Advanced RNA Synthesis as a Key Tool In RNA Biology Research.

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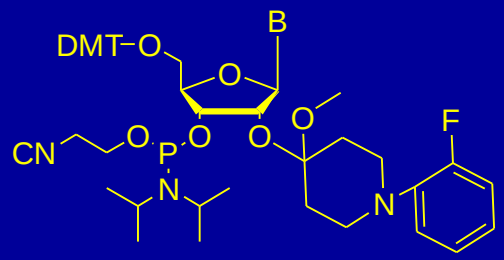
RNA Biology Challenges

- Optimizing chemistry and design of the corresponding small RNA
  (i.e. optimizing target sequence, chemical stability and nuclease resistance)
- Minimizing off target effect by optimizing antisense oligonucleotide-RISC complex
- Optimizing delivery system

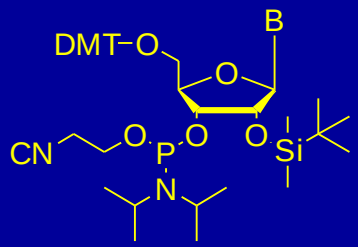
RNA Synthesis Challenges

- Monomer coupling efficiency
- Improving purity profile by minimizing n-1 and n+1 closed impurities
- Minimizing 3' ⇢ 2' phosphate backbone migration
- Optimizing attachment chemistry

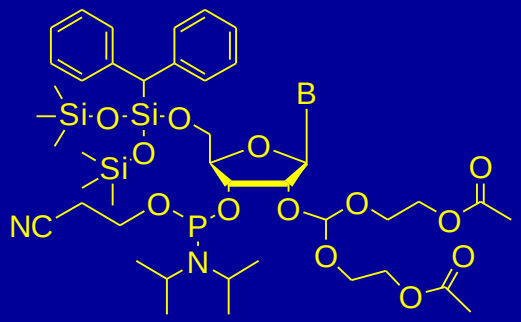
Classical Commercialized Strategies for RNA Synthesis



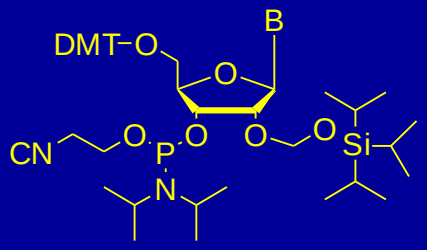
2'-*O*-Fpmp-3'-amidites;
C. Reese; J. Chem. Soc., Perkin Trans. 1, 43 - 55, 1993.



2'-*O*-TBDMS-3'-amidites;
K.K.Ogilvie; N.Usman; K.Nicoghosian; A.J. Cedergren; PNAS,USA; 85,5764-5768,1988.

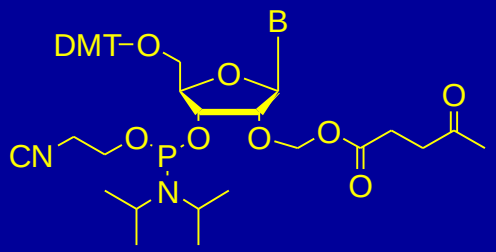


2'-*O*-ACE-3'-amidites;
S.A.Scaringe, F.E. Wincott, M.H.Caruthers ; JACS., 120, 11820-11821, 1998.

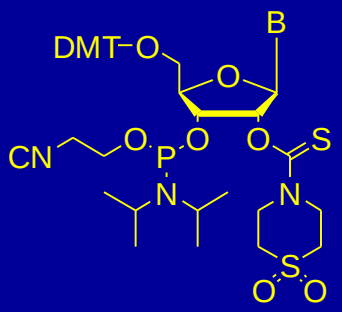


2'-*O*-TOM-3'-amidites;
S.Pitch; P.A.Weiss; X. Wu; D. Ackermann; T. Honegger, Helv, Chim. Acta, 82,1753-1761,1999.

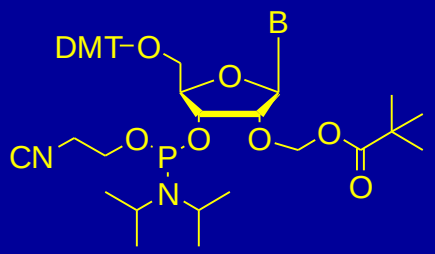
Relatively New Strategies for RNA Synthesis



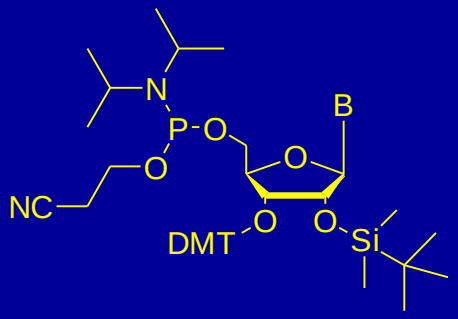
2'-*O*-ALE (Acetal levulinyl)-3'-amidites;
J.G. Lackey;D.Mitra; M.M.Somoza; F.Cerrina & M.J. Damha, NAR Sym Series, No. 52, pp51-52, 2008.



2'-Thio morpholino(TC)-3'-amidites;
D. Dellinger etal., Patent Application 12/118,655, 61/099,131, PCT/US08/63342

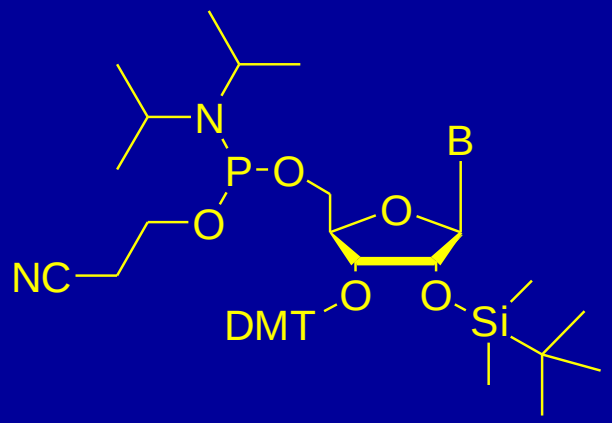


2'-*O*-PivOM-3'-amidites;
T. Lavergne, A. Martin, F. Diebart & J-J-Vasseur, NAR Sym Series, No. 52, pp51-52, 2008.



