

# Preparation and Analysis of Black Hole Quencher Probes

Joseph I. Knecht

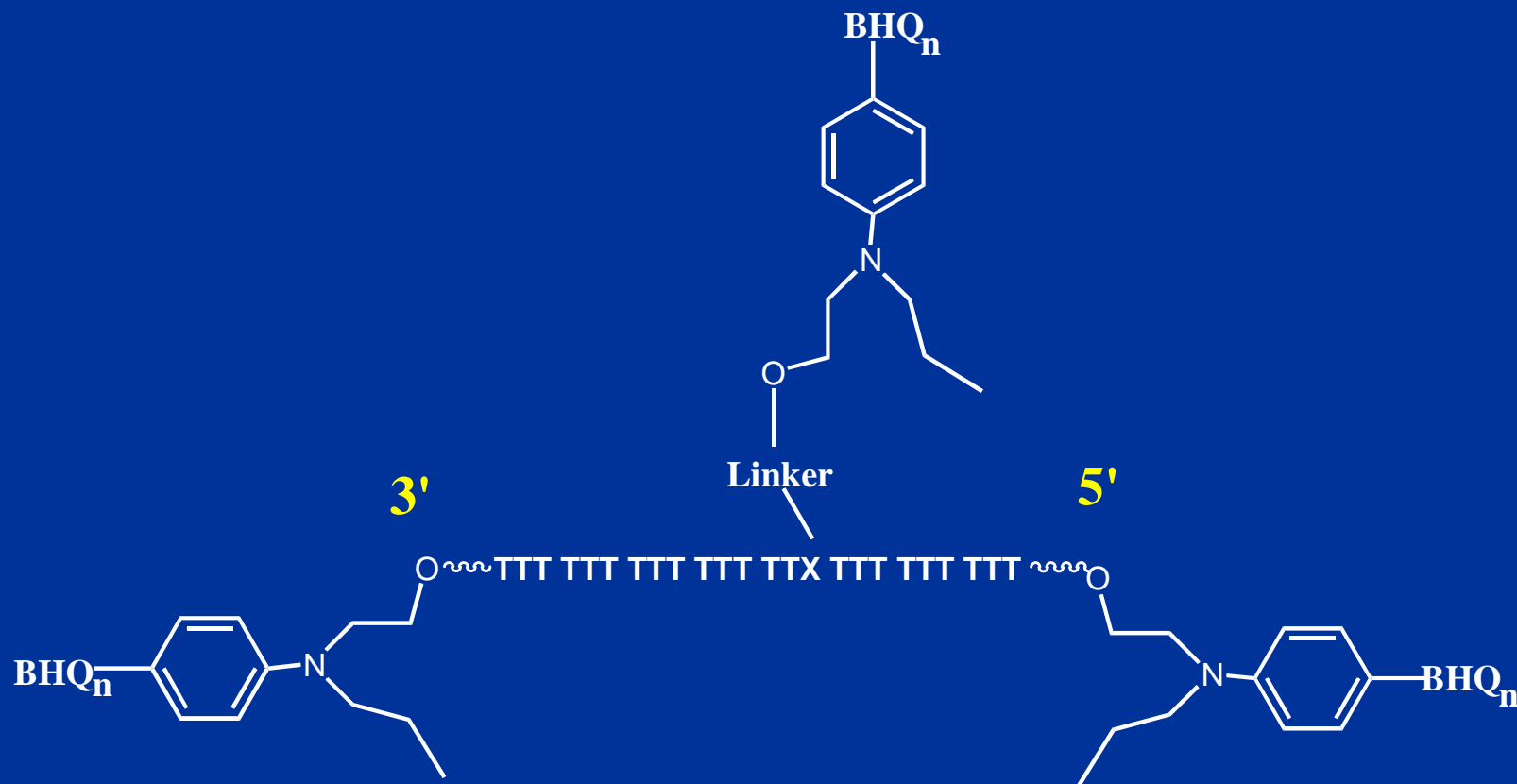


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BHQs may be placed at the 3' terminus, 5' terminus, or internally in an oligonucleotide



