## Spacers Introduction

Spacer modifcations C3, 9, C12 and 18 are used to insert a spacer arm in an oligonucleotide. These modifications can be added in multiple additions when a longer spacer is required. 3'-Spacer C3 CPG may also act as a blocker of exonuclease and polymerase activity at the 3'-terminus. dSpacer is used to introduce a stable abasic site within an oligonucleotide. See "Photo-Cleavable" modification category for photo-cleavable Spacer.

# Spacers Design Protocols

**Hydrophobic vs Hydrophilic Spacers—Design Considerations**

Spacer 9 and Spacer 18 are polyethylene glycol (PEG)-based, and thus hydrophilic, while Spacers C3, C12, PC Linker, and PC Spacer are aliphatic, and thus hydrophobic. Thus, when designing oligos that require one or more Spacer modifications, it is important to consider possible effects of Spacer hydrophobicity/hydrophilicity on the properties of the oligo. For example, post-synthetic labeling of an amino or thiol-modified oligo at the 5'-end with a dye, hapten or enzyme is commonly performed in aqueous solution. The active amino or thiol group typically is attached to the oligo via a hydrophobic linker, and when incorporated into long oligos or those with significant secondary structure, poor coupling of the label to the oligos is often observed. Consequently, optimization of the coupling reaction may require incorporation of a hydrophilic spacer (such as Spacer 18 or Spacer 9) next to the amino/thiol linker.

## Spacers Applications

Some common usages of Spacer phosphoramidites are to position various tags/labels at a desired length from an oligonucleotide, to immobilize oligonucleotides to a solid phase (like a microsphere or microarray slide), or to form non-nucleoside folds and hairpin loops within an oligonucleotide (1-3). Spacers can be placed anywhere within an oligo, and multiple incorporations of a Spacer can be performed to effectively generate spacers of virtually any desired length. For example, six successive incorporations of dSpacer were used to create the optimal separation for FRET between the donor and acceptor fluorophores in energy transfer fluorescent sequencing primers (4).

In addition to being used as a spacer arm, the Spacer C3 modification also can be placed at the 3'-end of an oligonucleotide to effectively block that end from enzymatic reactions (e.g., extension by PCR) (5). dSpacer and rSpacer also can be used to mimic abasic sites in oligonucleotides slated for use in DNA damage/repair studies (6). Photocleavable linkers and spacers can be used to introduce photocleavable tags/labels onto the ends of an oligo, or link two separate oligonucleotides through a short, photocleavable spacer arm for use in photo-triggered hybridization applications (7).

## References

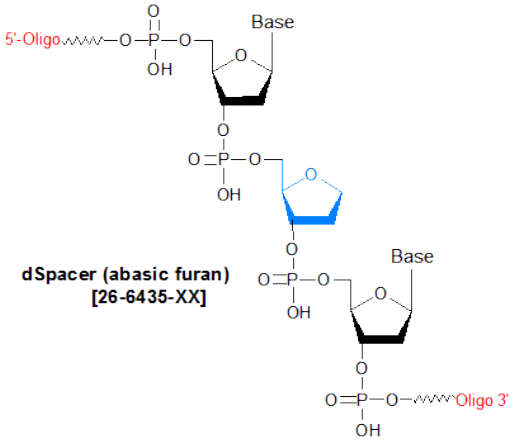
(1) Li, H., McGall, G. Photoactivatable Silanes: Synthesis and Uses in Biopolymer Array Fabrication on Glass Substrates. In Frontiers in Biochip Technology. X, W-L., Cheng, J. (Ed.) Springer Science+Business Media, Inc. (2006), pp. 176-190.
(2) Salunkhe, M., Wu, T.F., Letsinger, R.L. Control of folding and binding of oligonucleotides by use of non-nucleotide linker. J. Am. Chem. Soc. (1992), 114: 8768-8772.
(3) Durand, M., Chevrie, K., Chassignol, M., Thuong, N.T., Maurizot, J. Circular dichroism studies of an oligodeoxyribonucleotide containing a hairpin loop made of a hexaethylene glycol chain : conformation and stability. Nucleic Acids Res. (1990), 18: 6353-6359.
(4) Ju, J., Glazer, A.N., Mathies, R.A. Cassette labeling for facile construction of energy transfer fluorescent primers. Nucleic Acids Res. (1996), 24: 1144-1148.
(5) Zhou, L., Myers, A.N., Vandersteen, J.G., Wang, L., Wittwer, C.T. Closed-Tube Genotyping with Unlabeled Oligonucleotide Probes and a Saturating DNA Dye. Clin. Chem. (2004), 50: 1328-1335.
(6) Takeshita, M., Chang, C.N., Johnson, F., Will, S., Grollman, A.P. Oligodeoxynucleotides containing synthetic abasic sites. Model substrates for DNA polymerases and apurinic/apyrimidinic endonucleases. J. Biol. Chem. (1987), 262: 10171-10179.
(7) Ordoukhanian, P., Taylor, J-S. Design and synthesis of a versatile photocleavable DNA building block. Application to phototriggered hybridization. J. Am. Chem. Soc. (1995), 117: 9570-9571.