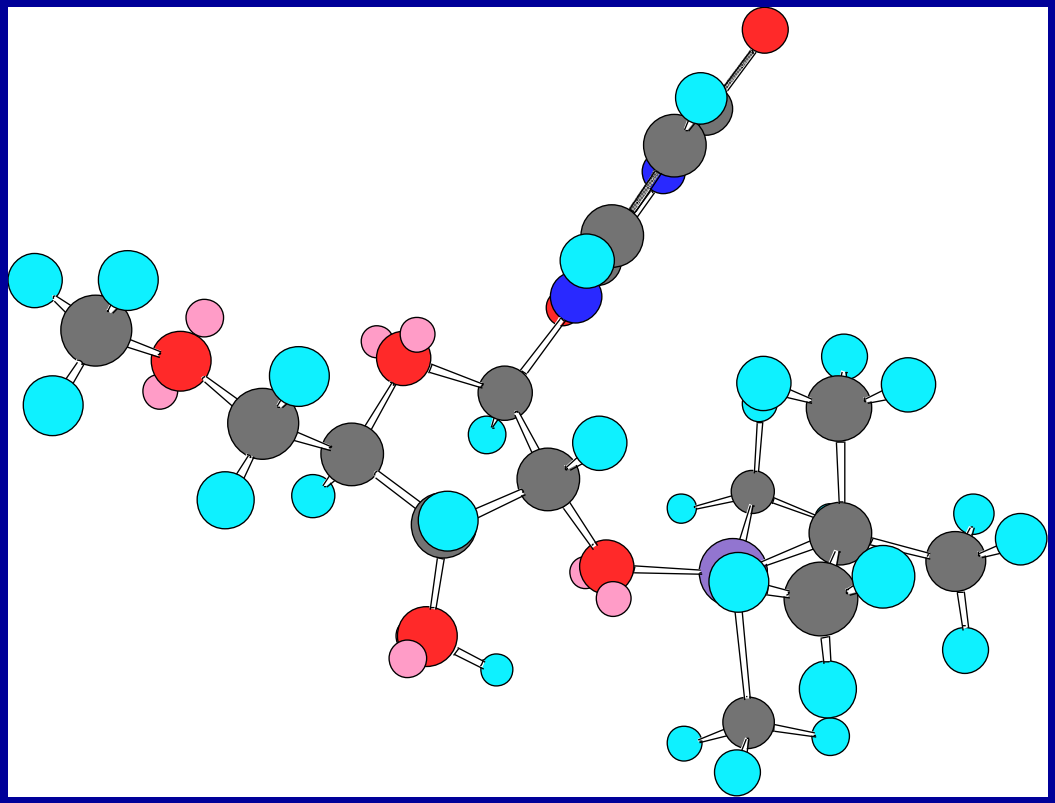
2'-*O*-TBDMS-5'-amidites;
S. Srivastava etal. Current Protocols in Nucleic Acid Chemistry, 2011