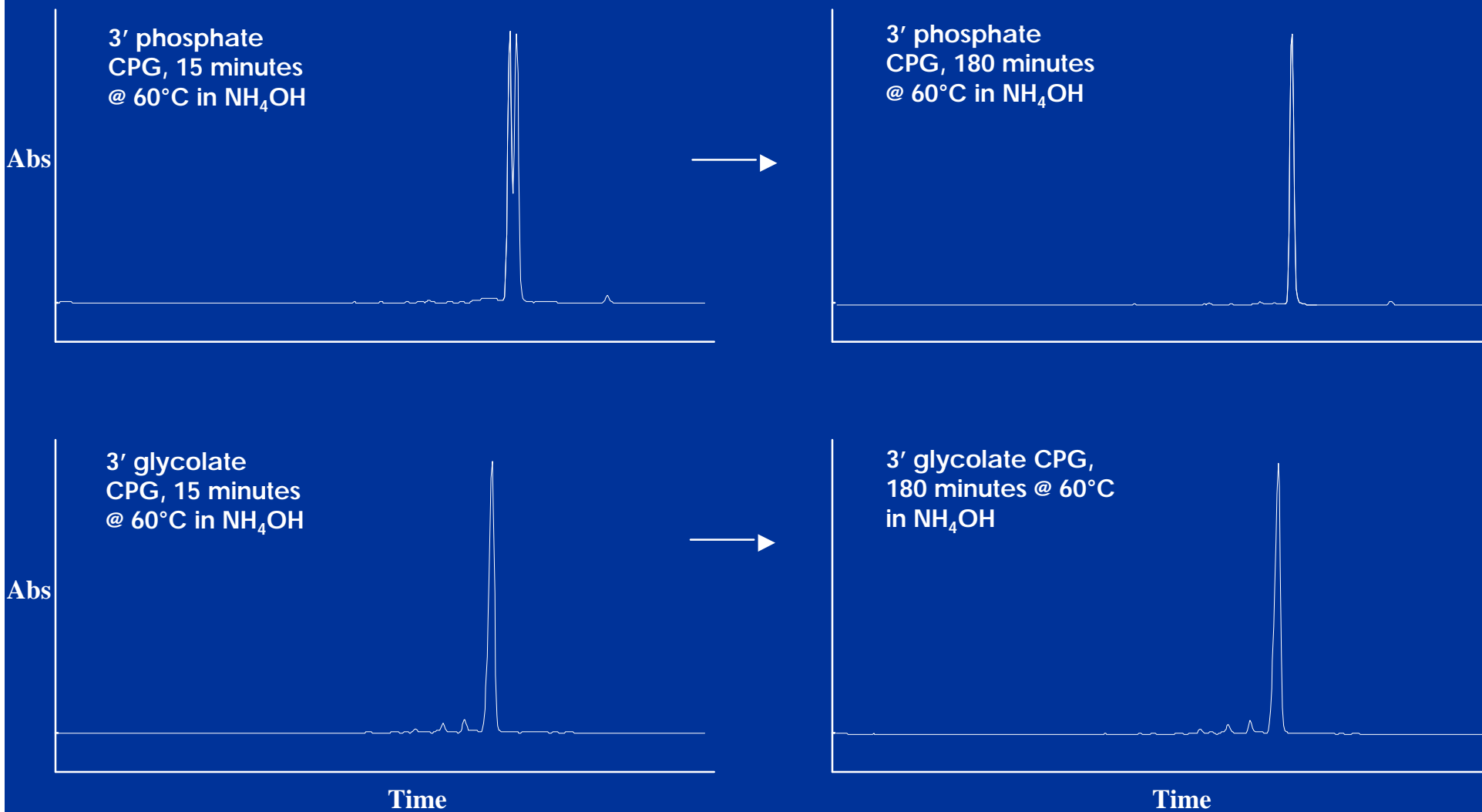
## **Two Classes of CPG available for 3' BHQ Labeling**

- **Sulfonyldiethanol (phosphate) linked BHQ CPGs**
- **Glycolate linked BHQ CPGs**

	<b>Advantages</b>	<b>Disadvantages</b>
<b>Phosphate</b>	<ul style="list-style-type: none"><li>• <b>Cleaves rapidly in NH<sub>4</sub>OH, TBA, K<sub>2</sub>CO<sub>3</sub>, AMA</b></li></ul>	<ul style="list-style-type: none"><li>• <b>Storage/shelf life issues</b></li><li>• <b>Slower deprotection</b></li></ul>
<b>Glycolate</b>	<ul style="list-style-type: none"><li>• <b>Cleaves Rapidly in NH<sub>4</sub>OH, AMA</b></li><li>• <b>Longer shelf life</b></li><li>• <b>Faster deprotection</b></li></ul>	<ul style="list-style-type: none"><li>• <b>Does not cleave rapidly in TBA, K<sub>2</sub>CO<sub>3</sub></b></li></ul>



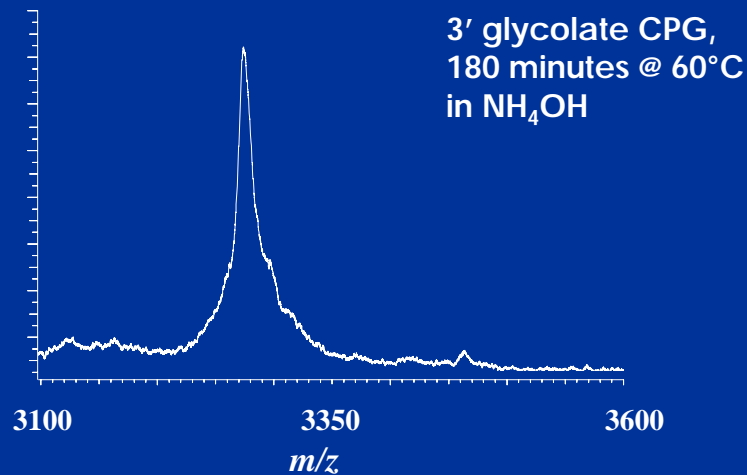
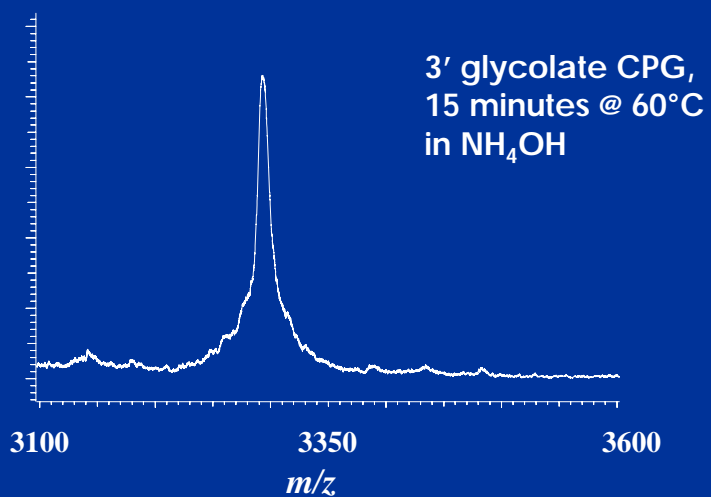
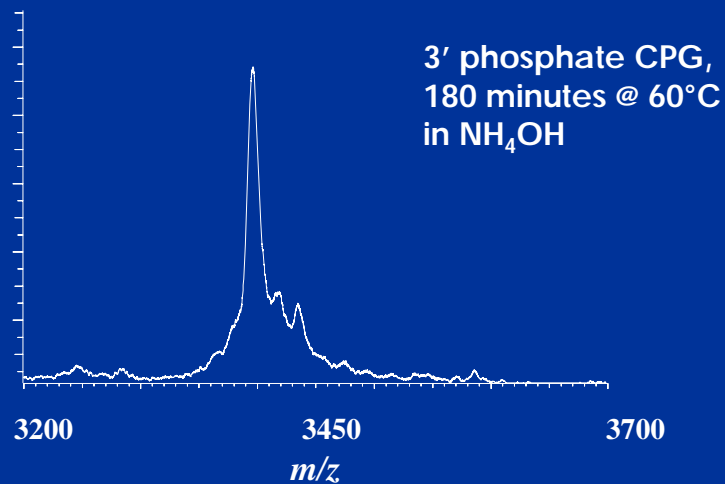
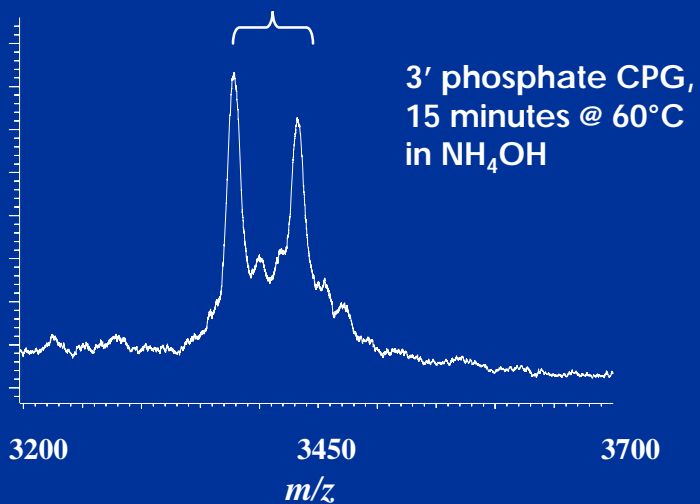
# 3' BHQ-1 T10s Analyzed by Anion Exchange HPLC