# Modificaton Code List

| Modification | Code | Catalog Number |
|---|---|---|
| Abasic Site (dSpacer abasic furan-THF) | [dABS] | 26-6435 |
| PC Linker (photocleavable) | [PCL] | 26-6888 |
| PC Spacer C3 (photocleavable) | [PC-Sp-C3] | 26-6889 |
| rAbasic Site (rSpacer abasic furan) | [rABS] | 26-6442 |
| Spacer 18 (hexaethyleneglycol, PEG6) | [Sp18] | 26-6447 |
| Spacer 9 | [Sp9] | 26-6440 |
| Spacer C12 | [SpC12] | 26-6441 |
| Spacer C2 | [SpC2] | 26-6637 |
| Spacer C3 3' | [SpC3-3] | 26-6439T |
| Spacer C3 Internal | [SpC3-Int] | 26-6439I |
| Spacer C3-5' | [SpC3-5] | 26-6439F |
| Spacer C4 | [SpC4] | 26-6638 |
| Spacer C6 | [SpC6] | 26-6945 |
| Spacer C9 | [SpC9] | 26-6639 |

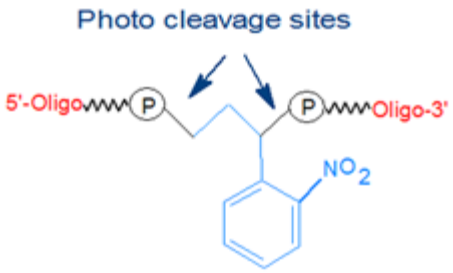
# Abasic Site (dSpacer tetrahydrofuran-THF)

| | |
|---|---|
| Category | Spacers |
| Modification Code | dABS |
| Reference Catalog Number | 26-6435 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 180.1 |



# PC Linker (photocleavable)

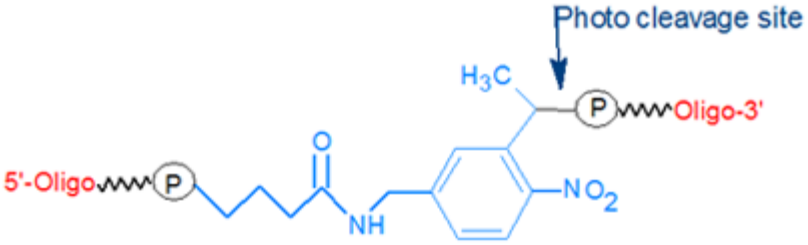
| | |
|---|---|
| Category | Photo Cleavable |
| Modification Code | PCL |
| Reference Catalog Number | 26-6888 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 259.15 |




**PC Linker (photocleavable)**
**[26-6888-XX]**

# PC Spacer (photocleavable)

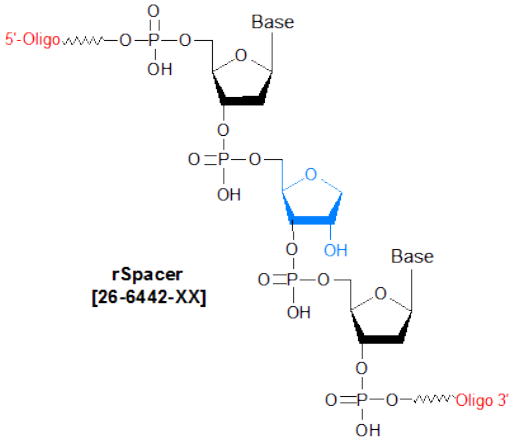
| | |
|---|---|
| Category | Photo Cleavable |
| Modification Code | PC-Sp-C3 |
| Reference Catalog Number | 26-6889 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 344.26 |




