

Practical Approaches for the Synthesis, Purification and Analysis of Dual-labeled Probes (FRET)

Brian Holloway

Biotechnology Core Facility

Centers for Disease Control and Prevention

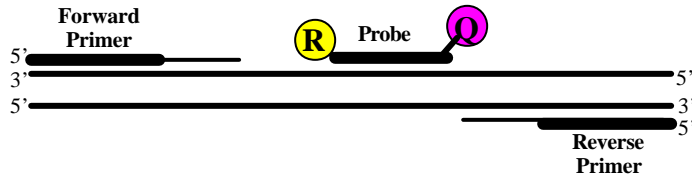


5' Exonuclease Assay

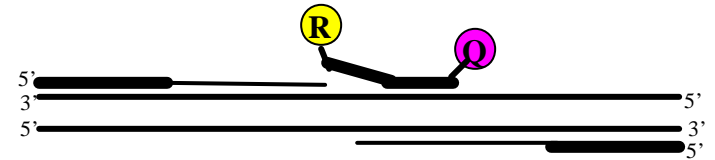
TaqMan™

I. Polymerization

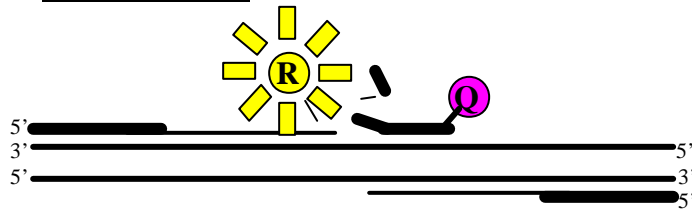
(R) - REPORTER
(Q) - QUENCHER



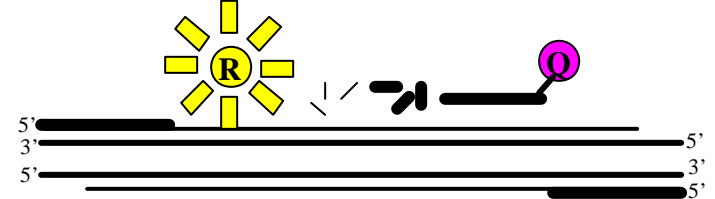
II. Strand displacement



III. Cleavage



IV. Polymerization- completed



Reasons for Synthesizing FRET probes

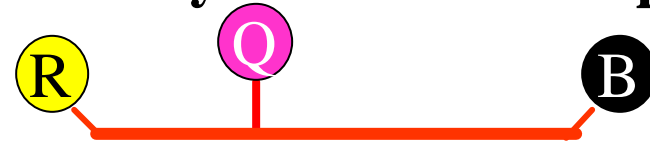
- **Institutional needs**
 - RT-QPCR, SNP, mutation detection and diagnostics
- **Cost**
 - Average commercial cost - \$ 250.00 - \$ 665.00
 - Actual reagent cost - \$ 60.00 - \$ 105.00
- **Turn-around time**
- **Flexibility of probe design**
 - Labeling and construction
- **Proprietary probes**
 - Protection of intellectual property

FRET probe - Design

End labeled FRET probe



Internally labeled FRET probe



R 5' Reporters:

- Any fluorescent dye compatible with RT-QPCR instrumentation
 - Phosphoramidite (incorporated during synthesis)
 - Succinimidyl ester (incorporated via an amine modifier)

Q Quenchers:

Fluorescent
TAMRA

Dark Quenchers(Non-fluorescent)

Dabcyl (Molecular Beacons only)
QSY-7, QSY-9, QSY-21
BHQ1, BHQ2, BHQ3
Eclipse-MGB (Proprietary)
Iowa Black (Proprietary)

B 3' Blockers:

- Phosphate-CPG
- Spacer-CPG

FRET probe - Synthesis

Modifications to standard protocol

1. Phosphoramidite

1. Final concentration 0.1 M, dissolved in dry ACN
2. Replace Guanidine^{ibu} with Guanidine^{dmf}

2. Cycle parameters (ABI 394)

1. Use the default cycles, make the probe trityl-on
2. After the cycles are completed, add the dye phosphoramidite or amine modifier to the instrument and run one more cycle with an extended coupling step of 10 minutes
3. Dry the support with inert gas before continuing to cleavage and deprotection procedures

