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AquaPhluor® 639

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Advantages of AquaPhluor® 639

- Excellent chemical stability for improved synthesis yield
- Excellent photostability for storage and applications
- Temperature-independent fluorescence

ELITechGroup (formerly Epoch Biosciences) has been developing improved fluorescent dyes for oligonucleotide labeling for over two decades. When in their labeling reagent form, AquaPhluor® dyes, one of our fluorophore lines, possess latent phosphonate groups in the linkers. Fully protected and neutral, these groups are compatible with phosphoramidite preparation and solid phase synthesis. In their ultimate form, upon completion of standard oligonucleotide synthesis, the dyes are negatively charged and thus significantly more hydrophilic. The phosphonate chemistry of AquaPhluor® dyes makes it possible to combine the versatility of automated oligonucleotide synthesis with the benefits of hydrophilicity. One particularly attractive AquaPhluor® dye is AquaPhluor® 639 (AP639).

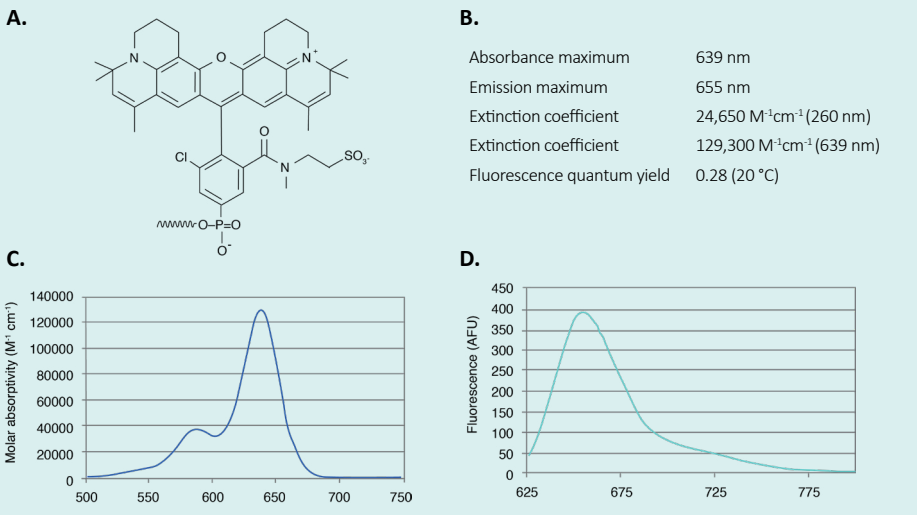

Figure 1. AquaPhluor® 639. A. AP639 chemical structure; B. AP639 spectral properties; C. AP639 molar absorptivity; D. AP639 fluorescence.

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See technical snippets on back cover

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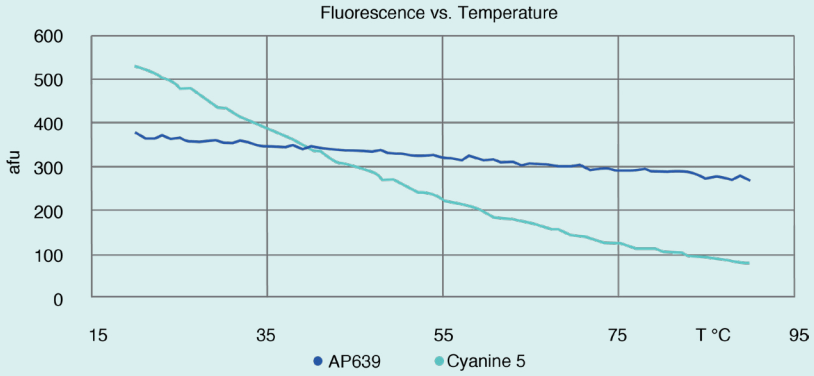


Figure 2. Comparison of fluorescence temperature dependencies between AP639 and Cyanine 5. Dye-T_8, 100 nM, Tris pH 8.

AP639 is a red-shifted rhodamine dye that exhibits many desirable properties (Figure 1). The dye is multiply charged with a net charge of -1, and with absorbance and extinction maxima at 639 and 655 nm, respectively. AP639 is relatively bright, with an extinction coefficient of 129,300 $M^{-1}cm^{-1}$ (639 nm) and a quantum yield of 0.28 (20 °C). In addition to sequence- and pH-independent (pH 5-8) fluorescence, AP639 also shows excellent chemical and photochemical stability, as well as temperature-independent fluorescence. These last three properties make AP639 an attractive alternative to Cyanine 5 and other similarly-structured Cyanine analogues in the red region of the visible spectrum.

AP639 differs from Cyanine 5 in three key aspects. Firstly, it displays relatively temperature-independent fluorescence that only decreases a modest 20 % when the temperature is increased from 20 to 80 °C (Figure 2). This is a stark contrast to Cyanine 5, which loses 80 % of its fluorescence intensity over the same temperature range. This difference allows AP639 to be particularly effective in higher temperature applications such as PCR, in which AP639 is at least twice as bright as Cyanine 5 under typical elongation conditions. AP639 is also very resistant to photobleaching. In one study, we monitored the fluorescence of AP639 and Cyanine 5 under constant exposure to visible light over time (Figure 3). Over a period of 2 hours, AP639 fluorescence was essentially unchanged, whereas Cyanine 5 fluorescence was reduced by about 50 %. This stability is particularly relevant for applications involving high intensity illumination, such as confocal microscopy. Finally, AP639 is much more stable to deprotection conditions. Unlike Cyanine 5, AP639 is stable to standard ammonium hydroxide conditions with <5 % of decomposition or side reactions. This allows for improved synthesis yields, as well as for fewer restrictions during oligonucleotide manufacturing.