RNA Synthesis in Reverse Direction

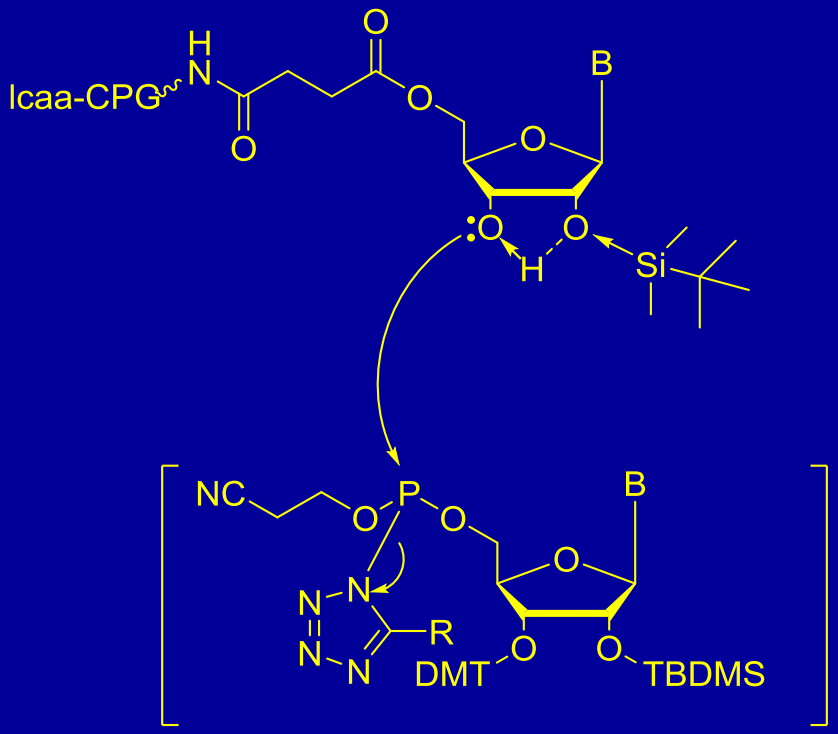


2’-TBDMS-3’-DMT-5’-Phosphoramidite

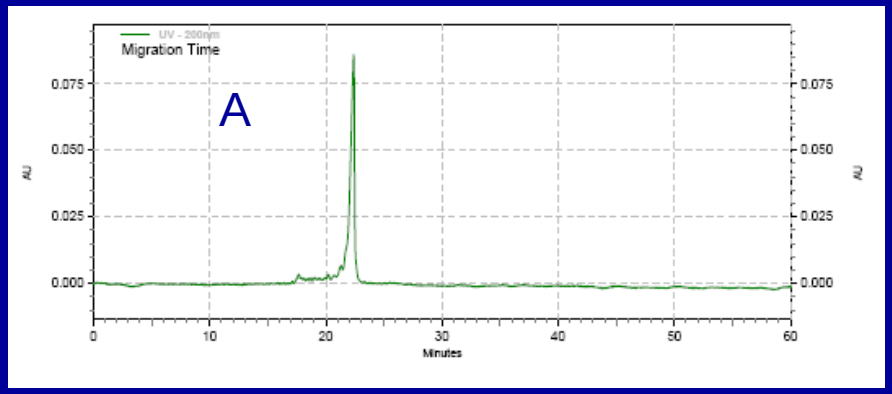
RNA Synthesis in Reverse Direction

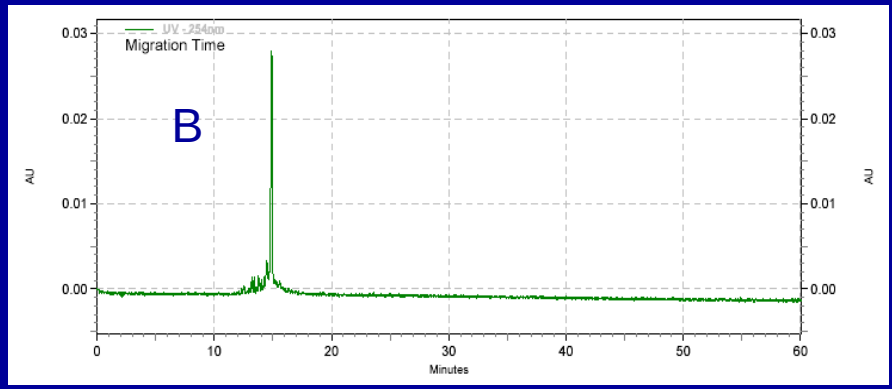


RNA Synthesis in Reverse Direction



RNA Synthesis in Reverse Direction





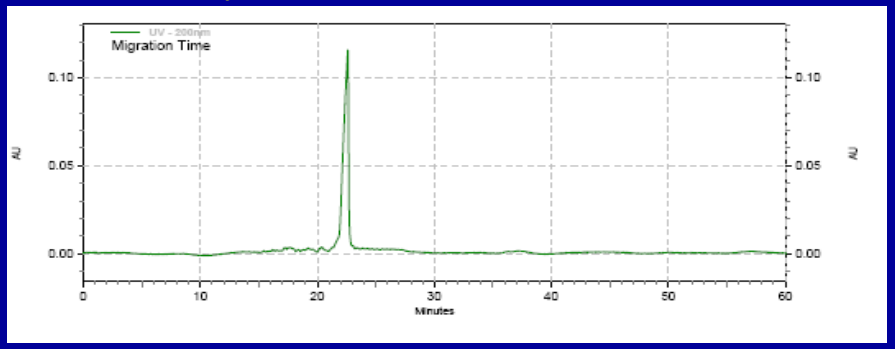
Typical CE traces of 20-mer RNA oligomers made by reverse synthesis (A) and by conventional method (B)

RNA Synthesis in Reverse Direction

CE Analysis of various RNAs (Crude desalted 20-mer) Synthesized with N+1 not observed

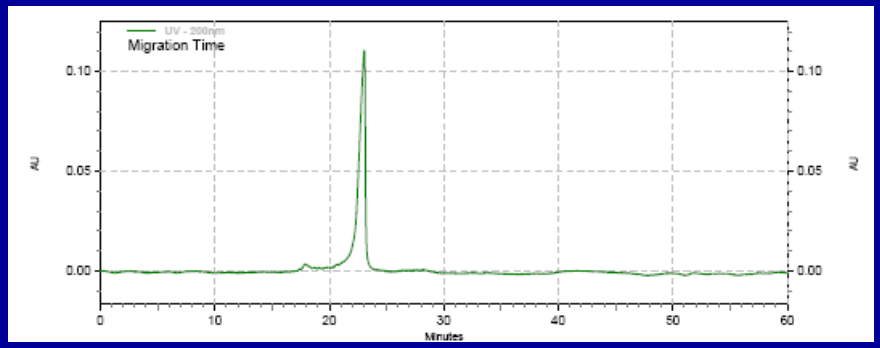
A) ETT; Coupling time 4 min (CE-Crude Purity:-91.29%)

2'-O-Methyl Chimera, 20mer



B) ETT; Coupling time 4 min (CE- Crude Purity: 89.09%)

Fully Thioated 2'-O-Methyl Chimera, 20 mer



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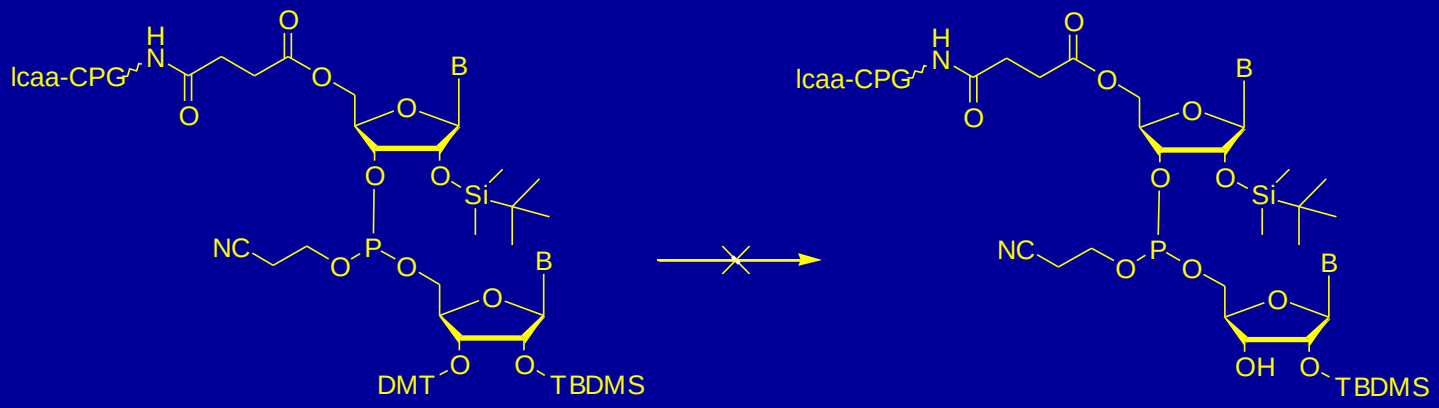
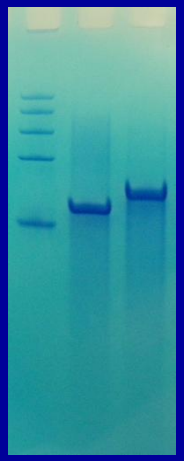
RNA Synthesis in Reverse Direction