**PC Spacer (photocleavable)**
**[26-6889-XX]**

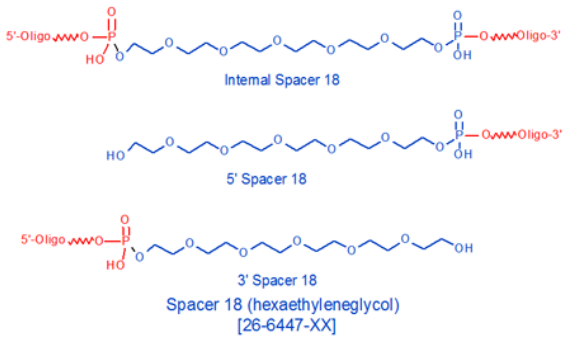
# rSpacer

| | |
|---|---|
| Category | Spacers |
| Modification Code | rABS |
| Reference Catalog Number | 26-6442 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 196.09 |



# Spacer 18

| | |
|---|---|
| Category | Spacers |
| Modification Code | Sp18 |
| Reference Catalog Number | 26-6447 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 344.3 |




Spacer 18 also known as PEG6 is a hexaethylene glycol chain that is 18 atoms long (12 carbons + 6 oxygen's), and is used to incorporate a long spacer arm into an oligonucleotide. Spacer 18 can be incorporated in consecutive additions whenever a longer spacer is required. Spacer 18 had been used to form bold folds and hairpin loops in oligonucleotides (1,2), and for solid-phase immobilization of hybridization probes (3). Spacer 18 has also been used to modify random primers used in whole genome amplification (WGA)-based applications, as a way to eliminate self-priming events that form spurious DNA products (that is, false-positive amplification) in the PCR reactions (4).
Gene Link offers spacers of various length, examples C2, C3, C6, C12 and Spacer 9 and 18. These can be inserted multiple times to yield a total much longer spacer length. **References**

1. Salunkhe, M., Wu, T.F., Letsinger, R.L. Control of folding and binding of oligonucleotides by use of non-nucleotide linker. *J. Am. Chem. Soc.* (1992), **114**: 8768-8772.
2. Durand, M., Chevrie, K., Chassignol, M., Thuong, N.T., Maurizot, J. Circular dichroism studies of an oligodeoxyribonucleotide containing a hairpin loop made of a hexaethylene glycol chain: conformation and stability.*Nucleic Acids Res.* (1990), **18**: 6353-6359.
3. Zhang, Y., Coyne, M.Y., Will, S.G., Levenson, C.H., Kawasaki, E.S. Single-base mutational analysis of cancer and genetic diseases using membrane bound modified oligonucleotides. *Nucleic Acids Res.* (1991), **19**: 3929-3933.
4. Brukner, I., Paquin, B., Belouchi, M., Labuda, D., Krajinovic, M. Self-priming arrest by modified random oligonucleotides facilitates the quality control of whole genome amplification.*Anal. Biochem.* (2005), **339**: 345-347.

Oligonucleotide PEGylation : Spacers vs. PEGylation Gene Link offers short PEG3 and PEG6 as direct coupling using automated chemistry. The PEG3 is termed as Spacer 9 and PEG6 as spacer 18. These are also used to introduce space between adjacent sequence and modifications. These can be inserted multiple times to increase the PEG units.
Larger 2, 5, 10 and 20 kDa PEGylation of oligonucleotides is inserted at any site of an oligonucleotide using a post synthesis amino group on the oligo with PEG-NHS.
PEGylation is the covalent attachment of polyethylene glycol (PEG) to oligonucleotides such as DNA, RNA, antisense, siRNA and aptamers.

It improves pharmacokinetics, reduces renal clearance, increases nuclease stability, and decreases immunogenicity. (1) The way PEG shields its conjugated payload offers new challenges and opportunities for oligonucleotide PEGylation. Other than aptamers, the target of most oligonucleotides is a complementary sequence.
Comparison of PEGylation Size & Biological Outcome

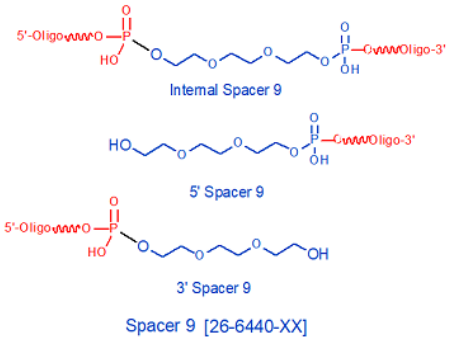
PEG Size (Ethylene Glycol Units)