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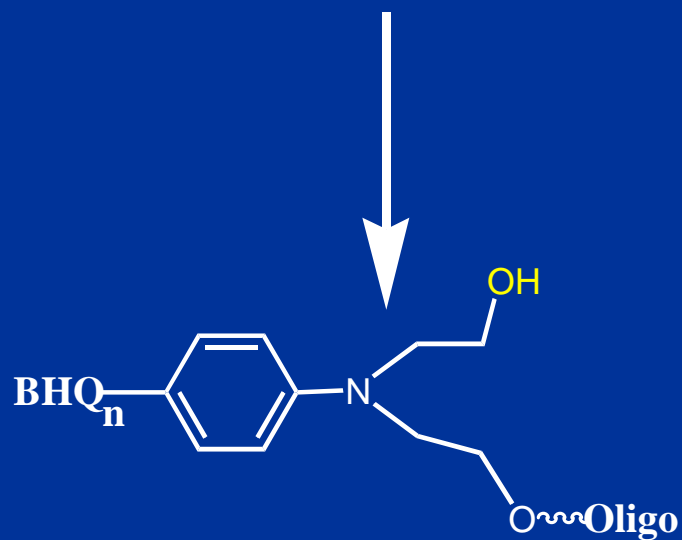
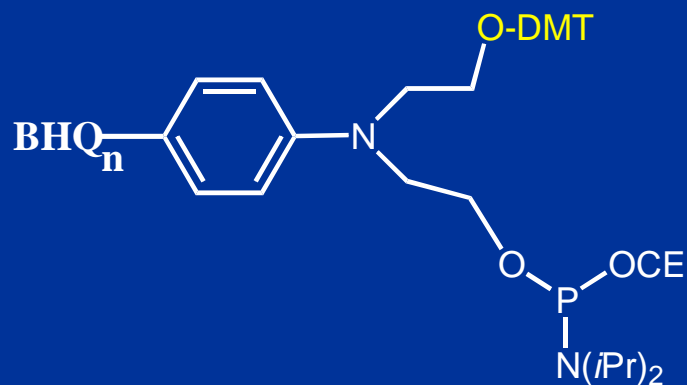
# BHQ-1 T10s Analyzed by MALDI-TOF MS: Incomplete Removal of Cyanoethyl from 3' Terminal Phosphate

? = 54 AMU

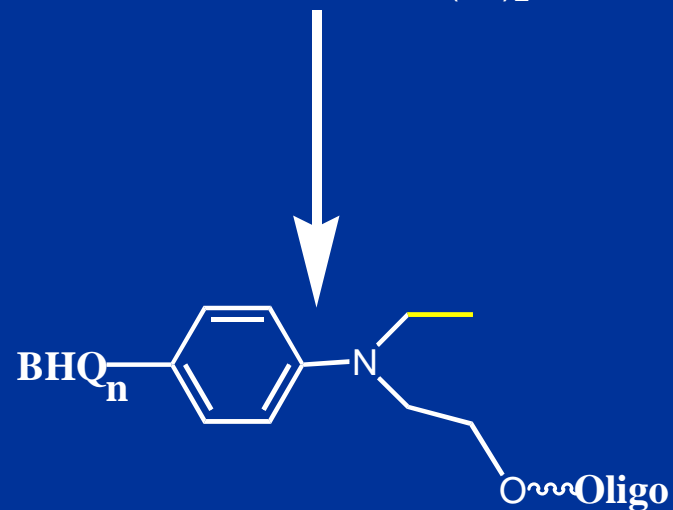
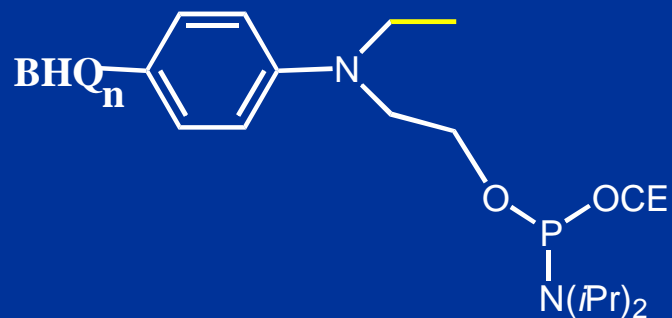