Absence of N+1 impurity in reverse RNA synthesis

RNA Synthesis in Reverse Direction

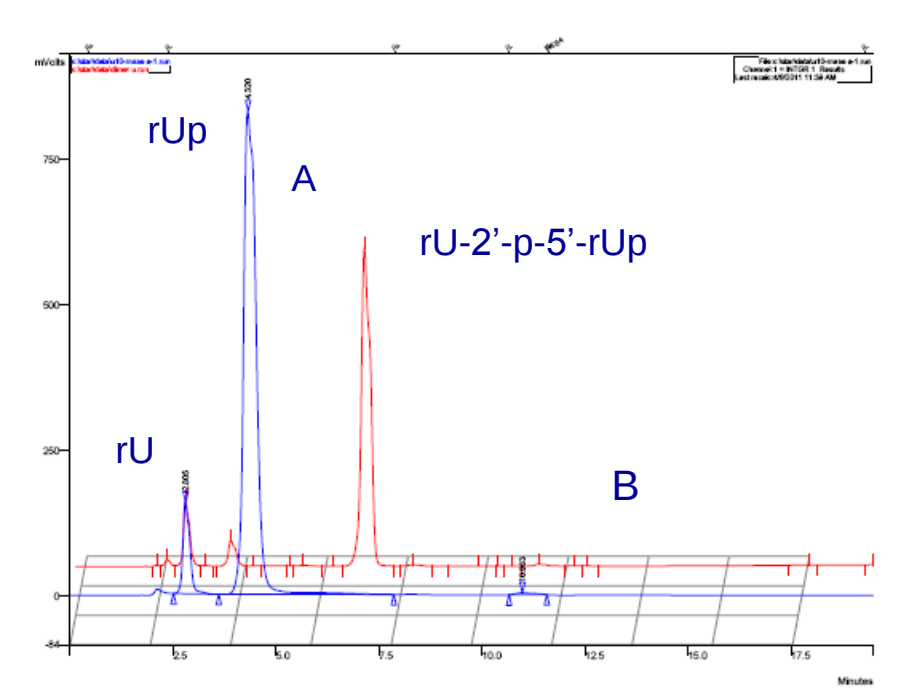


1 2 3

1. RNA lader 100/200/300/400/500; **2.** 125-mer poly-U ; **3.** 150-mer poly-U

PAGE of long poly-U crude RNAs via reverse RNA synthesis

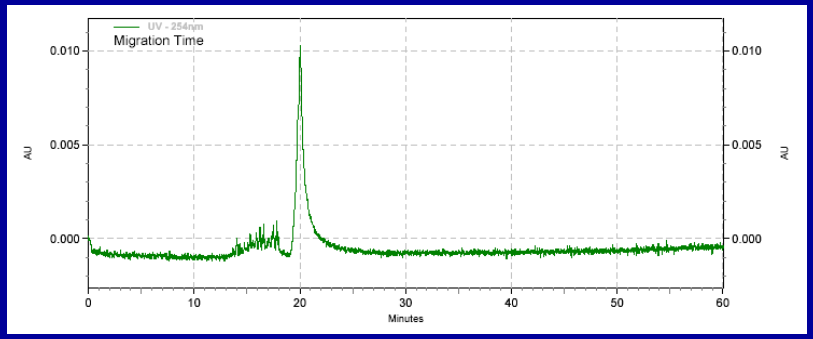
RNA Synthesis in Reverse Direction



Superposed HPLC traces of RNase A digested poly-U decamer (chromatogram A) and authentic sample of rU-2’-p-5’-rUp dimer (chromatogram B).

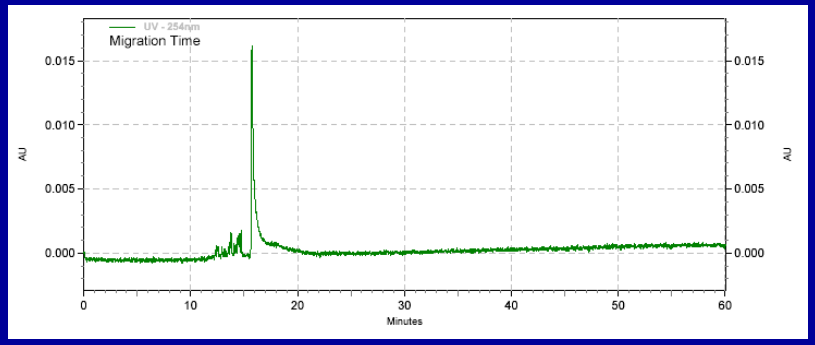
RNA Synthesis in Reverse Direction With 3’-end Modifications

21 mer 3’-Cholesterol TEG modified RNA Oligonucleotide, 1.0umol scale synthesis



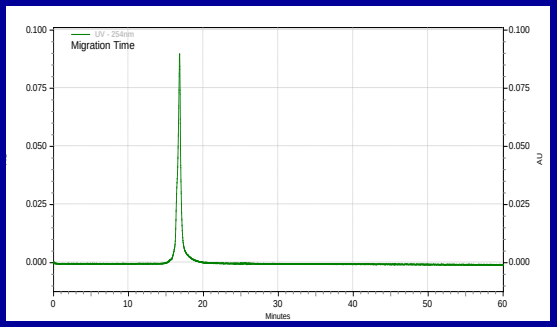
CE Analysis: Crude purity, ~ 75 %

21 mer 3’-Cholesterol TEG modified RNA Oligonucleotide synthesized using Conventional Method