FRET probe - Synthesis

Modifications to standard protocol

3. Cleavage and deprotection

- **BHQ and Amine-modified probes**
 1. Cleave with concentrated ammonium hydroxide
 2. Deprotect at 60°C for 2 hours or overnight at 25°C
 3. Remove cleavage solution on vacuum dryer
- **TAMRA labeled probes**
 1. Cleave with TAMRA cocktail
 2. Deprotect at 60°C for 16 hours
 3. Remove cleavage solution on vacuum dryer

FRET probe - Design

FAM-TAMRA probe (Traditional)

	Reagent Cost	6-FAM	C6dT -AL	TAMRA NHS	Phosphate CPG	TAMRA -CPG	C6dT -TAMRA	C7 -AL CPG
 <p>6-FAM TAMRA phos</p>	\$ 95	✓	✓	✓	✓			
 <p>6-FAM TAMRA</p>	\$ 60	✓			✓			
 <p>6-FAM TAMRA phos</p>	\$ 100	✓		✓		✓		
 <p>6-FAM TAMRA</p>	\$ 70	✓	✓				✓	

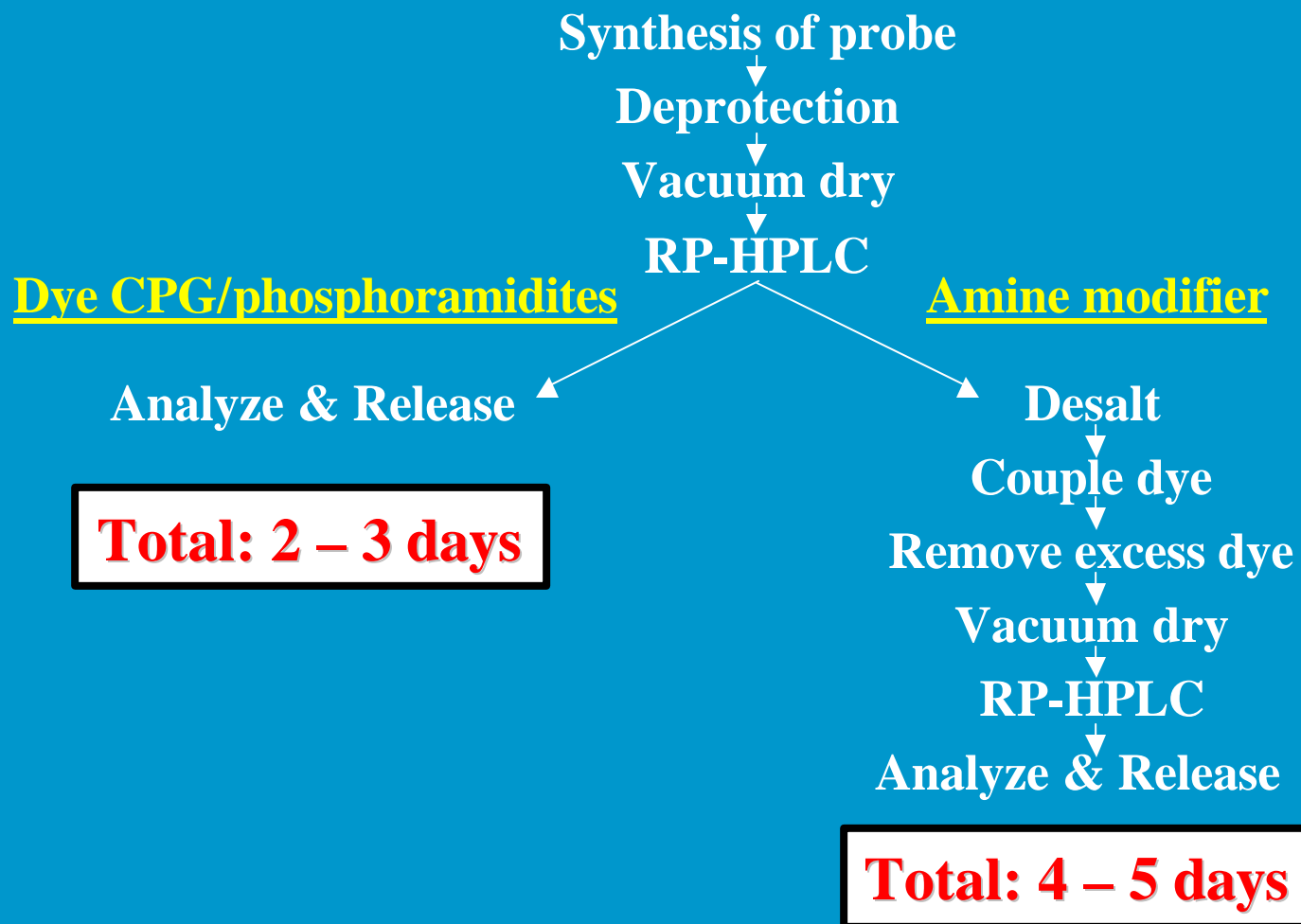
FRET probe - Design

FAM - Dark Quencher probe

	Reagent Cost	6-FAM	C6dT -AL	QSY-7 NHS	Phosphate CPG	BHQ-CPG	C7-AL CPG
	\$ 60	✓				✓	
	\$ 105	✓	✓	✓	✓		
	\$ 80	✓		✓			✓