## Two classes of Amidites for 5' BHQ labeling

5' BHQ Amidite



5' BHQ 'Terminating' Amidite



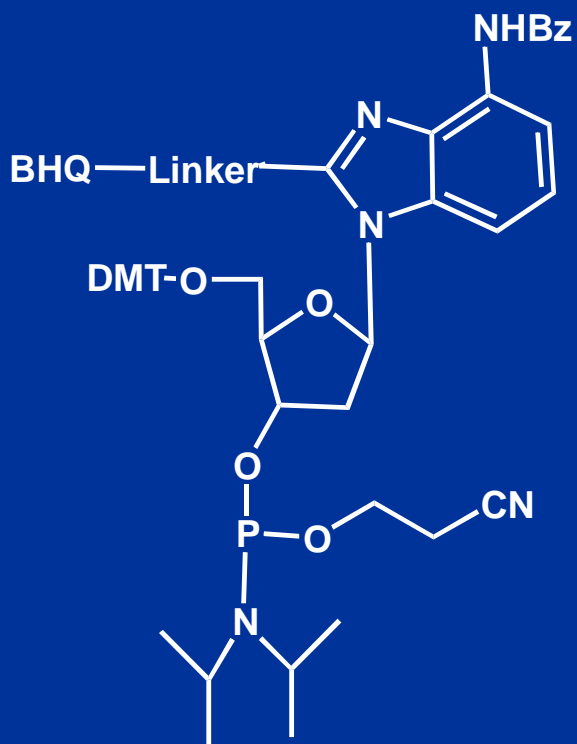
## **Three More Options for Internal BHQ Labeling**

- **BHQ phosphoramidites** - simplest approach to internal modification allows direct incorporation of quencher dye during automated synthesis
- **Levulinoyl Chemistry** - use of orthogonal protecting group strategies to allow coupling of terminating phosphoramidites at internal sites(levulinoyl dT amidite available)
- **Active Esters** - post synthetic labeling of internally amino modified oligos with BHQ esters

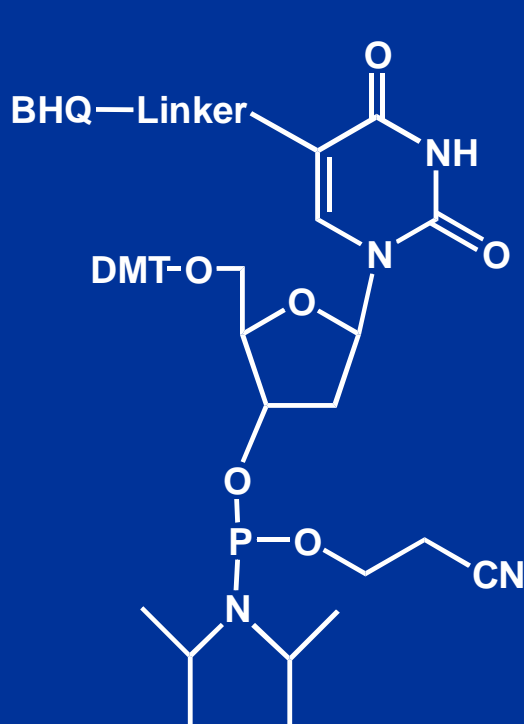
# Synthesis of Internally Labeled BHQ DNA