CE Analysis: Crude purity, ~ 70 %

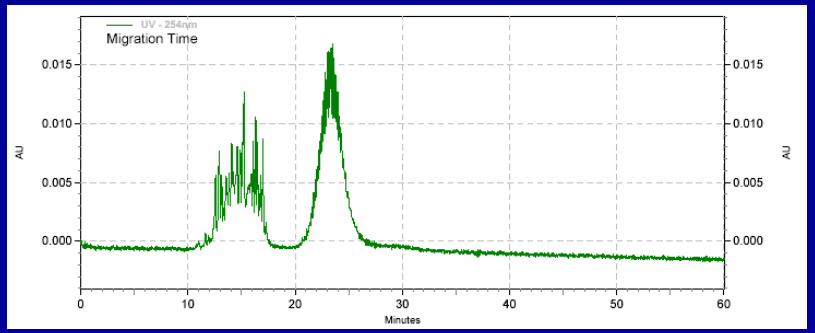
After Reverse Phase (C18) HPLC purification



CE Analysis: final purity, ~ 99 %

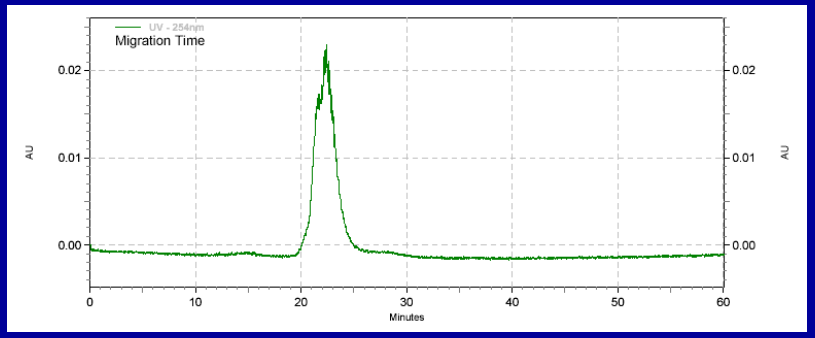
RNA Synthesis in Reverse Direction With 3’-end Modifications

21-mer RNA with 3’-PEG 2000, 1.0umol scale synthesis

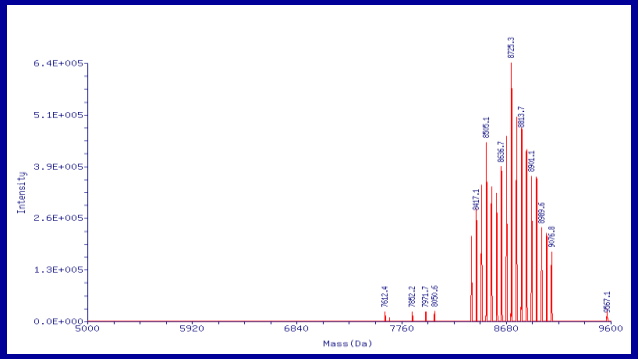


CE Analysis: crude purity ~ 65%

After RP-HPLC Purification



CE Analysis: purity > 99%



ESI Mass Spectral analysis: Calculated Molecular Weight: 8684.1
Observed Molecular Weight: 8681.1 & 8725.3 (most abundant ions)

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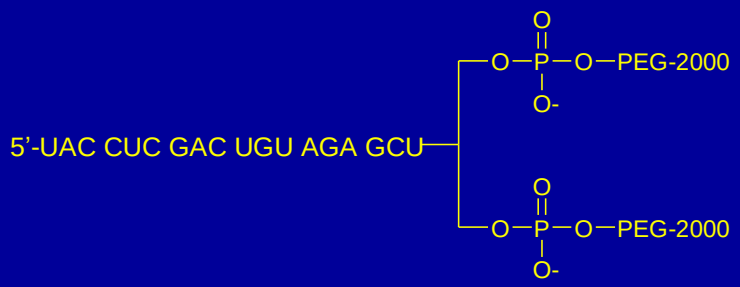
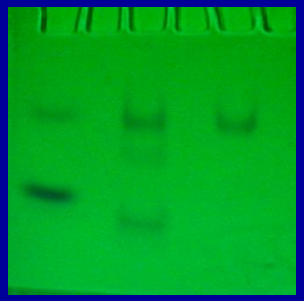
RNA Synthesis in Reverse Direction With 3’-end Modifications

18-mer RNA with 3’-Multiple PEGs (Branched PEGs)