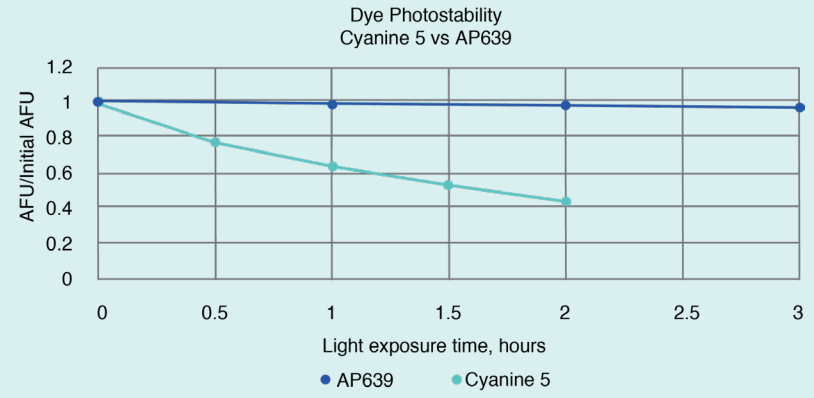


Figure 3. Comparison of photostability between AP639 and Cyanine 5. Dye-T_8, 500 nM, 1X TE buffer.

To illustrate the utility of AP639, a serially diluted set of target DNA was amplified by qPCR on an ABI7500 Fast Dx system, using a set of primers and an MGB TaqMan probe labeled either with AP639 or Cyanine 5 at the 5'-end (Figure 4). Clear signal improvement was observed for the AP639-labeled probe, with a final amplification fluorescence close to three times higher than that of the Cyanine 5-labeled probe. This difference can be especially important for the amplification and detection of low target samples, which require more amplification cycles when signals tend to drop in intensity.

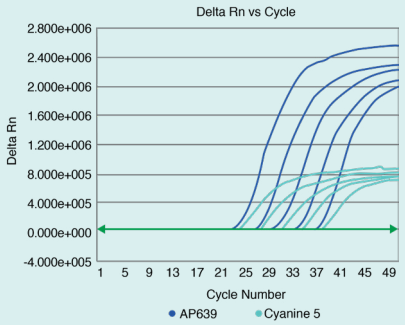
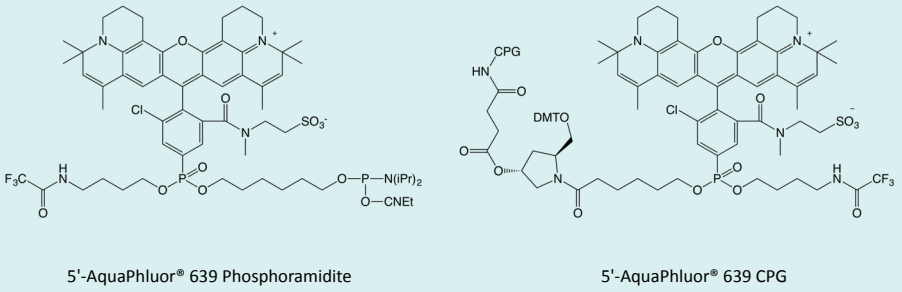


Figure 4. Real-time PCR amplification and detection using AP639 and Cyanine 5 labeled MGB TaqMan probes. Real-time PCR system: ABI7500 Fast Dx, probe sequence: AP639/Cyanine 5-TTTATGGAACGATGCTAAC-EDQ-MGB (5'–3'). EDQ = eclipse dark quencher, MGB = minor groove binder.

In summary, the new AquaPhluor® 639 dye offers numerous chemical stability and performance improvements over the traditional Cyanine 5. Over the years, Glen Research has brought several of our products to a wider customer base, and we hope that their customers will be able to realize the strengths of AP639 in fluorescence-based applications.

New Products — AquaPhluor® 639 5'-Phosphoramidite and CPG

AquaPhluor® 639 (AP639) is a rhodamine dye with an excitation and emission in the far red of the visible spectrum. Structurally, it closely resembles the non-Cyanine AquaPhluor® 593 we introduced a few years back, but functionally, it is an alternative to Cyanine 5. As described in the previous article, AP639 has several advantages over Cyanine 5, and in collaboration with ELITechGroup, Glen Research is pleased to offer the phosphoramidite and CPG versions of this dye for 5' or 3' labeling, respectively (Figure 1).



5'-AquaPhluor® 639 Phosphoramidite

5'-AquaPhluor® 639 CPG

Figure 1. New AquaPhluor® 639 Products

The use of these AP639 products is relatively straightforward. The phosphoramidite can be incorporated with a 3-minute coupling time, and the support should be used in the same way a normal protected nucleoside support would, as it contains a DMT group. For deprotection, most standard conditions are generally compatible.

In addition to having photostability and temperature-independent spectral properties superior to those of Cyanine 5, AP639 shows highly improved chemical stability under basic conditions. Those who have experience with Cyanine 5 will know that it must be deprotected gently. In the presence of standard deprotection conditions, Cyanine 5 will slowly go from a deep blue, to green, to eventually yellow. UltraMild deprotection is ideal, but ammonium hydroxide at room temperature for up to 36 hours will also provide acceptable amounts of intact Cyanine 5. On the other hand, deprotection stability is generally not an issue for AP639. In fact, it is the opposite of Cyanine 5, as UltraMild deprotection will not suffice in removing the AP639 TFA-aminobutyl group. Deprotection for AP639 was evaluated in a range of different conditions (Table 1), and all were suitable. In more aggressive deprotection conditions, an extra non-fluorescent peak or two may be present on the baseline in addition to the major desired product, but these are all minor in intensity and not unusual for most dye-labeled oligonucleotides. For example, with AMA, we observed a small non-fluorescent, more hydrophobic peak by RP-HPLC. According to ESI-MS, it matches the displacement of the N-methyl taurine amide by methylamine, and the absence of fluorescence is likely due to the formation of an additional lactam ring. The excellent chemical stability of AP639 should prove particularly useful in highly modified oligonucleotides for which mild deprotection methods are not possible.