Nucleoside Amidites Modified with Quencher Moiety

Adenosine



Thymidine



Guanosine



## Recommended Cleavage/Deprotection Conditions for BHQ Probes

### 'Fast' Deprotection System

A<sup>Bz</sup> C<sup>Ac</sup> G<sup>dmf</sup> T

### 'Normal' Deprotection System

A<sup>Bz</sup> C<sup>Bz</sup> G<sup>ibu</sup> T

**FAM/BHQ1**

NH<sub>4</sub>OH 60°C 45min  
 NH<sub>4</sub>OH RT 15h  
 AMA 60°C 10min

NH<sub>4</sub>OH 60°C 3h  
 NH<sub>4</sub>OH RT 18h  
 AMA 60°C 15min

**TAMRA/BHQ2**

TBA 60°C 6h  
 NH<sub>4</sub>OH 60°C 45min

TBA 60°C 15h  
 NH<sub>4</sub>OH 60°C 3h

**BHQ3/Cy5\*\*<sup>1</sup>**

NH<sub>4</sub>OH 60°C 45min  
 NH<sub>4</sub>OH RT 15h

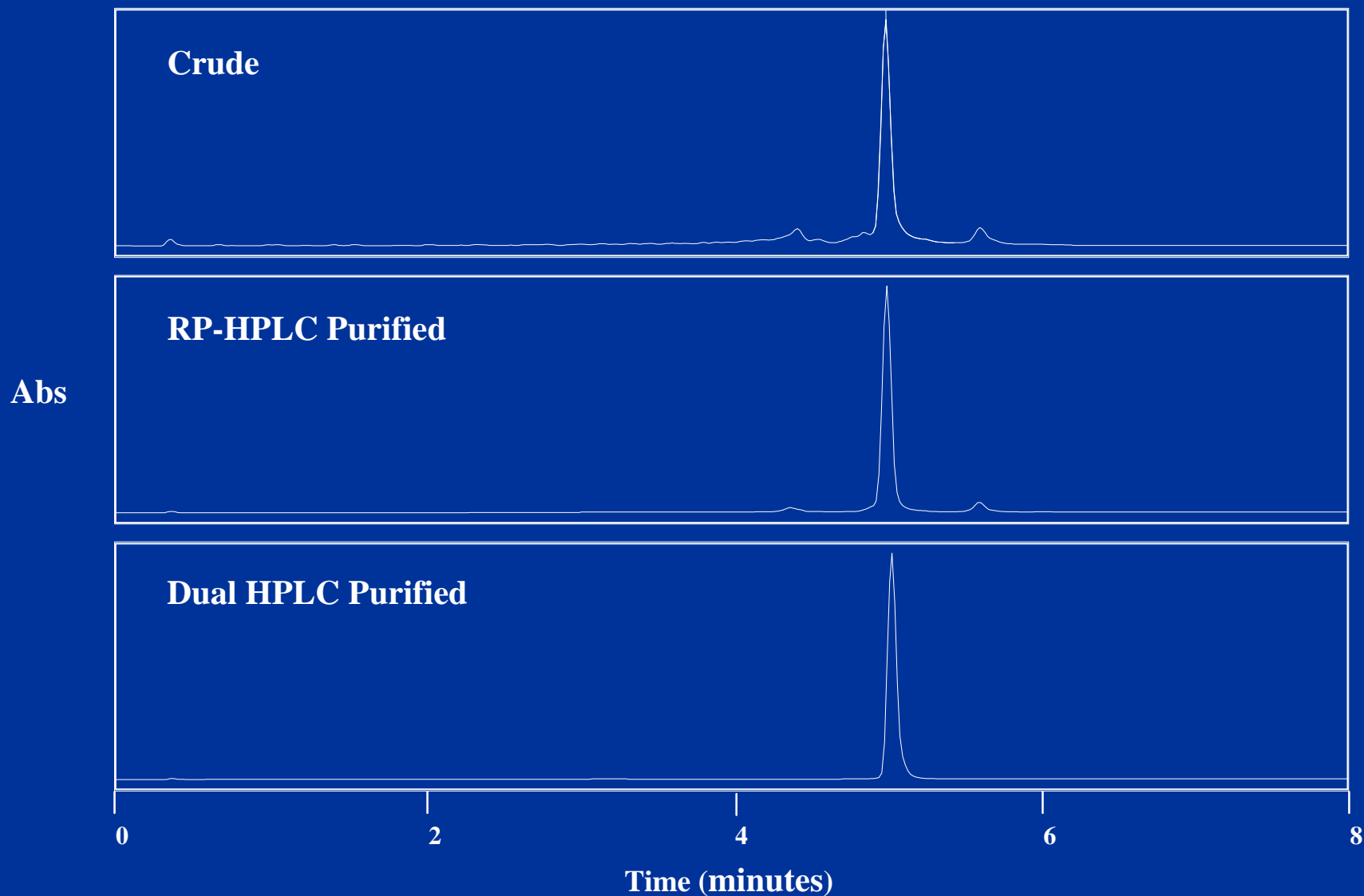
NH<sub>4</sub>OH RT 18h

<sup>1</sup> K<sub>2</sub>CO<sub>3</sub> and TBA Deprotection systems are not compatible with BHQ3