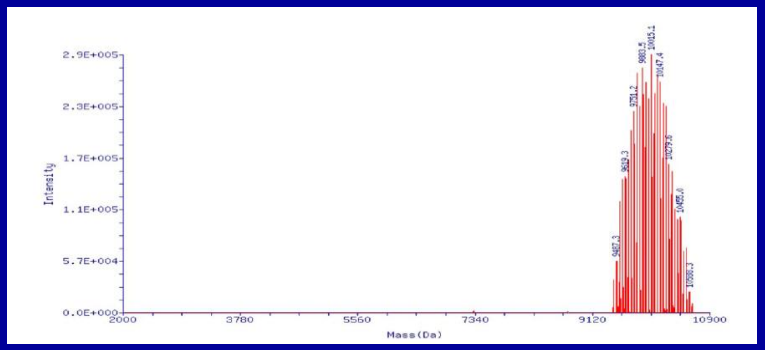




1 2 3

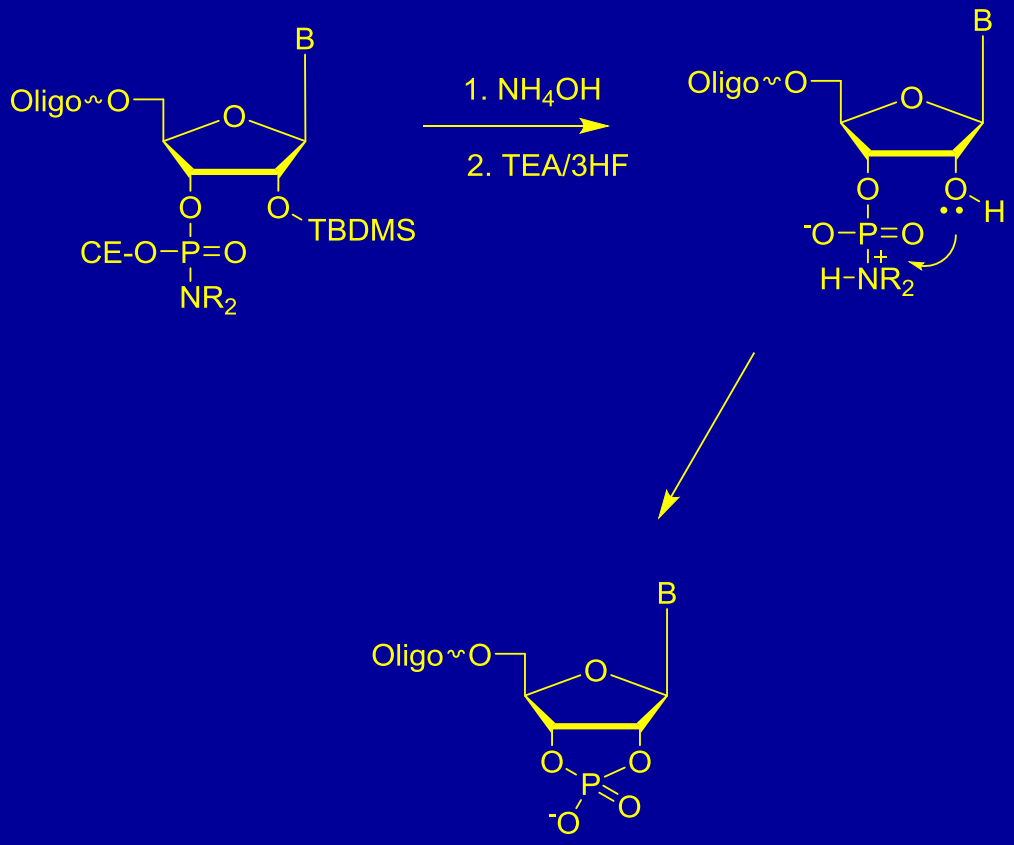
Lane 1 – BPB and Xylene Cynol; Lane 2 – Crude Oligo.;
Lane 3 – R P HPLC Purified Oligonucleotide.

RP HPLC purified 18-mer RNA with 3’-Branched PEG-2000



ESI/MS of RP HPLC Purified 3’-Branched PEG-2000 attached RNA,
Calculated Molecular Weight: 9926.5
Observed Molecular Weight: 9927.6 & 10015.1 (most abundant ions)

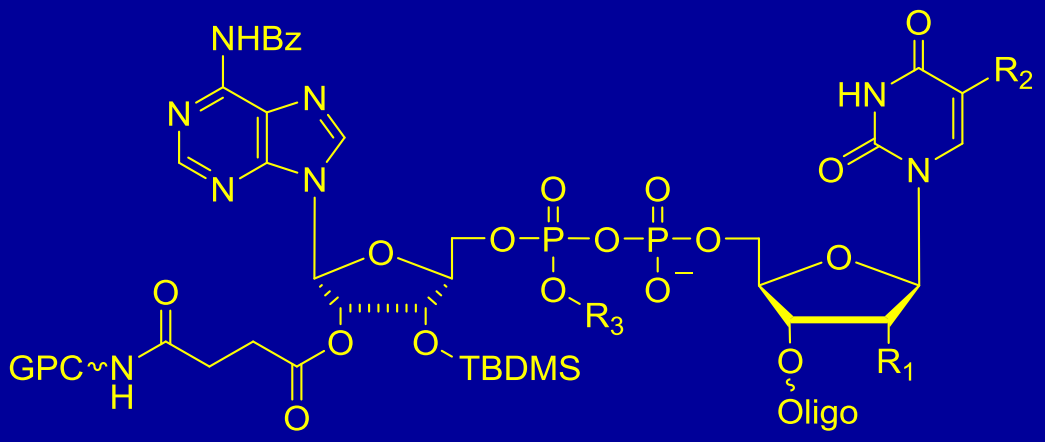
Unique Modifications by Using Reverse Synthesis: 2',3'-Cyclic Phosphate



