Highly Efficient Enzymatic Fluorescent Labelling of DNA Probes

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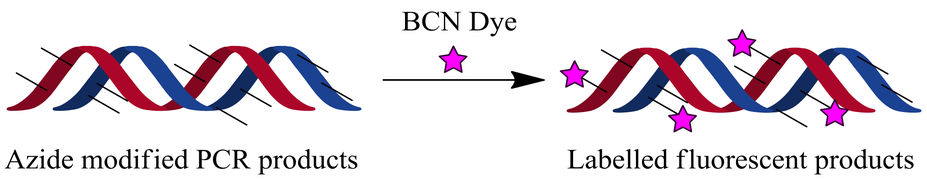
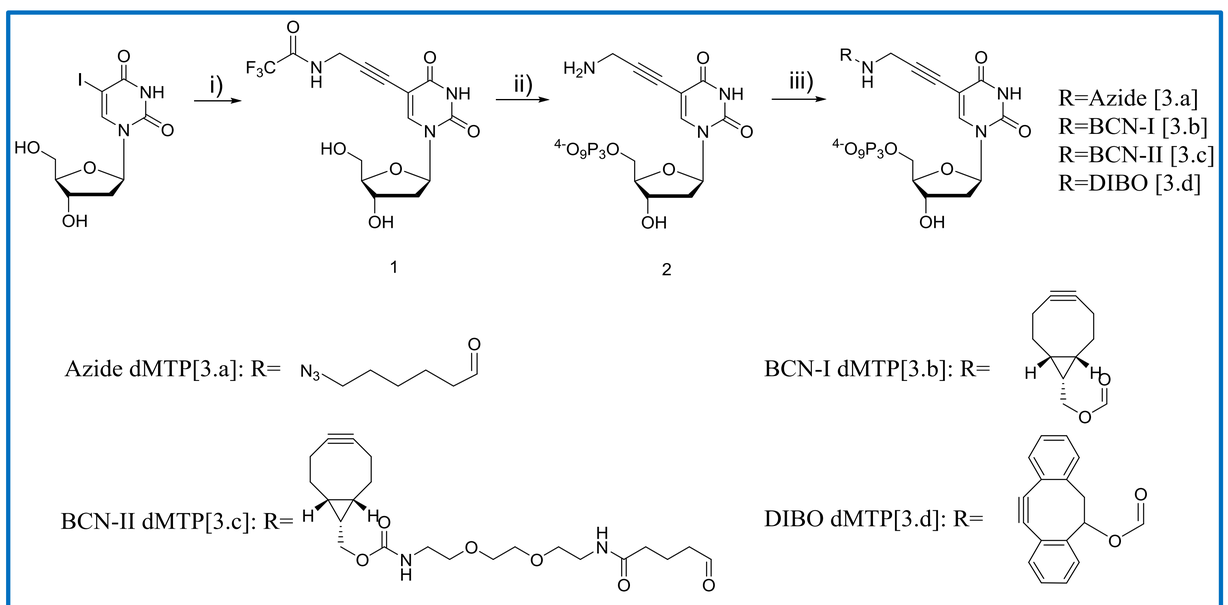


Azide and alkyne functionalised nucleotides are finding wide applications in DNA labelling, biotechnology and nanotechnology[1-2]. We are developing new technologies for use in these applications. To this end 5-propargylamino-2'-deoxyuridine-5'-triphosphate was synthesised and labelled with alkyne and azide *N*-hydroxysuccinimide active esters (DIBO, BCN-I, BCN-II and azide NHS esters) to prepare modified triphosphates (dMTPs). Azide dMTP was successfully incorporated into oligonucleotides by primer extension and PCR with high efficiency. The azide-modified PCR products exhibited 100 % correct nucleobase sequences and high fluorescence signals after labelling with Cy3-BCN via the highly efficient and mild SPAAC reaction[3-4].

Modified Triphosphate Synthesis



Scheme 1. The synthesis of 5-propargylamino-2'-deoxyuridine-5'-triphosphate and labelling the amino-dMTP with four active NHS esters quantitatively to obtain DIBO, Azide, BCN-I and BCN-II modified triphosphates.
Reagents: i) TFA-protected propargylamine, CuI, $Pd(PPh_3)_4$, Et_3N, DMF, 3 h, RT, 44.3 %; ii) $POCl_3$, proton sponge, $(MeO)_3PO_4$, 1 h, 0 ^oC; Tri (tetrabutylammonium) hydrogen pyrophosphate, Bu_3N, DMF, 3 h; 1 M TEAB buffer, 30 min; Ammonia (33 %), 3 h, 9.3 %; iii) DMF and TEAB buffer, 4 h, 55 ^oC.