\*\* Placing the BHQ3 at the 5' end simplifies purification

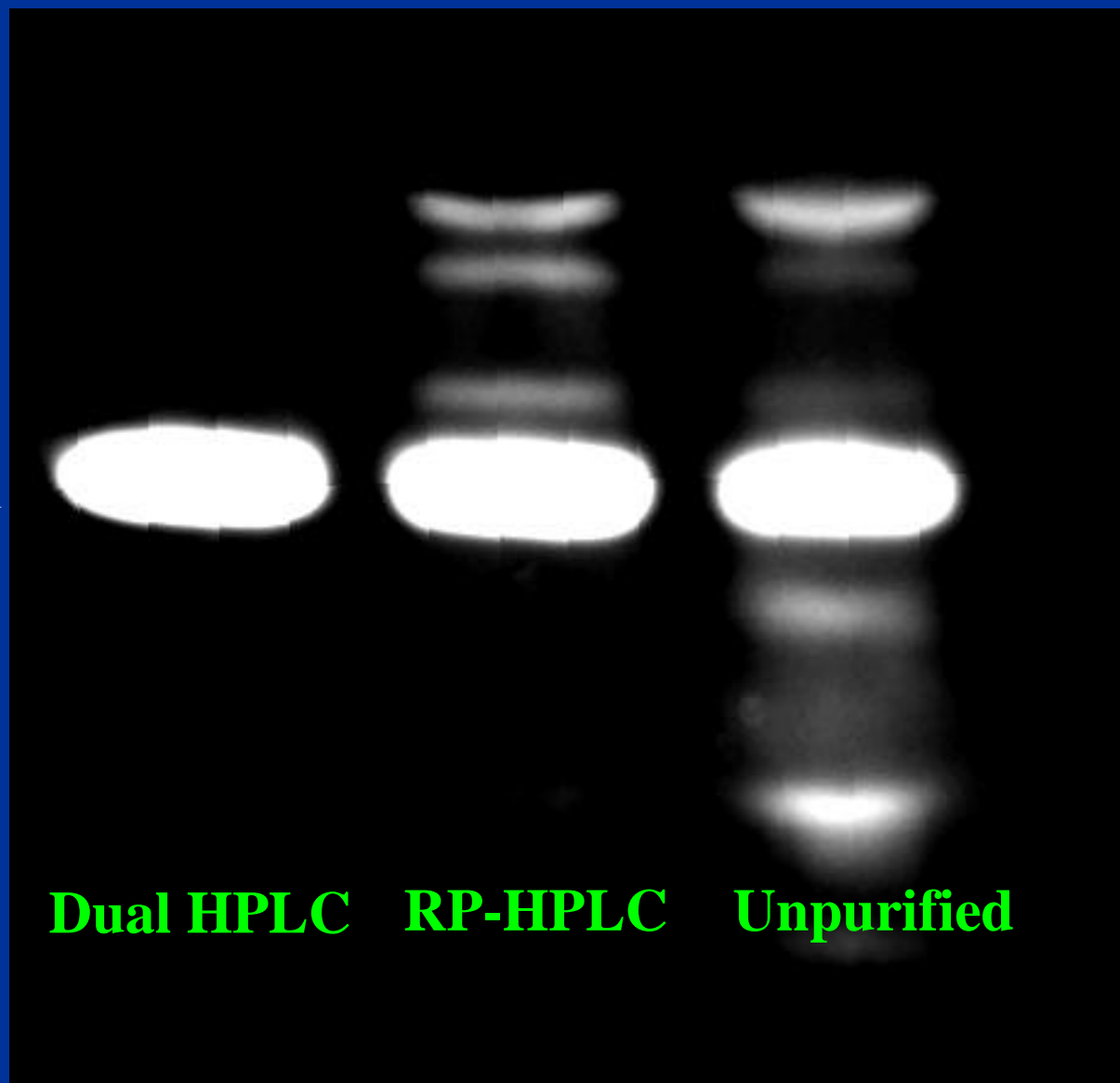
## HPLC Purification of FAM-BHQ 1 Probe

Anion – Exchange Data illustrating the different levels of purity

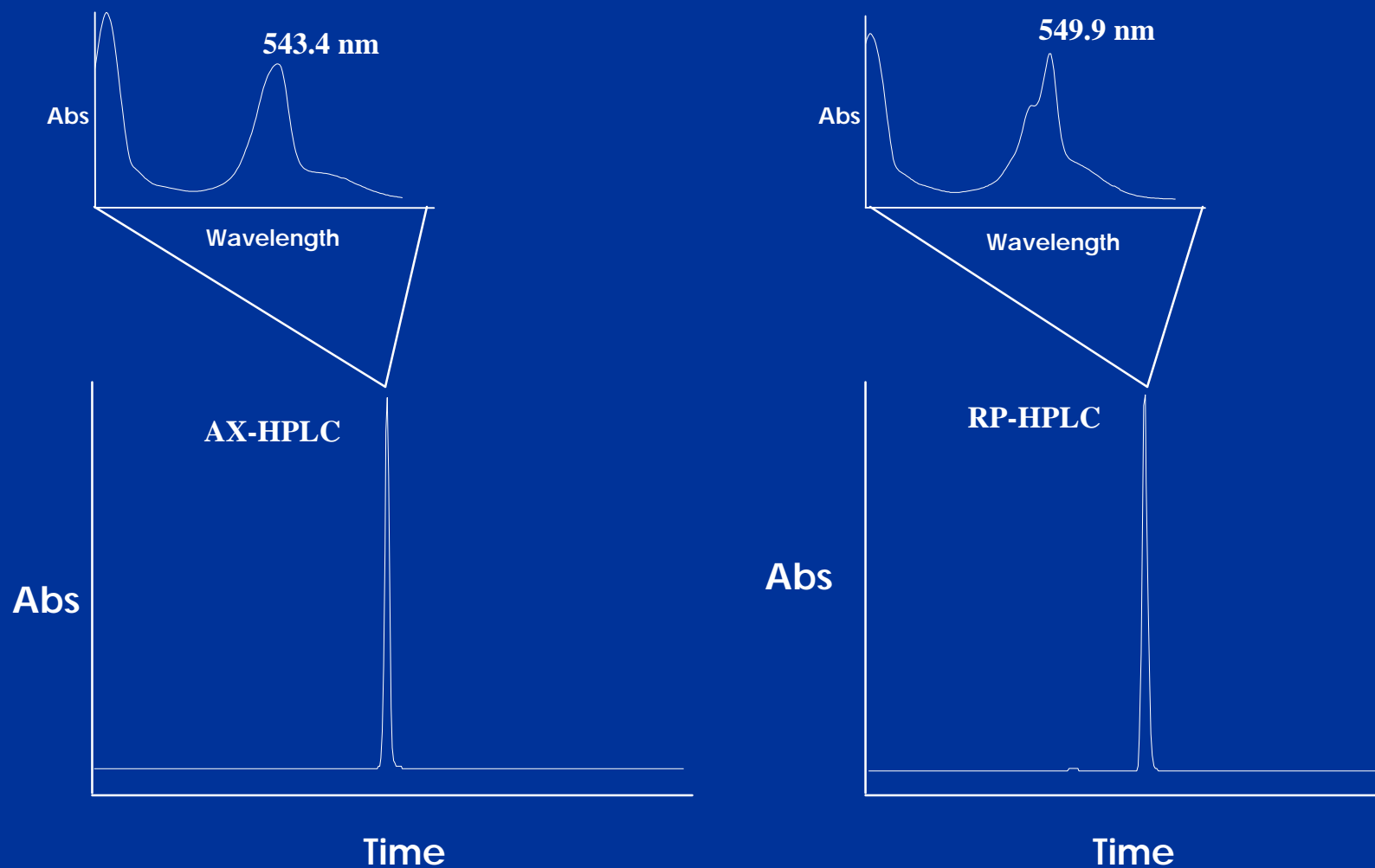


## Purification Options Analyzed by PAGE

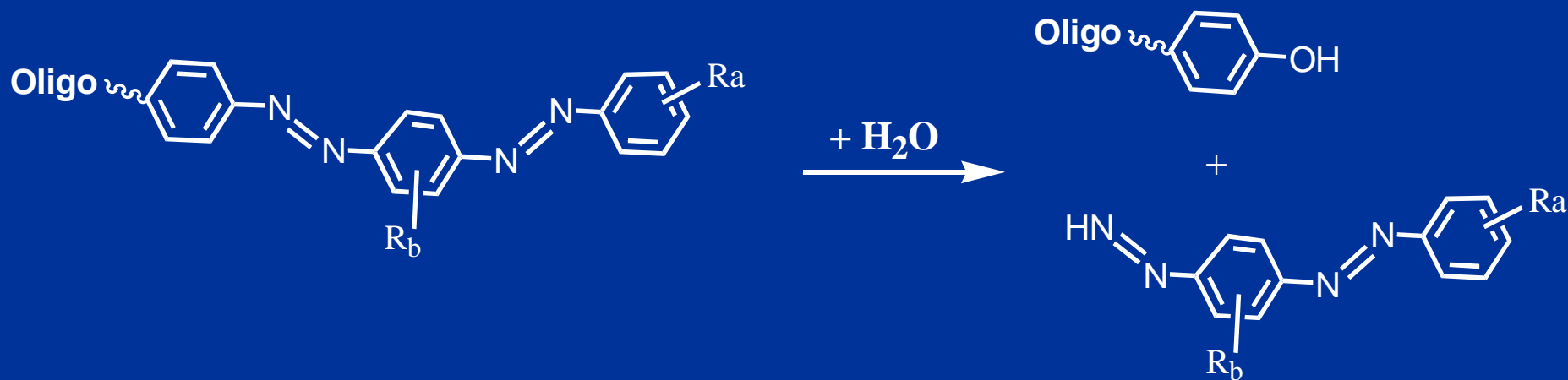