Where R is iPr

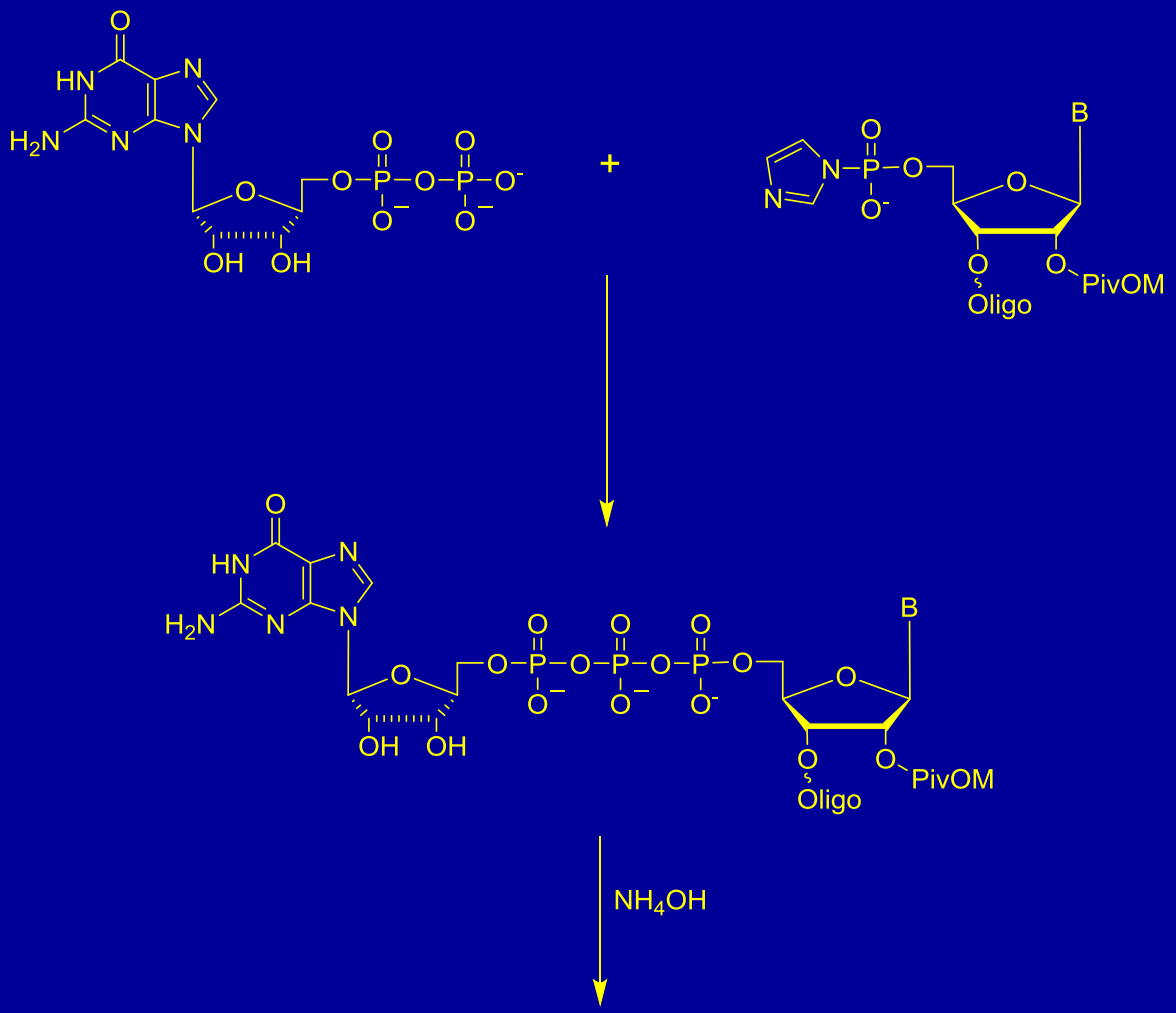
A. Laikhter et.al. US Patent 8,618,279

Unique Modifications by Using Reverse Synthesis: Adenylated Oligonucleotides



Where R_1 is H or O-TBDMS
R_2 is H or Me
R_3 is Salicylic acid

Conventional 5'-G-Capped Oligonucleotide Synthesis



Y. Thillier, E. Decroly, F. Morvan, B. Canard, J. Vasseur, and F. Debart, *RNA*, **2012**, *18*, 856-868

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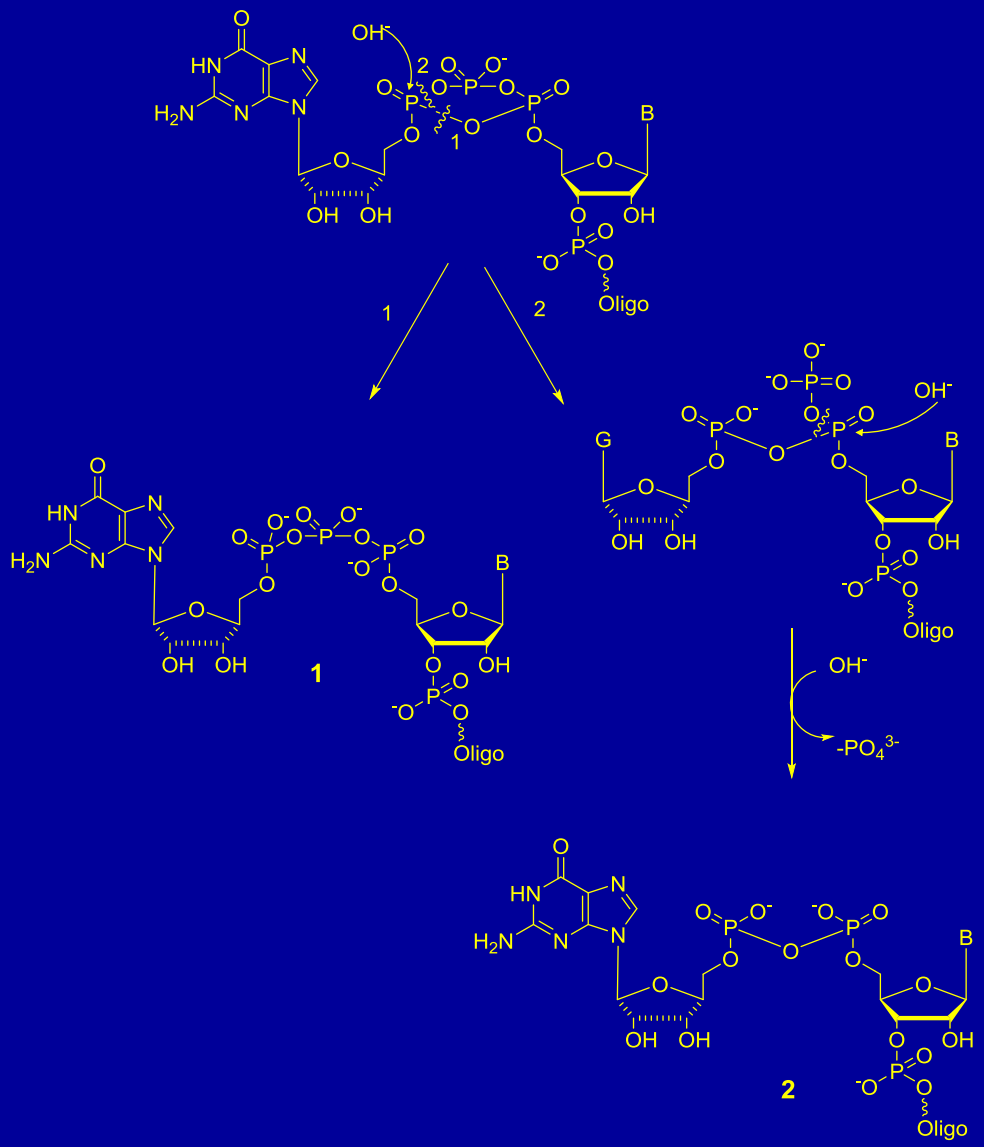
Conventional 5'-G-Capped Oligonucleotide Synthesis