Table 1. Deprotection Conditions Evaluated for AP639

Solution	Temperature (°C)	Time (h)
NH_4OH	RT	17
	55	17
	65	2
AMA	65	0.17 (10 min)
$tBuNH_2/H_2O$ 1:3	60	6

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Item	Pack Size	Catalog No.
5'-AquaPhluor® 639 Phosphoramidite	50 µmol	10-5926-95
	100 µmol	10-5926-90
	0.25 g	10-5926-02
AquaPhluor® 639 CPG	0.1 g	20-5926-01
	1.0 g	20-5926-10
1 µmol columns	Pack of 4	20-5926-41
0.2 µmol columns	Pack of 4	20-5926-42
10 µmol column (ABI)	Pack of 1	20-5926-13
15 µmol column (Expedite)	Pack of 1	20-5926-14

New Products — AKTA Capping Reagents

The AKTA OligoPilot Plus (10/100) oligonucleotide synthesizers are popular for small to medium scale operations. The instruments are supported by UNICORN control software, and its HPLC-driven system makes it flexible and reagent-efficient. Many of our customers use AKTA OligoPilot systems, and we have a set of standard AKTA-compatible reagents that can be found at the following link: https://www.glenresearch.com/browse/akta-oligopilot.

For capping, Glen Research has historically offered the following:

Cap A 20 % Methylimidazole in Acetonitrile (40-4015)

Cap B 20 % Acetic Anhydride and 30 % 2,6-Lutidine in Acetonitrile (40-4028)

The Cap B mixture is actually an equal mix of two solutions: 40 % acetic anhydride/ acetonitrile and 60 % 2,6-lutidine/ acetonitrile. Ideally, these two would be mixed by the end user prior to loading onto the instrument, but regulatory restrictions make such an option challenging. Instead, the mixing is performed at Glen Research just prior to shipping. The resulting mixture has a relatively short half-life and we recommend that the solution is used within 8 weeks of the mixing date.

To address this short shelf life, Glen Research is introducing a new set of capping reagents for the AKTA instrument line:

Cap A 30 % 2,6-Luditine/20 % Methylimidazole/Acetonitrile

Cap B 20 % Acetic Anhydride in Acetonitrile

Cap B 5 % Phenoxyacetic Anhydride in Acetonitrile

These Cap B formulations are much more stable than our traditional offerings, with a reanalysis date of one year following release. In addition to standard capping, we are also making an UltraMild Cap B available for syntheses that require UltraMild conditions. We hope that users of AKTA instruments find these new formulations useful in the capping step of the oligonucleotide synthesis cycle.

Item	Pack Size	Catalog No.
Cap Mix A, Acetonitrile/Methylimidazole	1 L	40-4015-71
Cap Mix B*, Acetonitrile/ Acetic Anhydride/ 2,6-Lutidine	1 L	40-4028-71*
Cap Mix A, 20% 1-Methylimidazole in Acetonitrile/ 2,6-Lutidine	1 L	40-4115-71
Cap Mix B, 20% Acetic Anhydride in Acetonitrile	1 L	40-4224-71
Cap Mix B, 5% Phenoxyacetic Anhydride in Acetonitrile	1 L	40-4128-71

**Cap Mix B is a two-part formulation that is combined immediately before shipment*

Very Fast CRISPR (vfCRISPR) "On Demand"

Author: Gerald Zon, Ph.D.
Trilink Biotechnologies

- Annual CRISPR Publications Continue to Increase
- vfCRISPR, a Novel CRISPR-Variant, Is Transformative
- The Key is Light-Induced "Uncaging" of CRISPR Guide RNA (gRNA)

In modern molecular biology and biotechnology, very few methods have been met with as much success as CRISPR, as evidenced by this chart of publications indexed to CRISPR in the NIH PubMed database. The rate of annual increase indicates that there will be a projected

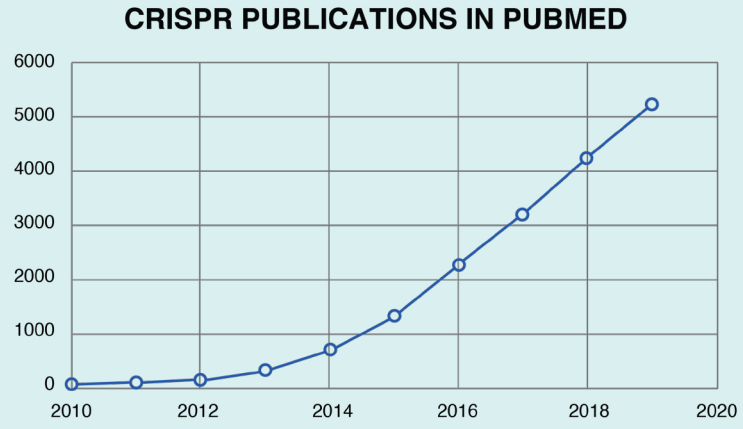

Figure 1. PubMed search of CRISPR publications

6,200 CRISPR-related publications added to PubMed for the year of 2020, amounting to an incredible average of 17 articles each day, 7 days per week (Figure 1)! And of course, the 2020 Nobel Prize in Chemistry was awarded to the discovery of CRISPR.

There are many variants of CRISPR-based methods that have been applied to virtually every facet of molecular biology and biotechnology, such as gene editing, functional genomics, diagnostics, epigenetic editing, and more. This

versatility is demonstrated by the over 600 review articles that have appeared in PubMed during only the past year. This article will cover light-induced vfCRISPR reported on June 20, 2020, by Liu et al. at Johns Hopkins University School of Medicine.[1] A perspective article in the prestigious Science magazine described vfCRISPR as "transformative for understanding" kinetic aspects following DNA double-strand breaks (DSBs).[2] According to Science metrics, this publication by Liu et al. was viewed over 44,000 times in only 10 days, indicating a remarkable level of interest by the scientific community. Photochemical "uncaging" is the key component for this methodological advance, and this chemistry will be introduced in the next section before proceeding to its application for vfCRISPR.