Hydrodynamic Effect

Typical Outcome 2 kDa (~44) Minimal Slight stability increase 5 kDa (~114) Moderate Partial half-life improvement 10 kDa (~227) Strong Reduced renal clearance 20 kDa (~455) Very strong Long circulation 40 kDa (~910) Extreme Depot-like behavior Pharmacokinetics depend on hydrodynamic diameter, not molecular weight of the oligo. Unmodified 20-mer oligo ≈ ~7 kDa. Kidney filtration cutoff ≈ 40-60 kDa hydrodynamic equivalent Oligo PEG size controls circulation time versus tissue penetration. Optimal design balances exposure and activity
Messenger RNA (mRNA) delivery strategies are required to protect biologically fragile mRNA from ribonuclease (RNase) attacks to achieve efficient therapeutic protein expression. To tackle this issue, most mRNA delivery systems have used cationic components.
A cation-free delivery strategy by hybridization of PEGylated RNA oligonucleotides with mRNA. The PEG strands on the mRNA sterically and electrostatically shields the mRNA, improving mRNA nuclease stability 15-fold and the PEGylated mRNA induced nearly 20-fold higher efficiency of reporter protein expression than unhybridized mRNA in cultured cells (2).
PEGylation has been used to improve the biopharmaceutical properties of protein drugs since the 1990s, and over a dozen PEGylated pharmaceuticals are currently on the market (2). PEG creates a large hydration shell, which sterically blocks other biomacromolecules from penetrating through the polymer layer and binding with the interior substrate (3, 4). Binding requires displacing the PEG by the incoming molecule, generally making such binding less thermodynamically favorable. These properties usually result in weaker interactions between the receptor and the conjugated molecule, but increased drug solubility, prolonged blood circulation, and increased drug stability often offset by the reduced binding affinity. PEGylated oligonucleotides can be an exception to this generalization, with increased binding to a complementary sequence compared to unmodified ONs. The effect is attributed to macromolecular volume exclusion (6).
**PEGylation References**
1. Li WJ; Zhan P; De Clercq E; Lou HX; Liu XY Current drug research on PEGylation with small molecular agents. Prog. Polym. Sci 2013, 38, 421-444.
2. Yoshinaga, N; Naito, M; Tachihara, Y; Boonstra, E; Osada, K; Cabral, H and Uchida, S. PEGylation of mRNA by Hybridization of Complementary PEG-RNA Oligonucleotides Stabilizes mRNA without Using Cationic Materials. Pharmaceutics 2021, 13, 800.
3. Harris JM; Chess RB Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov 2003, 2, 214-221. [PubMed: 12612647]
4. Harris JM; Martin NE; Modi M Pegylation. Clin. Pharmacokinet 2001, 40, 539-551. [PubMed: 11510630]
5. Plesner B; Fee CJ; Westh P; Nielsen AD Effects of PEG size on structure, function and stability of PEGylated BSA. Eur. J. Pharm. Biopharm 2011, 79, 399-405. [PubMed: 21620970]
6. Nakano S-I; Karimata H; Ohmichi T; Kawakami J; Sugimoto N The effect of molecular crowding with nucleotide length and cosolute structure on DNA duplex stability. J. Am. Chem. Soc 2004, 126, 14330-14331. [PubMed: 15521733]

# Spacer 9

| | |
|---|---|
| Category | Spacers |
| Modification Code | Sp9 |
| Reference Catalog Number | 26-6440 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 212.14 |




Spacer 9 [26-6440-XX]

Spacer 9 is a triethylene glycol (PEG30 chain that is 9 atoms long (6 carbons + 3 oxygens), and is used to incorporate a spacer arm into an oligonucleotide. Spacer 9 can be incorporated in consecutive additions whenever a longer spacer is required. Spacer 9 has been used to form non-nucleotide bridges in hairpin loops in oligonucleotides (1), for linking oligonucleotides to epitopes for drug development (2), and for solid-phase immobilization of hybridization probes (3). Multiple incorporation of Spacer 9 has been used to form long, flexible linker arms between the two domains (double-helix forming and triple-helix forming, respectively) of a bifunctional DNA oligonucleotide, in order to maximize the binding flexibility of the two domains for their respective targets (4). This oligo was used to form a peptide nucleic acid (PNA)-DNA conjugate for use in site-directed recombination applications.
Gene Link offers spacers of various length, examples C2, C3, C6, C12 and Spacer 9 and 18. These can be inserted multiple times to yield a total much longer spacer length. **References**