FRET probe - Synthesis

Dye CPG/phosphoramidites vs. NHS dyes



Post Synthesis of FRET Probes

FRET probe - Post Synthesis

Major products seen after synthesis

I. Full-length probe with Reporter and Quencher



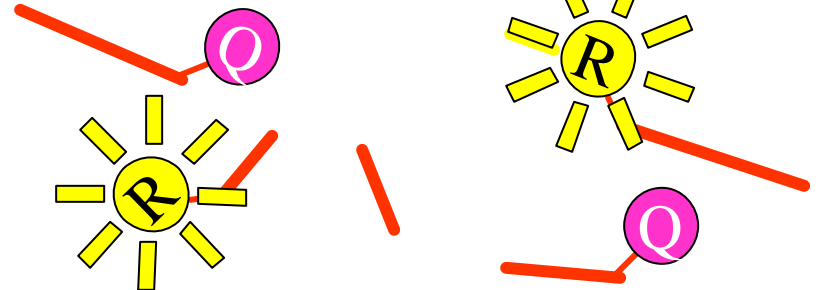
II. Full-length probe with Quencher but no Reporter



III. Full-length probe with Reporter but no Quencher



IV. Mixture of failure products



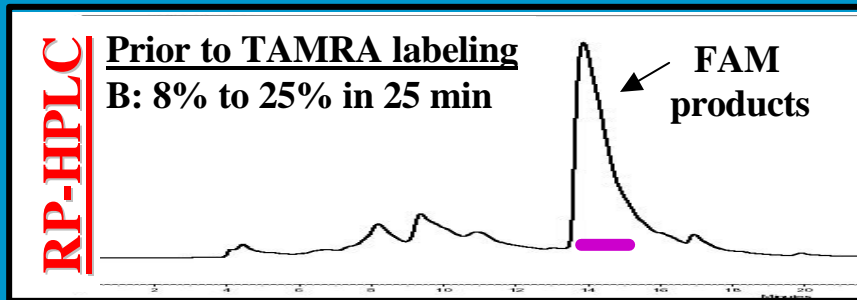
FRET probe - Post Synthesis

Instrumentation requirements

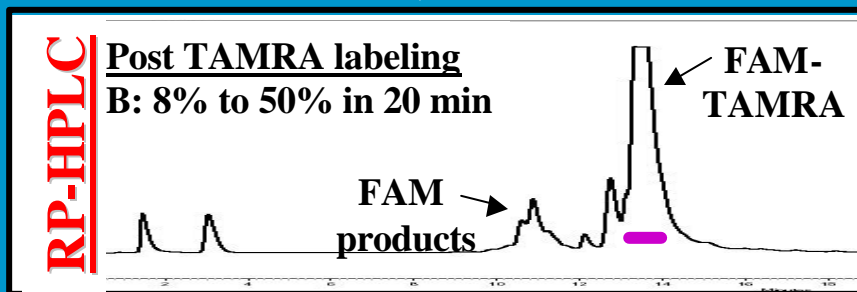
- **Essential equipment**
 - Oligo purification cartridge (OPC)
 - UV spectrophotometer
 - Gel electrophoresis
- **Optional equipment**
 - HPLC (highly recommended)
 - Capillary electrophoresis (CE)
 - Mass spectroscopy