Photochemical Uncaging

Photoremovable (sometimes called photoreleasable, photocleavable, or photoactivatable) protecting groups (PPGs) provide spatial and temporal control over the release of a wide variety of chemical moieties, as reviewed elsewhere.[3] In one of the first applications for a biochemical molecule, namely adenosine 5'-triphosphate (ATP), Kaplan et al. used 2-nitrobenzyl as the PPG and introduced the term "caged" to designate a compound protected by a PPG.[4] Thirty years later, in 2007, a structural variant of this PPG was reported by Deiters and coworkers for light-mediated uncaging (i.e. activation) of a DNAzyme.[5]

In that work, the oligonucleotide comprising the DNAzyme was chemically synthesized using a 6-nitropiperonyloxymethyl (NPOM) derivative of deoxythymidine (NPOM-caged-dT, 10-1534) (Figure 2). The NPOM group at a single site, which functionally blocked (i.e. caged) the DNAzyme activity, could be removed in only a few minutes using UV light at 365nm to produce the T moiety and thus release (i.e. uncage) the DNAzyme activity.

As discussed in GR23.2, Deiters and coworkers have also demonstrated "an astonishing array of applications of this strategy" in the fields of PCR and polymerase activity, antisense and gene silencing, regulation of restriction endonuclease activity, enzyme-free mutagenesis, and photochemical control of DNA decoy function to regulate gene transcription.

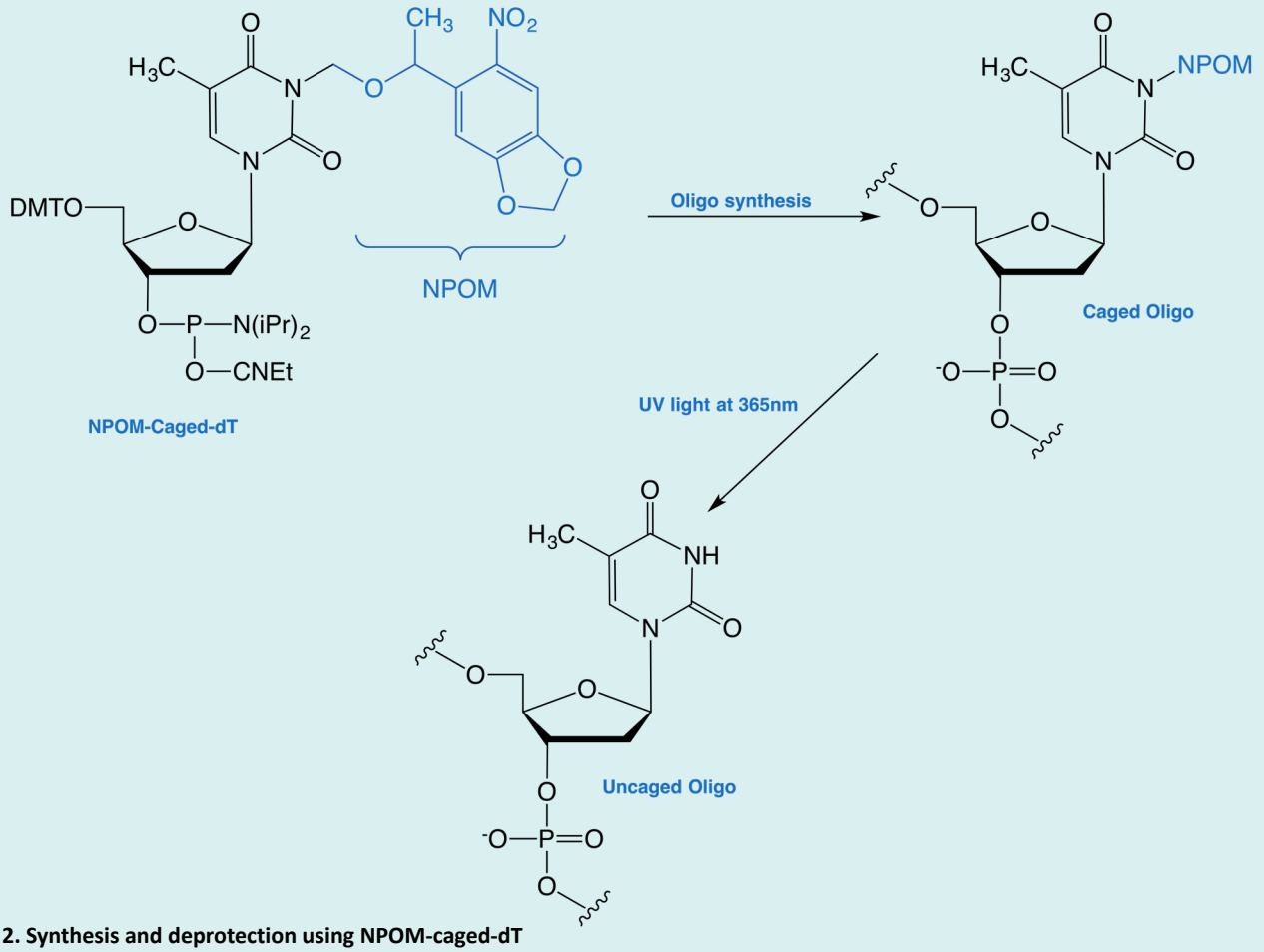

Figure 2. Synthesis and deprotection using NPOM-caged-dT

The minimum number and location of NPOM-caged-T moieties incorporated into an oligonucleotide to achieve functional inactivity is sequence- and condition-dependent, and is best approached by systematic empirical determinations. With the desired sequence, photo-uncaging of an oligonucleotide is easily carried out with UV light at 365 nm for seconds to minutes, and this can be readily achieved with a UV transilluminator, a hand-held UV light, or a fluorescence microscope to uncage the oligo in a specific location within the cell.

The mechanism of NPOM (and other) "on demand" photochemical uncaging is discussed in a comprehensive review by Klán et al. titled: Photoremovable Protecting Groups in Chemistry and Biology: Reaction Mechanisms and Efficacy.[3] Briefly, irradiation with UV light leads to fragmentation of NPOM-caged-T into the N-hydroxymethyl derivative of T and the nitroso ketone depicted here (Figure 3). The former fragment then spontaneously decomposes to T and formaldehyde.