**puc19**  
**FAM.BHQ1**



# Comparison of Cy3-BHQ2 probe absorption spectra in anion exchange and reversed phase HPLC buffers

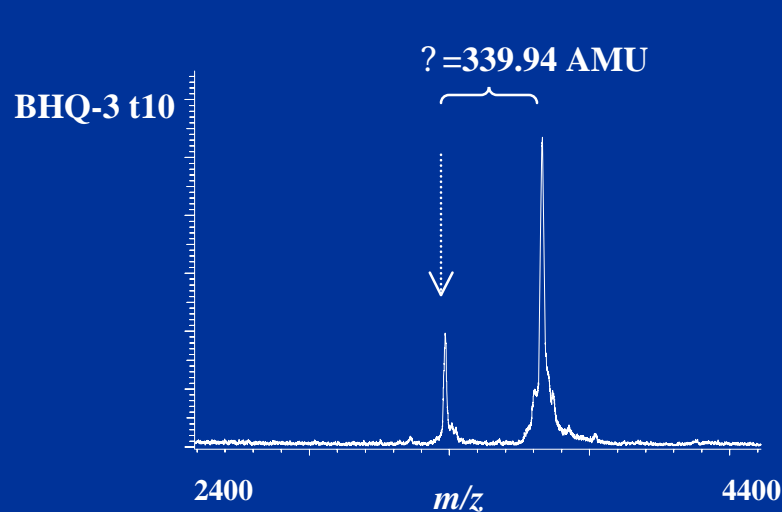
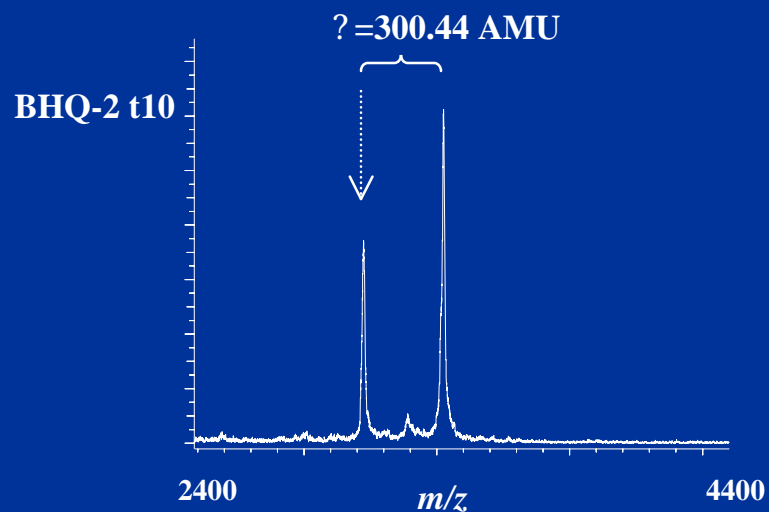
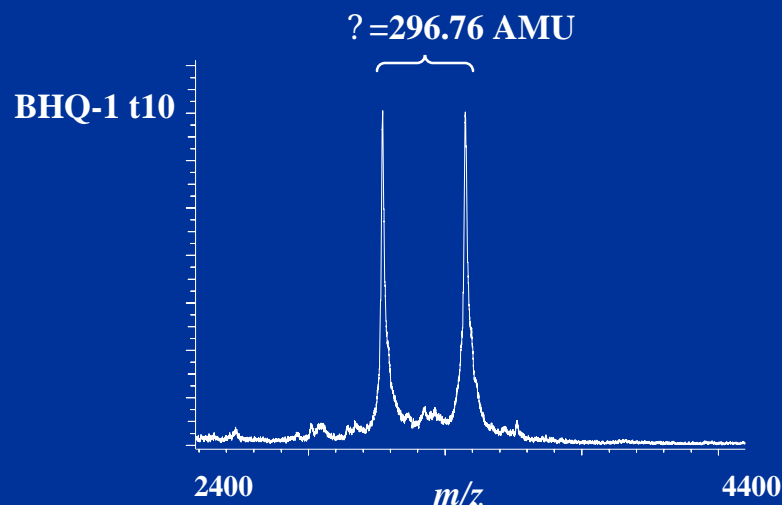
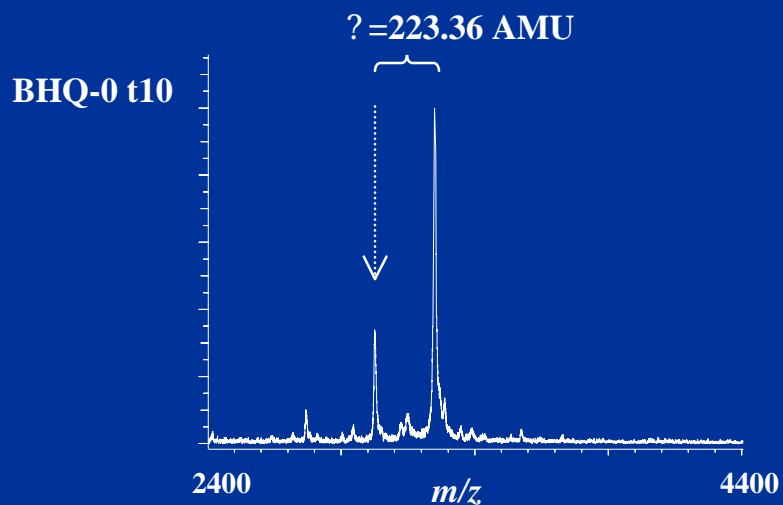