1. Nelson, J.S., Giver, L., Ellington, A.D., Letsinger, R.L. Incorporation of Non-Nucleotide Bridge into Hairpin Oligonucleotides Capable of High-Affinity Binding to the Rev Protein of HIV-1. *Biochemistry.* (1996), **35**: 5339-5344.
2. Palma, E., Klapper, D.G., Cho, M.J. Antibodies as Drug Carriers III: Design of Oligonucleotides with Enhanced Binding Affinity for Immunoglobulin G. *Pharm. Res.* (2005), **22**: 122-127.
3. Beattie, W.G., Meng, L., Turner, S.L., Varma, R.S., Dao, D.D., Beattie, K.L. Hybridization of DNA targets to glass-tethered oligonucleotide probes. *Mol. Biotechnol.* (1995), **4**: 213-225.
4. Rogers, F.A., Vasquez, K.M., Egholm, M., Glazer, P.M. Site-directed recombination via bifunctional PNA-DNA conjugates. *Proc. Natl. Acad. Sci. USA* (2002), **99**: 16695-16700.

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1. Li WJ; Zhan P; De Clercq E; Lou HX; Liu XY Current drug research on PEGylation with small molecular agents. Prog. Polym. Sci 2013, 38, 421-444.
2. Yoshinaga, N; Naito, M; Tachihara, Y; Boonstra, E; Osada, K; Cabral, H and Uchida, S. PEGylation of mRNA by Hybridization of Complementary PEG-RNA Oligonucleotides Stabilizes mRNA without Using Cationic Materials. Pharmaceutics 2021, 13, 800.
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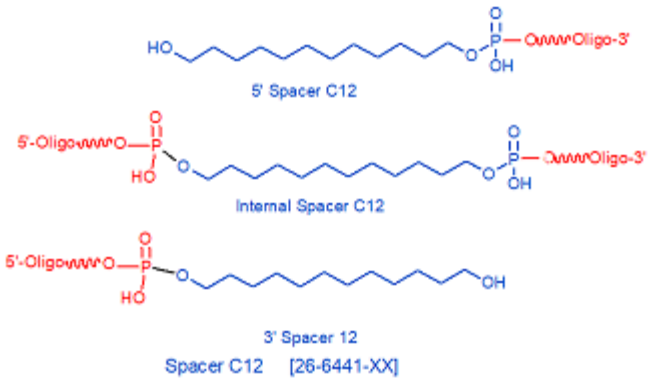
4. Harris JM; Martin NE; Modi M Pegylation. Clin. Pharmacokinet 2001, 40, 539-551. [PubMed: 11510630]
5. Plesner B; Fee CJ; Westh P; Nielsen AD Effects of PEG size on structure, function and stability of PEGylated BSA. Eur. J. Pharm. Biopharm 2011, 79, 399-405. [PubMed: 21620970]
6. Nakano S-I; Karimata H; Ohmichi T; Kawakami J; Sugimoto N The effect of molecular crowding with nucleotide length and cosolute structure on DNA duplex stability. J. Am. Chem. Soc 2004, 126, 14330-14331. [PubMed: 15521733]

## Spacer C12

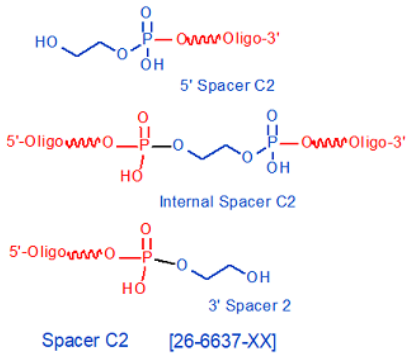
| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC12 |
| Reference Catalog Number | 26-6441 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 264.3 |




Spacer C12 [26-6441-XX]

# Spacer C2

| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC2 |
| Reference Catalog Number | 26-6637 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 125.04 |




Spacer C2 [26-6637-XX]