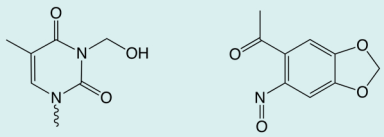

Figure 3. Fragments produced by light-induced uncaging of NPOM-caged-T in an oligonucleotide

NPOM-Mediated vfCRISPR On Demand

Rationale

According to Liu et al., after genomic DNA cleavage by CRISPR-Cas9, DNA damage response (DDR) proteins are recruited to initiate complex repair processes.[1] They add that, although DDR is known to be influenced by factors such as target sequence, cell cycle, and chromatin dynamics, the precise timing and sequence of cellular events require further investigation. Cas9 has potential as a tool for the study of DDR dynamics, but it currently lacks the necessary level of control to initiate precise DNA damage on demand. Liu et al. therefore posit that "[t]o unveil the nature of Cas9-induced DDR events in living cells, an inducible Cas9 system with the spatiotemporal resolution that matches the rapidity and subcellularity of DDR would be powerful."

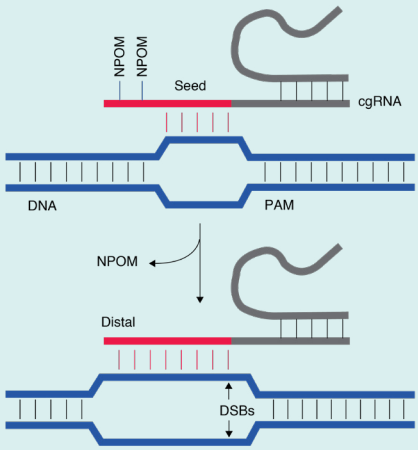

Figure 4. Schematic depiction of photoactivation leading to vfCRISPR

The design principle of vfCRISPR is based on the Streptococcus pyogenes Cas9 (Cas9) cleavage mechanism. According to Liu et al., after protospacer adjacent motif (PAM) recognition by CRISPR-associated 9 (Cas9), the caged guide RNA (cgRNA) forms base pairs with the target DNA at the PAM-proximal "seed" sequences (Figure 4). However, distal cgRNA sequences are prevented from binding by steric hindrance from the NPOM-caged-T moieties. UV irradiation at 365 nm causes photolytic removal of NPOM groups, leading to uncaged T nucleotides for distal base pairing, which triggers a conformational change of the Cas9 HNH endonuclease domain (not shown). This activates DNA cleavage by both nuclease domains leading to DSBs.

Note that this "cgRNA" construct is actually a chimeric RNA/DNA oligonucleotide comprised of mostly RNA, but it also incorporates two or three DNA moieties, i.e. NPOM-modified T. The significance of the chimeric nature of the cgRNA and reduced off-target cleavage is discussed below.

According to Liu et al., although the Cas9/cgRNA complex retains the ability to bind its target DNA, it cannot cleave because the steric hindrance imposed by the caging groups prevents full DNA unwinding and nuclease activation. Upon light irradiation at 365 nm, the caging groups are removed and the pre-bound, now-activated Cas9/cgRNA complex rapidly cleaves target DNA. This pre-binding without formation of DSBs allows for subsequent "on demand" rapid photolytic uncaging and Cas9 HNH endonuclease-mediated DSBs.

Demonstration of vfCRISPR Using NPOM-Modified crRNA

An electrophoretic mobility shift assay (Figure 5) confirmed that Cas9/cgRNA stably bound to target DNA in the absence of light, and no cleavage was observed. After uncaging with UV light for incremental periods of time between 1 second and 30 minutes, this assay showed asymptotically increased percent cleavage of DNA by the Cas9/cgRNA complex, with ~50 % cleavage after only 5 seconds and ~90 % after 30 minutes.

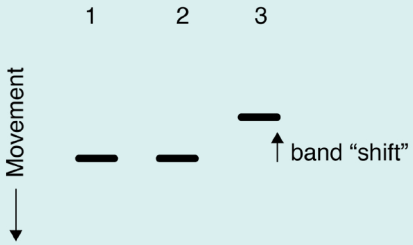

Figure 5. Exemplary depiction of an electrophoretic mobility shift assay. Lane 1 = DNA; Lane 2 = DNA plus irrelevant (control) protein; Lane 3 = DNA plus specific DNA-binding protein causing band "shift" by slower eluting (higher molecular weight) DNA-protein complex.

Next, Liu et al. characterized the activity of vfCRISPR in human embryonic kidney 293 cells by targeting four endogenous loci (ACTB, IFT88, MYC, PPP1R2) in the genomic DNA. Light-induced indel efficiency up to 97 % was found, whereas cells without light exposure had almost no detectable indels. Importantly, cells exposed to this dosage of