Primer Extension

Five synthesised modified nucleotide T-analogue triphosphates (amino, DIBO, BCN-I, BCN-II and azide dMTPs) were incorporated into the oligonucleotide strands through primer extension experiments. Family B (Therminator II, Gotaq and KOD) and family A (Klenow large fragment) polymerases were tested. Even with four consecutive adenines in the template (T1) (**Figure 2 and Table 3**) **the amino and azide modified dMTPs with small steric undemanding modifications were efficiently incorporated by all polymerases.**

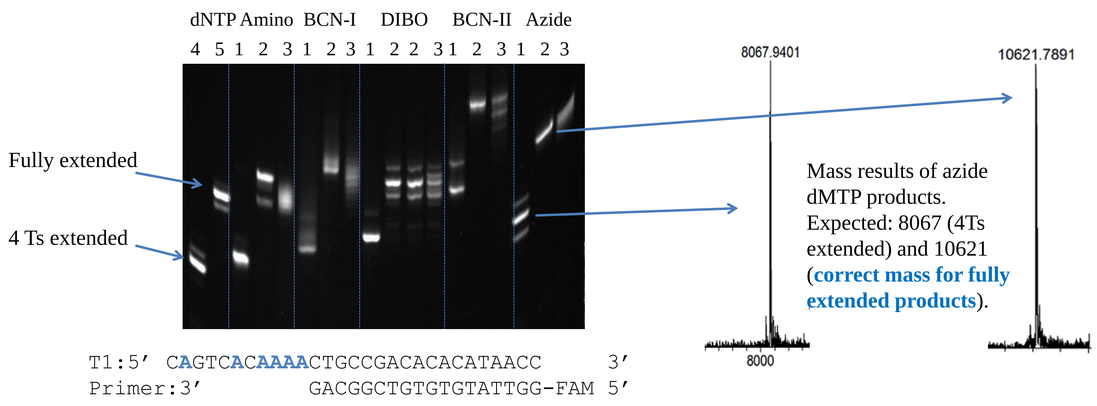


Figure 2. Primer extension reactions with five different dMTPs using KOD polymerase. Lane 1, dMTP; lane 2, dMTP+dATP+dCTP+dGTP; lane 3, dMTP+dNTPs (1:1); lane 4, dTTP; lane 5, dNTP (dATP, dGTP, dCTP, dTTP). (20 % PAGE gel)

Table 3. Summary of the compatibility of five modified triphosphates (Amino, BCN-I, BCN-II, DIBO and azide) in the presence of template T1 with various DNA polymerases in primer extension reactions (confirmed by Mass Spec.).

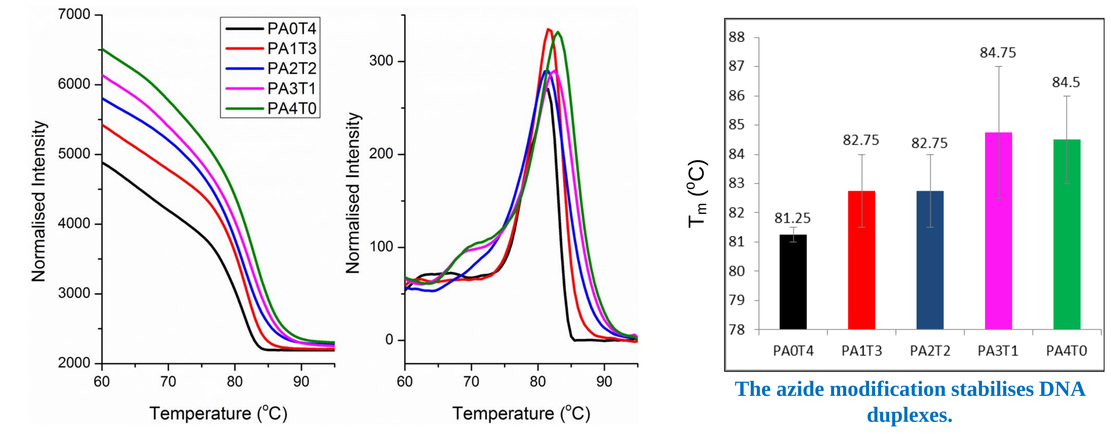
Polymerase	Compatibility of dMTPs for T1
KOD	Amino=Azide=BCN-I=BCN-II>**DIBO**
Therminator II	Amino=Azide=BCN-I=BCN-II>**DIBO**
Gotaq	Amino=Azide>BCN-II> **DIBO** > **BCN-I**
Klenow	Amino=Azide=BCN-I>BCN-II>**DIBO**

Note: The bold label means that for this dMTP, no fully extended products were produced.