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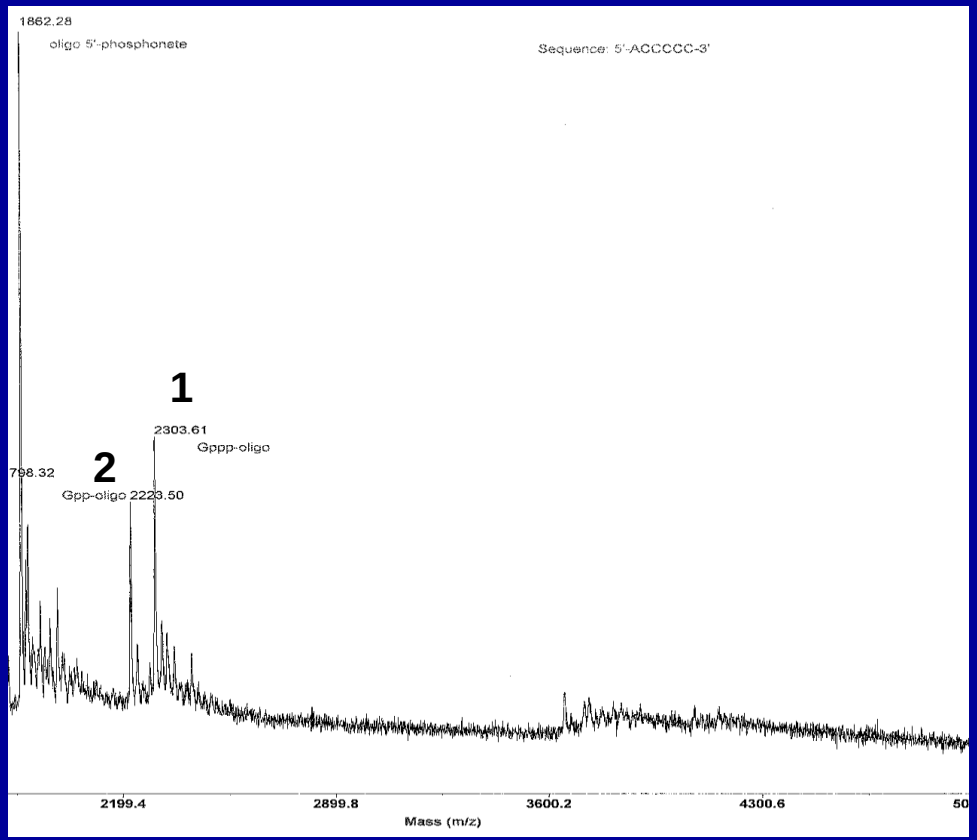
Conventional 5’-G-Capped Oligonucleotide Synthesis



MALDI TOFI Mass Spectral analysis:
Calculated Molecular Weight: 2298.3
Observed Molecular Weight: 2303.6

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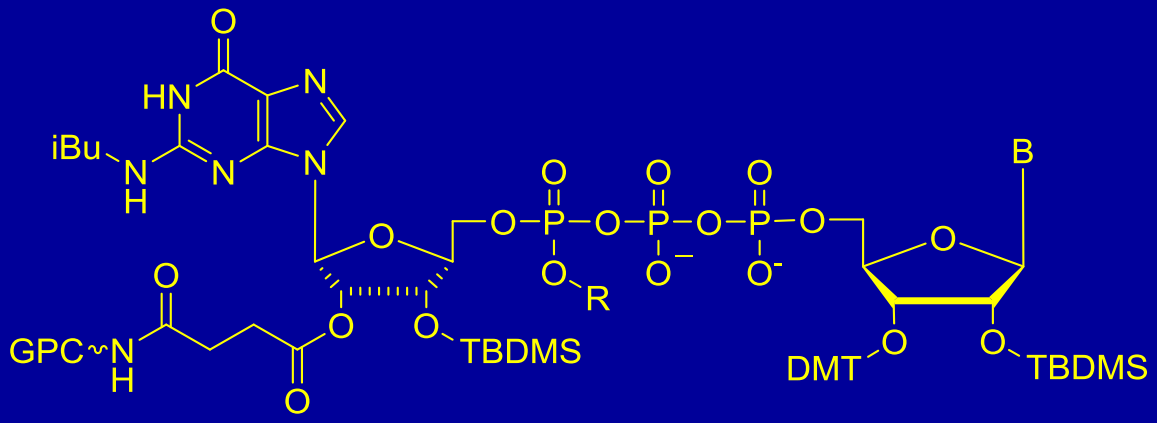
5’-G-Capped Oligonucleotides Using Reverse Synthesis Approach



Conclusions

1. Isomeric impurities essentially not present in 2'-TBDMS-3'-DMT-5'-Phosphoramidites
2. Coupling efficiency per step >99% (i.e. purity of crude 20-mers ranges between 83-92%)
3. Coupling time reduced three times from 12min to 4 min in comparison with conventional method
4. Dramatic reduction in n+1 impurities that leads to greater recovery of full length product
5. 3'-End modified oligonucleotides are extremely high purity
6. New unique modifications have been developed using reverse synthesis approach

Acknowledgements

Bio-synthesis, Inc:
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Naveen Srivastava
Navneet Singh