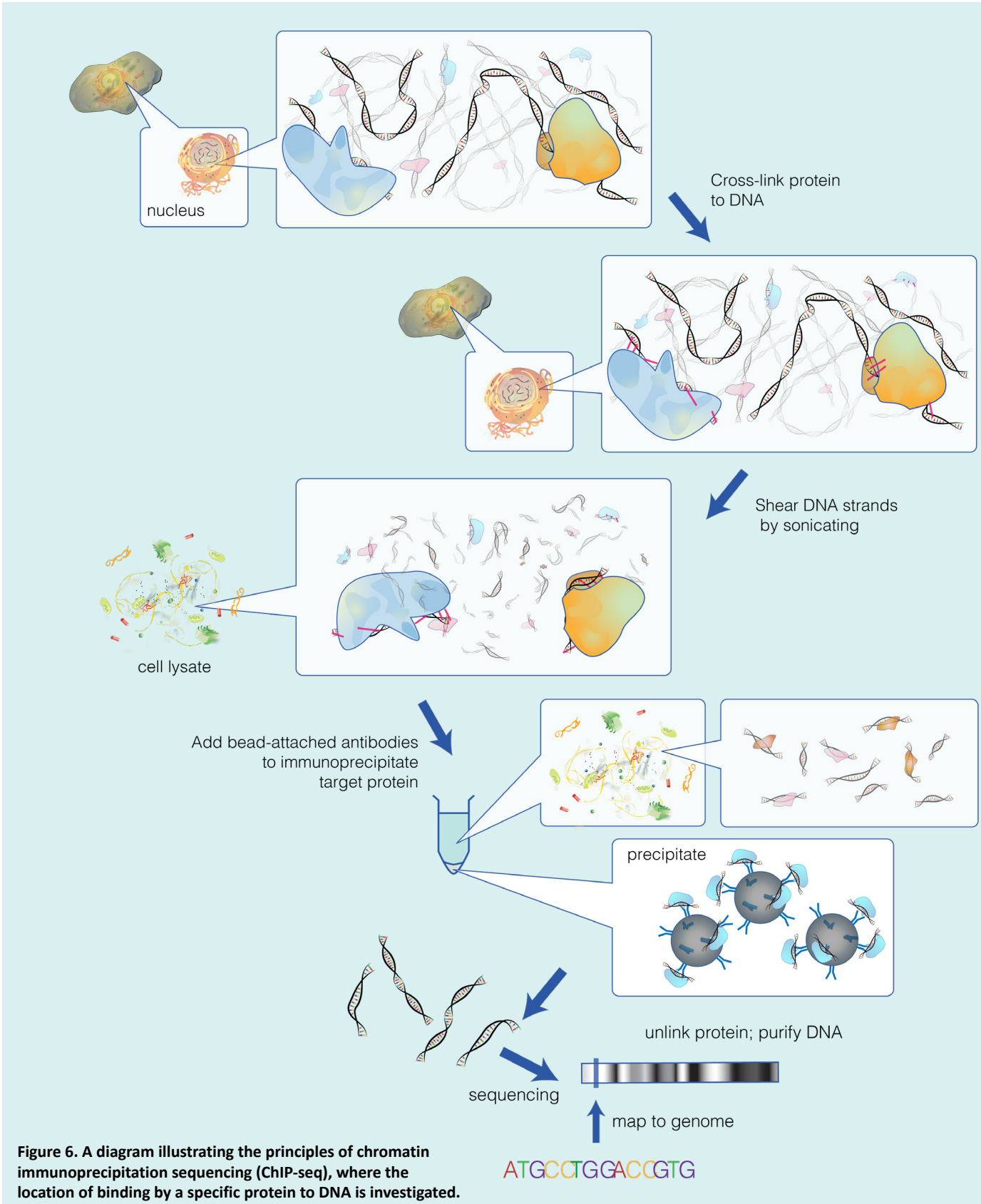

Figure 6. A diagram illustrating the principles of chromatin immunoprecipitation sequencing (ChIP-seq), where the location of binding by a specific protein to DNA is investigated.

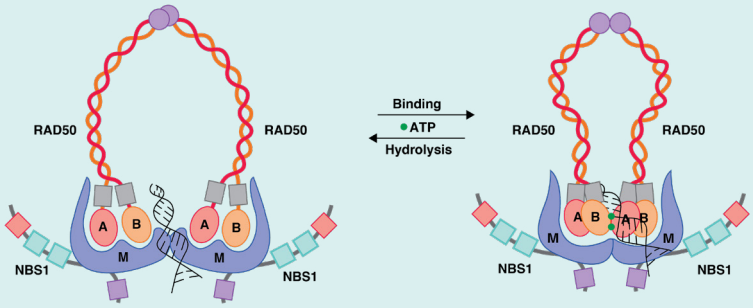

Figure 7. Structure and conformation of MRN complex. MRN complex goes through conformational changes when ABC-ATPase domains bind to ATP and form a head-to-tail dimer. This compact, rigid, and closed conformation blocks access to MRE11 active sites. Upon hydrolysis and removal of ATP, MRN complex switches to an open form, exposing the active sites of MRE11. Zinc hook domain (purple sphere) of RAD50 facilitates the formation of dimers, as depicted.

light exhibited no apparent phototoxicity. Approximately 50 % of DNA cleavage was found within 30 seconds of light activation. Compared with other Cas9 induction techniques, such as Rose et al.'s chemically inducible method using a protein-engineered variant of Cas9,[6] vfCRISPR exhibited much faster cleavage kinetics and higher cleavage efficiency.

Liu et al. attributed the very fast kinetics achieved via light-activated cgRNA to skipped nuclear localization or target-searching steps, and the higher cleavage efficiency to the use of wild-type Cas9. Genome-wide analysis of off-target editing using GUIDE-seq also revealed reduced off-target activity compared to wild-type gRNA, consistent with improved specificity from deoxyribonucleotide (T) incorporation into the guide RNA. This, alongside previously reported work by others, demonstrated that partial replacement of 3' end RNA nucleotides with DNA nucleotides in wild-type gRNA retains efficient target cleavage and significantly reduces off-target genome editing.

Kinetic and Mechanistic Studies Using vfCRISPR

With a precisely defined time for cleavage, vfCRISPR allowed Liu et al. to investigate the generation and repair kinetics of Cas9-mediated DSBs. They measured the percentage of DSBs and indels as a function of time after Cas9 light-activation at multiple target sites, and adopted mathematical models to describe the kinetics of DSB and indel formation, as detailed in the Supplementary Materials.

Using vfCRISPR for highly synchronized DNA cleavage of the ACTB site, Liu et al. performed time-resolved chromatin immunoprecipitation (ChIP), followed by sequencing (ChIP-seq, Figure 6).