## BHQ Fragmentation by MALDI-TOF MS



Quencher	Predicted Mass Difference
BHQ-0	221
BHQ-1	296
BHQ-2	298
BHQ-3	339

# BHQ Fragmentation by MALDI-TOF MS



## **In Summary**

- **Strategies are available for placement of BHQs at the 5' terminus, 3' terminus, or internally in oligonucleotides**
- **BHQs (with the exception of BHQ-3) are very stable to aggressive deprotection conditions such as  $\text{NH}_4\text{OH}$  and AMA**
- **Dual HPLC Purification is recommended with BHQ probes**
- **Some properties of BHQs that can complicate analysis were discussed**



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## **Acknowledgements**

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**Ron Cook, Ph.D., President and CEO**

**<http://www.biosearchtech.com>**

## Black Hole Quencher Dyes may be used to prepare a wide variety of fluorescence quenched probes

- Taqman
- Molecular Beacons
- Sunrise primers
- Scorpions primers
- Quenched fluorescent peptides
- Others

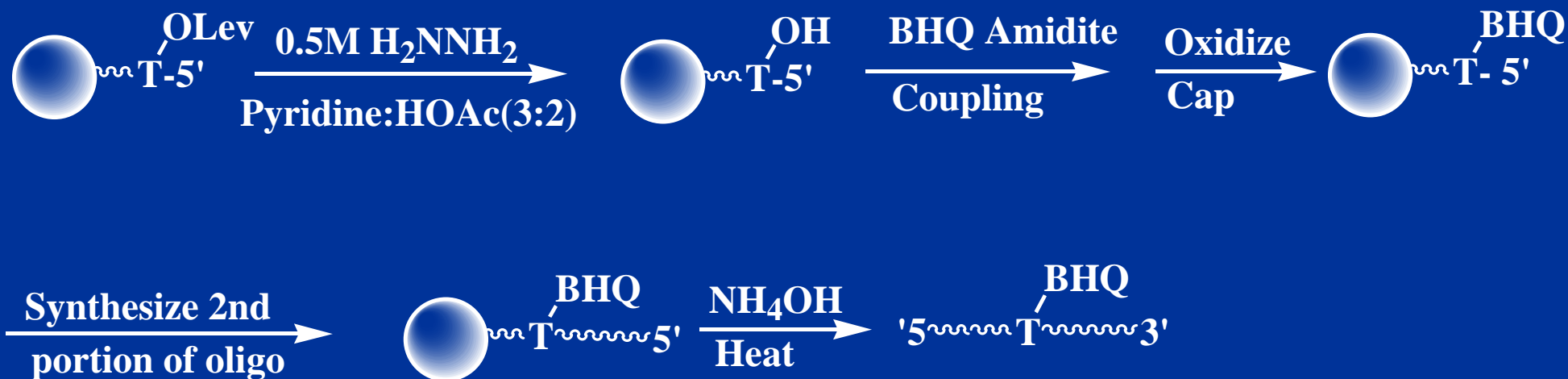
**Focus of presentation will be preparation of oligonucleotides modified with BHQs.**

**Topics will include:**

- Structures available for incorporating BHQs into oligos
- Recommended workup conditions for BHQ oligos
- Purification Strategies
- Methods for analysis

## Synthesis of Internally Labeled BHQ DNA

Modified nucleosides using levulinoyl protected hydroxyl as attachment point



Internal label must be 'terminating amidite'

Hydrazine cocktail is not compatible with all fast deprotect amidites, use G<sup>ibu</sup>, and A<sup>bz</sup>

BHQ-3 requires ultra-mild deprotection so remove levulinoyl and couple amidite after DNA synthe

Generating Internally Labeled BHQ DNA

Amine - Active ester solution coupling:

Deprotected and Desalted Oligo with  
internal amine modification

Internally Modified Oligo

5'-TTTTT-T-TTTTT-3'

Linker  
NH<sub>2</sub>

→ +BHQ Active ester

5'-TTTTT-T-TTTTT-3'

Linker  
BHQ

## HPLC Methods For Purification of BHQ Probes

### Anion Exchange HPLC

Column: Dionex DNAPac® PA-100 9 x 250 mm

Gradient: Linear Gradient over 20min  
10%:70% B  
A = .038M Tris + 15% ACN  
B = A + 1M NaBr

### Reversed Phase HPLC

Column: Hamilton PRP-1 10 x 250 mm 7  $\mu$ m

Gradient: Linear Gradient over 20min  
0%:75% B  
A = .1M TEAA + 5% ACN  
B = ACN

## Comparison of Cy3-BHQ2 probe absorption spectrum in single stranded vs double stranded DNA