Polymerase Chain Reaction

Different ratios of azide dMTP and dTTP with dGTP+dCTP+dATP were used to amplify template plasmid hydGΔCTD to give 500 bp PCR products using KOD polymerase (**Figure 4**). The melting temperature increased with higher percentage of azide dMTP (**Figure 5**) confirming that **the modification is compatible with strong Watson-Crick base pairing.**

Figure 4. PCR using plasmid template, azide dMTP and KOD polymerase. **PAXTY**= ratio of Azide dMTP/dTTP +dATP+dCTP+dGTP. The total concentration of azide dMTP+dTTP was 200 µM. N is negative control without template and L is 100 bp ladder. (1.5 % Agarose gel)



The azide modification stabilises DNA duplexes.

Figure 5. Left. Duplex melting curves of amplified azide PCR products (500 bp). **Centre.** Derivative of the melting curve. PAXTY as in **Figure 4**, i.e. different ratios of azide dMTP to dTTP. **Right:** Summary of T_m values.

Fluorescent Labelling

The 100% azide containing PCR product (i.e. no dT) was successfully labelled with Cy3-BCN in 20 min at RT (**Figure 6**). The dNTP PCR product (no azide dMTP) was used as negative control and as expected, no fluorescent bands were observed after reaction with Cy3-BCN (without ethidium staining). **The Cy3-labelled azide PCR product exhibited strong fluorescence without ethidium staining.**

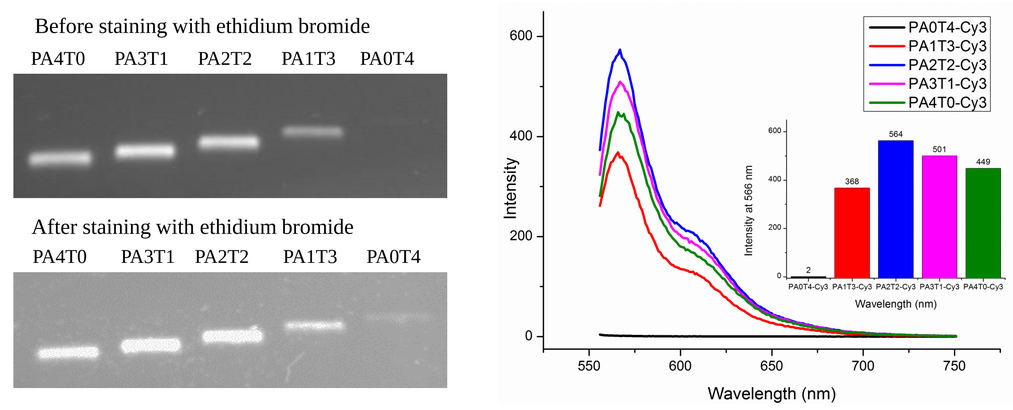
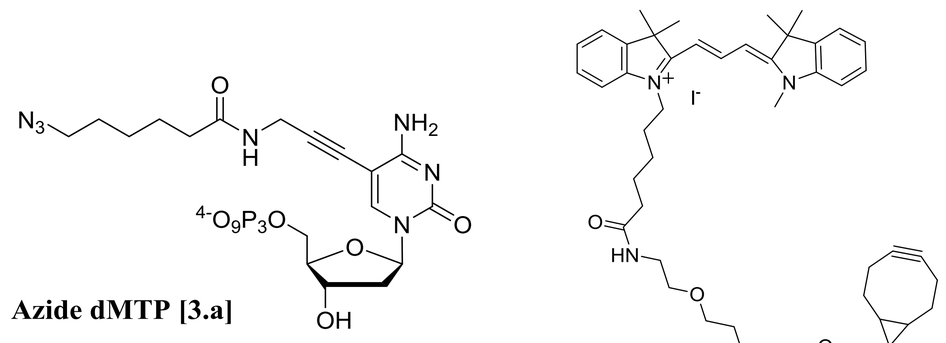


Figure 6. Labelling azide amplified PCR products (500 bp) with Cy3-BCN via SPPAC reactions. PAXTY as in **Figure 4**. **Left:** 1.5 % Agarose gel of labelled products before and after staining with Ethidium Bromide. **Right:** Fluorescent spectra of labelled products. Excited at 547 nm, voltage=750 V, ex and em slit are all 5. **Cy3 PCR products are highly fluorescent.**

Conclusions

Azide modified triphosphate 3.a (T-analogue) was successfully incorporated in DNA by primer extension and PCR. Its base pairing properties and incorporation efficiency are similar to the natural thymidine triphosphate. After PCR, highly fluorescent products were obtained by reaction with Cy3-BCN. This technique overcomes the instability of azide group in solid phase synthesis and efficiently labelled oligonucleotides via copper free click reactions.

Future work: Synthesis of densely labelled fluorescent probes for detection of specific genomic DNA sequences in single cells.



Azide dMTP [3.a] **Cy3-BCN**

References

1. El-Sagheer, A. H.; Brown, T., *Chem. Soc. Rev.* **2010,** *39* (4), 1388-1405.
2. El-Sagheer, A. H.; Brown, T., *Acc. Chem. Res.* **2012,** *45* (8), 1258-1267.
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