By doing so, the researchers were able to elucidate events related to the MRN complex, a protein complex consisting of MRE11, Rad50, and Nbs1 that plays an important role in the initial processing of double-strand DNA breaks. As illustrated in the generalized scheme (Figure 7),[7] the MRN complex binds avidly to double-strand breaks and serves to tether broken ends prior to repair. Liu et al. were able to track the recruitment of MRE11, which forms the MRN complex with Rad50 and Nbs1 to the ACTB cleavage site. Liu et al. observed rapid MRE11 recruitment that reached half-maximal signal between 5 and 15 minutes. Readers interested in this and additional mechanistic details revealed by vfCRISPR and ChIP-seq can consult Lui et al., as this topic requires deeper discussion of the results than what is provided here.

Conclusions

With the help of NPOM caged-dT, vfCRISPR provides the highest spatial and temporal resolutions to induce site-specific DSBs in living cells. This study sets the blueprint for further systematic studies of the DDR that combine vfCRISPR with time-resolved biochemical, sequencing, and imaging readouts. The use of cgRNA with other Cas9-based systems such as nickases, base editors, and prime editors may facilitate the study of single-strand break, base excision or mismatch, and flap repair, respectively. Combining vfCRISPR with subcellular photoactivation potentially enables precise genome editing with single-allele specificity and elimination of off-target activity

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Application Note — NPOM-Caged-dT

NPOM-Caged-dT substitutions allow for the function of an oligonucleotide to be controlled by light. The nucleotide cannot participate in Watson-Crick base pairing when the bulky NPOM group is attached to the N3 of thymidine. However, on exposure to low energy UV light, the NPOM group is removed, releasing the native thymidine. This simple and effective mechanism known as 'photocaging' has proven to be relatively popular in biological settings. In addition to vfCRISPR,[1] this nucleotide has recently played key roles in facilitating the mapping of single cell transcriptomes[2] and the deep sequencing of non-enzymatic RNA primer extension.[3]

Oligonucleotides with caged thymidines are relatively easy to prepare. NPOM Caged-dT-CE Phosphoramidite will be used in place of dT-CE Phosphoramidite in select locations, and no changes to coupling times or deprotection procedures are necessary. As with any light-sensitive modification, exposure to light should be minimized.

References

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Item	Pack Size	Catalog No.
NPOM Caged-dT-CE Phosphoramidite	50 µmol	10-1534-95
	100 µmol	10-1534-90
	0.25 g	10-1534-02

Technical Brief — NHS Esters

N-Hydroxysuccinimide (NHS) esters are a family of conjugation reagents first used almost sixty years ago. Not only are they relatively easy to prepare, but they are also reactive, selective, stable for storage, and functional in an aqueous environment. These properties make NHS ester conjugation one of the most common labeling and bioconjugation strategies.

NHS esters selectively react with primary aliphatic amine groups. While NHS esters can also react with other nucleophiles, such as hydroxyl and sulfhydryl groups, the resulting esters and thioesters are not very stable and can be hydrolyzed or displaced by amines. Typical NHS esters consist of an NHS ester group, a label, and often a linker as well (Figure 1). In the reaction, the carbonyl of the ester group is attacked by a primary aliphatic amine, resulting in a tetrahedral intermediate and the elimination of the NHS as a leaving group. The coupling reaction causes NHS (a weak acid) to be released, and an amide (a very stable covalent linkage) to be formed.

This labeling strategy has been used extensively, and it is highly effective for oligonucleotides labeled with amino-modifiers. NHS esters can be used with any one of the 25 amino modifiers in the Glen Research catalog. Most of these are for the 5'-terminus, but other versions can introduce amine groups to the 3'-terminus, as well as to several nucleobases. Generally, the NHS ester is dissolved in a small amount of dry DMF or DMSO, and the solution is added to the amine-labeled oligonucleotide in a non-nucleophilic buffer (pH 7–9). After a short reaction time, the amine gets quantitatively labeled and no other part of the oligonucleotide is affected. If there are multiple amino modifiers present, each and every one can be completely labeled as well. A general protocol can be found below.

For a 0.2 µmole synthesis of an amine-modified oligo:

1. Dissolve oligo in 500 µL of 0.1 M sodium bicarbonate.
2. Dissolve 5–10 eq of NHS ester in 25 µL DMF or DMSO.
3. Add NHS ester solution to oligo solution.
4. Agitate the mixture and incubate at room temperature for 1–2 hrs.
5. Separate oligo-conjugate from salts and excess label by size exclusion on a Glen Gel-Pak™ desalting column or equivalent.

Although these reactions are usually pretty straightforward, there are a few areas to be mindful of. First and foremost, the amino-modified oligonucleotide needs to be of good quality, as previously detailed (https://www.glenresearch.com/media/productattach/import/tbn/TB_Avoid_Amine_Alkylation.pdf). In addition, NHS esters can react with water and they should be stored appropriately. The moment the NHS ester is added to the oligo, the water begins to react with the NHS ester, directly competing with labeling. For this reason, several equivalents are required. As primary amines are much better nucleophiles than water, hydrolysis of NHS ester is generally not an issue, but if low conjugation efficiency is observed, perhaps due to a bulkier NHS ester that is slower to react, lowering the volume of buffer by two- or four-fold will make the reaction with the amine more

