2.** Denaturing polyacrylamide gel electrophoresis (PAGE) analysis of unpurified click- ligated products: Analysis of the click ligation reaction mixtures by 6% polyacrylamide/7M urea gel electrophoresis at a constant power of 20 watts for 1.5 h, using 1 X TBE buffer. a) Lane1; ON18 (186-mer) unpurified reaction mixture, lane2; ON13 (60-mer). b) Lane1; ON21 (151-mer) unpurified reaction mixture, lane2; ON13 (60-mer).

**Figure S3.** Reversed-phase HPLC chromatogram of CuAAC unpurified click-ligated ON4 (28-mer) a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI') of the ligated product (calculated: 8043, found: 8042).
Figure S4. Reversed-phase HPLC chromatogram of CuAAC unpurified click-ligated ON6 (72-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product after 21 h at 55 °C (calculated: 22232, found: 22232).

Figure S5. Reversed-phase HPLC chromatogram of incomplete CuAAC unpurified click-ligated ON6 (72-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product after 7 h at 55 °C showing the ligated product (calculated: 22232, found: 22232) and staring oligonucleotide (calculated:18872, found: 18872).

This study (Figures S4 and S5) confirms that it is possible by HPLC-MS to differentiate between a successful click ligation reaction and an incomplete reaction and that the reaction proceeds in high yield if left for a sufficient period of time.
Figure S6. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON10 (28-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product (calculated: 8735, found: 8736).
Figure S7. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON15 (120-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product after 21 h at 40°C (calculated: 37617, found: 37618).

Figure S8. Reversed-phase HPLC chromatogram of incomplete SPAAC unpurified click-ligated ON15 (120-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product after 7 h at 40 °C showing the ligated product (calculated: 37617, found: 37618) and staring oligonucleotide (calculated: 18769, found: 18770).

This study (Figures S7 and S8) confirms that it is possible by HPLC-MS to differentiate between a successful click ligation reaction and an incomplete reaction and that the reaction proceeds in high yield if left for a sufficient period of time.
Figure S9. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON17b (120-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product (calculated: 37615, found: 37615).

Figure S10. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON18 (186-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product (calculated: 58417, found: 58418).
Figure S11. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON20b (85-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product (calculated: 26798, found: 26798).

Figure S12. Reversed-phase HPLC chromatogram of SPAAC unpurified click-ligated ON21 (151-mer)
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the ligated product (calculated: 47600, found: 47601).
Figure S13. Reversed-phase HPLC chromatogram of starting oligonucleotide ON1 (17-mer) 
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the crude oligonucleotide 
(calculated: 5043, found: 5044).

Figure S14. Reversed-phase HPLC chromatogram of starting oligonucleotide ON2 (17-mer) 
a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the crude oligonucleotide 
(calculated: 5043, found: 5044).
Figure S15. Reversed-phase HPLC chromatogram of starting oligonucleotide ON5 (61-mer) a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the crude oligonucleotide (calculated: 18872, found: 18872). In the mass spectrum, the peak after the molecular weight peak is the Et$_3$N adduct.

Figure S16. Reversed-phase HPLC chromatogram of starting oligonucleotide ON13 (60-mer) a; reversed-phase HPLC chromatogram and b; mass spectrum (ESI) of the crude oligonucleotide (calculated: 18769, found: 18770). In the mass spectrum, the peak after the molecular weight peak is the Et$_3$N adduct.
Table S1 Sequences and mass spectrometry of oligonucleotides used in this study

For CuAAC reactions (Scheme 1 in main paper): X = 3’-propargyl 5-MedC, 5’N3 = 5’-azido, tz1 = biocompatible triazole linker.

For SPAAC reactions (Fig 3 in main paper): 5’BCN is added using phosphoramidite 2, 3’BCN from 1+4, 3’N3 from 3+4. Structures of tz2, tz3 and tz4 are also shown in Fig 3 in main paper.

*a mass was recorded for the azide oligos ON17b or ON20b

Mass spectra were recorded on a Bruker micrOTOF™ II focus ESI TOF MS instrument in ES⁺ mode

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References