# Spacer C3 3'

| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC3-3 |
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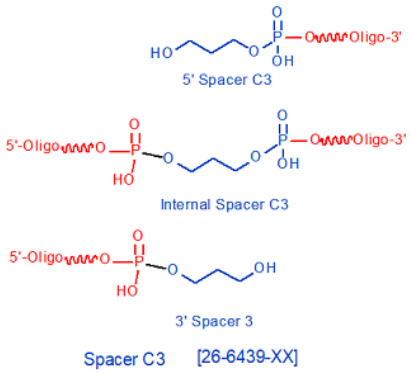
| 5 Prime | N |
| 3 Prime | Y |
| Internal | N |
| Molecular Weight(mw) | 138.06 |




Spacer C3 [26-6439-XX]

# Spacer C3 Internal

| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC3-Int |
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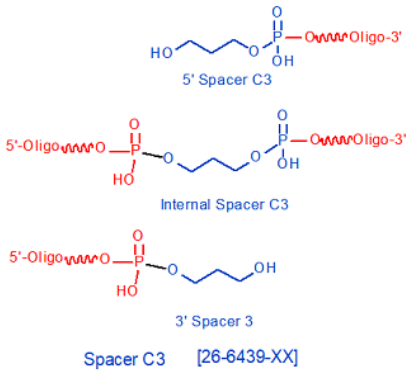
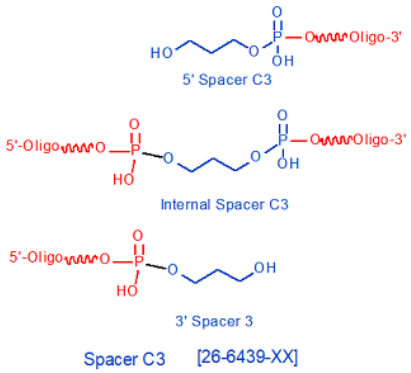
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 138.06 |




Spacer C3 [26-6439-XX]

## Spacer C3-5'

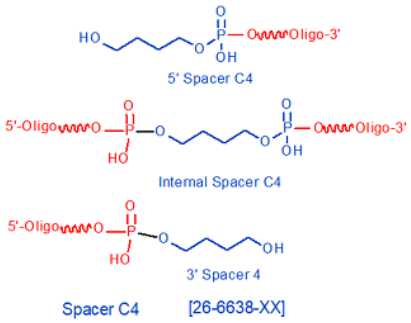
| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC3-5 |
| Reference Catalog Number | 26-6439F |
| 5 Prime | Y |
| 3 Prime | N |
| Internal | N |
| Molecular Weight(mw) | 138.06 |




Spacer C3 [26-6439-XX]

# Spacer C4

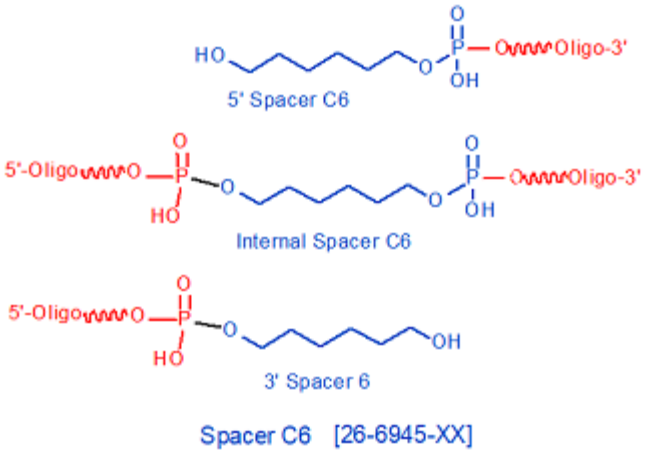
| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC4 |
| Reference Catalog Number | 26-6638 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 153.09 |




Spacer C4 [26-6638-XX]

## Spacer C6

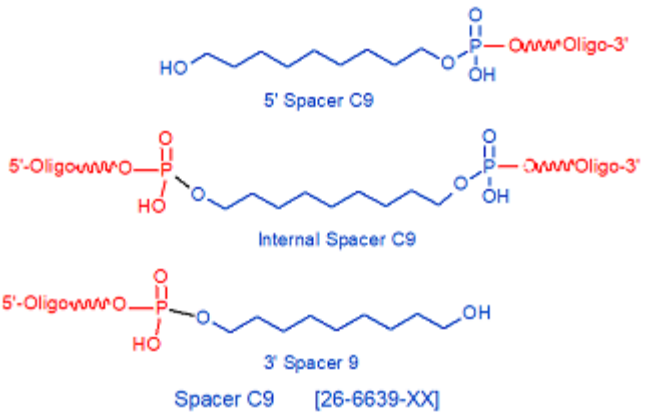
| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC6 |
| Reference Catalog Number | 26-6945 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 180.06 |