Continued on Page 10

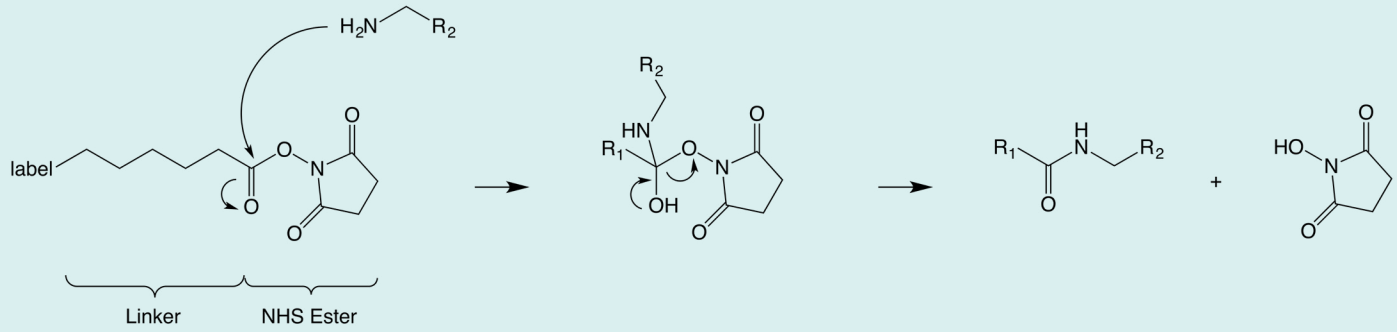
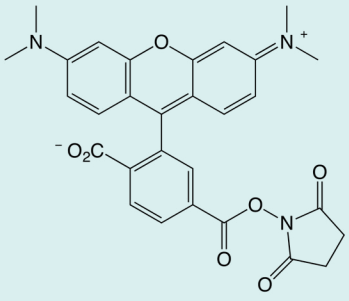
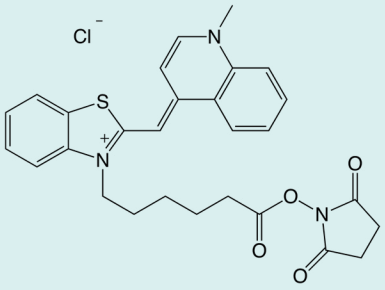


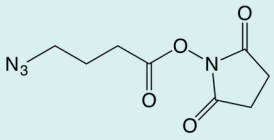
Figure 1. NHS ester coupling mechanism



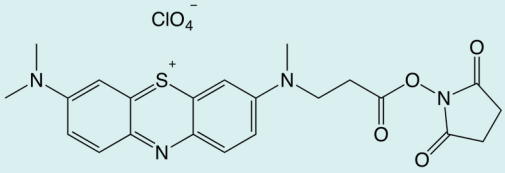
TAMRA NHS Ester



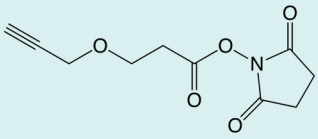
Thiazole Orange NHS Ester



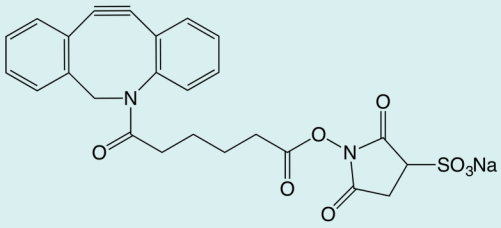
Azidobutyrate NHS Ester



Methylene Blue NHS Ester



Alkyne-NHS Ester



DBCO-sulfo-NHS Ester

Figure 2. NHS Esters

favorable. Finally, there should not be any other competing nucleophiles present in the reaction mixture, as these will interfere with labeling. Oligonucleotides that are deprotected with amine-based deprotection solutions and dried down are usually isolated as ammonium salts. These oligos need to be converted to the sodium salt, a process that can be achieved with ethanol precipitation or an equivalent method prior to labeling. Tris buffer and other nucleophilic modifications are also not compatible.

In terms of work flow, incorporating modifications using phosphoramidites over post-synthesis/deprotection modification with NHS esters is generally advantageous. This is particularly true for high throughput operations, in which manual/additional steps are not desirable. However, there are many situations in which additional steps are preferred. If a desired modification/label is not stable during oligonucleotide synthesis or deprotection, then a post-synthesis conjugation would be the only option. Even if stability is not an issue, custom phosphoramidite synthesis is often complicated and expensive, and the NHS ester functionalization route might be more efficient in the early stages of a project, as it allows access to the reagent much sooner, while saving synthesis costs on structures that ultimately prove to not be useful. This strategy can also be used to aliquot a single amine-functionalized oligo into multiple aliquots for conjugation to a series of different NHS esters. When all the details of the application are finalized, an amidite that would give the exact same chemical structure obtained via the NHS ester labeling method can be synthesized.

Glen Research offers several NHS ester products (Figure 2). Some of these are available only as the NHS ester while others are also available as a phosphoramidite. The latter products include methylene blue and DBCO. Aside from those on this NHS ester list, we have a couple of additional NHS ester phosphoramidites for a different and previously discussed application (https://www.glenresearch.com/reports/gr19-19)

Item	Pack Size	Catalog No.
Alkyne-NHS Ester	2.3 mg	50-1905-23
	23 mg	50-1905-24
Azidobutyrate NHS Ester	2.3 mg	50-1904-23
	23 mg	50-1904-24
DBCO-sulfo-NHS Ester	5.2 mg	50-1941-23
	52 mg	50-1941-24
Methylene Blue NHS Ester	5.4 mg	50-1960-23
TAMRA NHS Ester	60 µL	50-5910-66
Thiazole Orange NHS Ester	5.4 mg	50-1970-23

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Technical Snippets

What special phosphoramidites are required for phosphorothioate synthesis?

The answer is none. The standard method of introducing phosphorothioates is to use standard phosphoramidites and sulfurization reagent in place of standard iodine oxidation. Sulfurization requires a contact time of at least one minute on the synthesizer. Sulfurization also needs to occur before capping, in the opposite order when compared to standard iodine oxidation. This is to prevent trace amounts of peroxides in capping reagents from performing undesired oxidation.

Products: Sulfurizing Reagent II, 40-4037
0.05M Sulfurizing Reagent II in pyridine/acetonitrile, 40-4137

How long are phosphoramidites good for on the synthesizer?

It could be anywhere between one day and two or more weeks. The actual time frame will depend on a host of factors including the phosphoramidite, phosphoramidite concentration, solvent, instrument, humidity, temperature, water content and more. Special or rare phosphoramidites should generally be consumed within a 24-hour time frame, while standard DNA phosphoramidites should be good for at least a week. If a bottle of phosphoramidite that was coupling well initially starts showing reduced coupling efficiency, that's a sign that it is time to replace that bottle.

Products: All phosphoramidites