FRET Probe - Post Synthesis

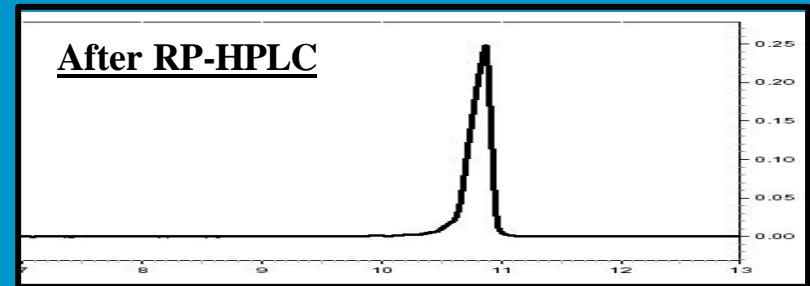
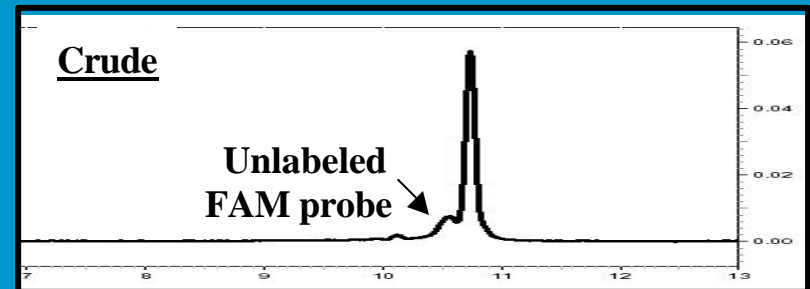
FAM-TAMRA NHS Ester



TAMRA NHS labeled



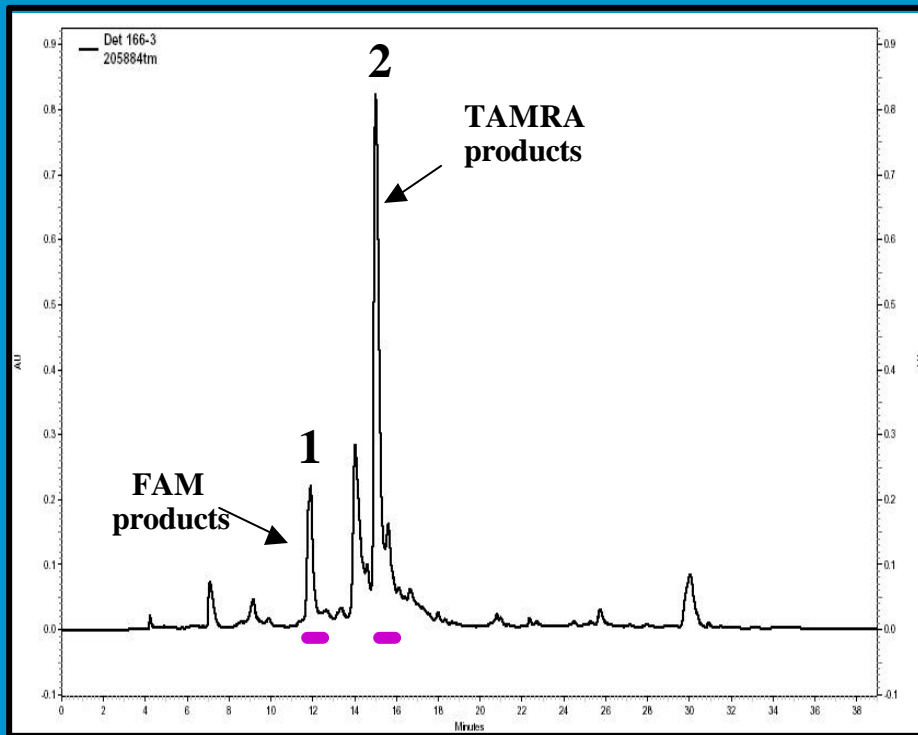
Capillary Electrophoresis (CE)



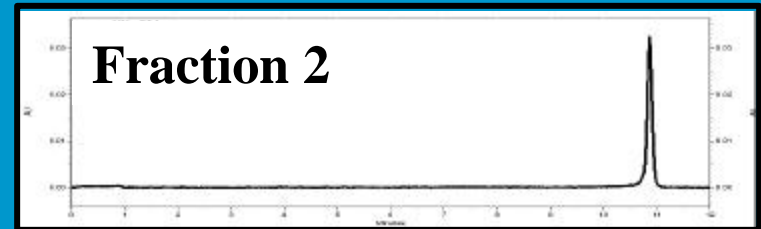
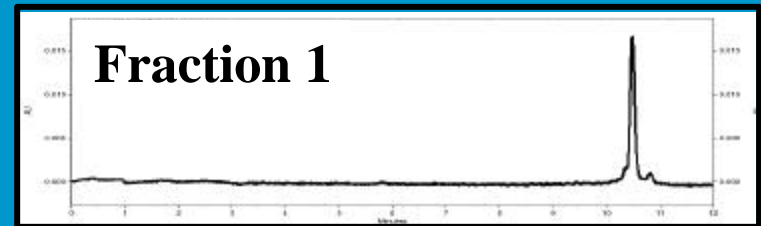
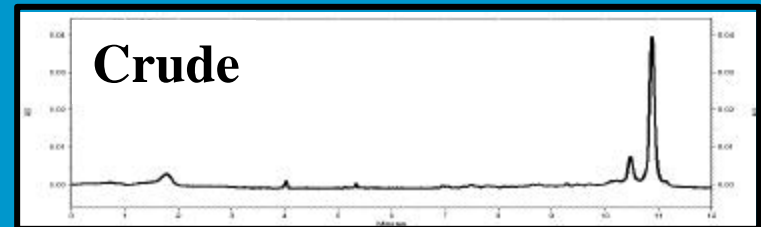
FRET Probe - Post Synthesis