Spacer C6 [26-6945-XX]

# Spacer C9

| | |
|---|---|
| Category | Spacers |
| Modification Code | SpC9 |
| Reference Catalog Number | 26-6639 |
| 5 Prime | Y |
| 3 Prime | Y |
| Internal | Y |
| Molecular Weight(mw) | 222.3 |




Spacer C9 [26-6639-XX]

Spacer modifications C2, C3, C4, C6, C9, C12 and triethylene glycol Spacer 9 (PEG30 and 18 (PEG6)are used to insert a spacer arm in an oligonucleotide. These modifications can be added in multiple additions when a longer spacer is required. 3'-Spacer C3 CPG may also act as a blocker of exonuclease and polymerase activity at the 3'-terminus. dSpacer is used to introduce a stable abasic site within an oligonucleotide. See "Photo-Cleavable" modification category for photo-cleavable Spacer.

Spacer C9 is a 9-carbon spacer that is used to incorporate a long spacer arm into an oligonucleotide. Spacer C12 can be incorporated in consecutive additions if a longer spacer is required. Spacers are frequently used for solid-phase immobilization of DNA probes or aptamers for microarray applications (1,2), but can be used for any oligonucleotide-based application requiring a long spacer arm. **References**

1. Reese, M.O., van Dam, R.M., Scherer, A., Quake, S.R. Microfabricated Fountain Pens for High-Density DNA Arrays. *Genome Res.* (2003), **13**: 2348-2352.
2. Lao, Y-H., Peck, K., Chen, L-C. Enhancement of Aptamer Microarray Sensitivity through Spacer Optimization and Avidity Effect. *Anal. Chem.* (2006), **81**: 1747-1754.

**Oligonucleotide PEGylation : Spacers vs. PEGylation Gene Link offers short PEG3 and PEG6 as direct coupling using automated chemistry. The PEG3 is termed as Spacer 9 and PEG6 as spacer 18. These are also used to introduce space between adjacent sequence and modifications. These can be inserted multiple times to increase the PEG units.**
**Larger 2, 5, 10 and 20 kDa PEGylation of oligonucleotides is inserted at any site of an oligonucleotide using a post synthesis amino group on the oligo with PEG-NHS.**
**PEGylation is the covalent attachment of polyethylene glycol (PEG) to oligonucleotides such as DNA, RNA, antisense, siRNA and aptamers. It improves pharmacokinetics, reduces renal clearance, increases nuclease stability, and decreases immunogenicity. (1) The way PEG shields its conjugated payload offers new challenges and opportunities for oligonucleotide PEGylation. Other than aptamers, the target of most oligonucleotides is a complementary sequence.**
**Messenger RNA (mRNA) delivery strategies are required to protect biologically fragile mRNA from ribonuclease (RNase) attacks to achieve efficient therapeutic protein expression. To tackle this issue, most mRNA delivery systems have used cationic components.**

A cation-free delivery strategy by hybridization of PEGylated RNA oligonucleotides with mRNA. The PEG strands on the mRNA sterically and electrostatically shields the mRNA, improving mRNA nuclease stability 15-fold and the PEGylated mRNA induced nearly 20-fold higher efficiency of reporter protein expression than unhybridized mRNA in cultured cells (2). PEGylation has been used to improve the biopharmaceutical properties of protein drugs since the 1990s, and over a dozen PEGylated pharmaceuticals are currently on the market (2). PEG creates a large hydration shell, which sterically blocks other biomacromolecules from penetrating through the polymer layer and binding with the interior substrate (3, 4). Binding requires displacing the PEG by the incoming molecule, generally making such binding less thermodynamically favorable. These properties usually result in weaker interactions between the receptor and the conjugated molecule, but increased drug solubility, prolonged blood circulation, and increased drug stability often offset by the reduced binding affinity. PEGylated oligonucleotides can be an exception to this generalization, with increased binding to a complementary sequence compared to unmodified ONs. The effect is attributed to macromolecular volume exclusion (6).

**PEGylation References**

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