FAM-TAMRA CPG

RP-HPLC



Capillary Electrophoresis (CE)

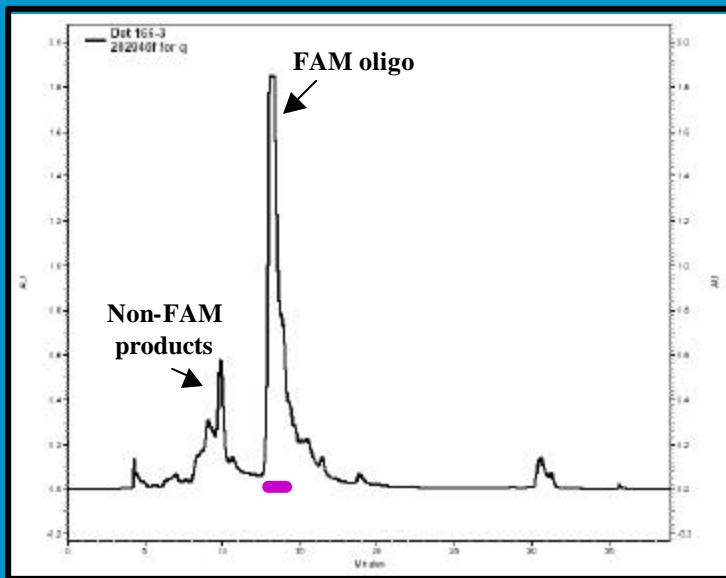


Jupiter C₁₈ column - 5 micron, 4.6 X 250 mm
Buffer A: 0.1 M TEAA, pH 7.0; Buffer B: 100% ACN
Gradient from 8% B to 50% B in 20 min @ 1.0 ml/min

FRET Probe - Post Synthesis

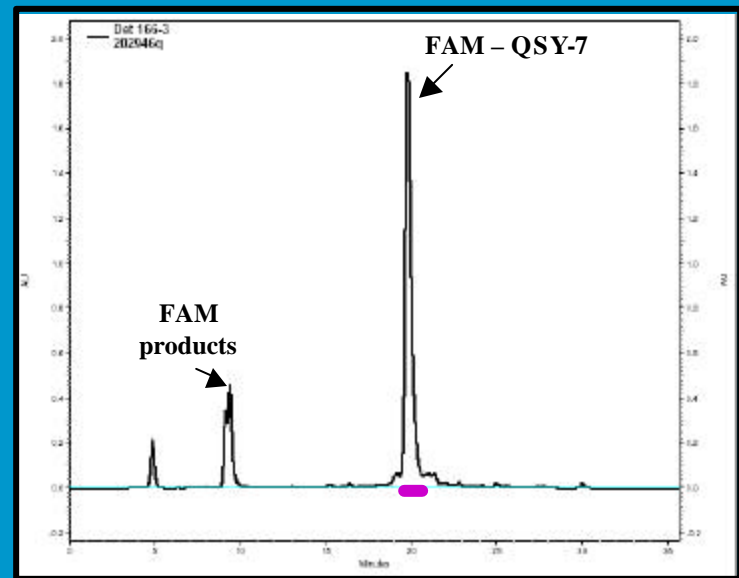
RP-HPLC of a QSY-7 NHS Ester probe

Purification of 6-FAM probe
prior to QSY-7 labeling



Buffer B: 8% to 25% in 25 min

Purification of FAM - QSY-7 probe



Buffer B: 8% to 50% in 20 min

Jupiter C₁₈ column- 5 micron, 4.6 X 250 mm

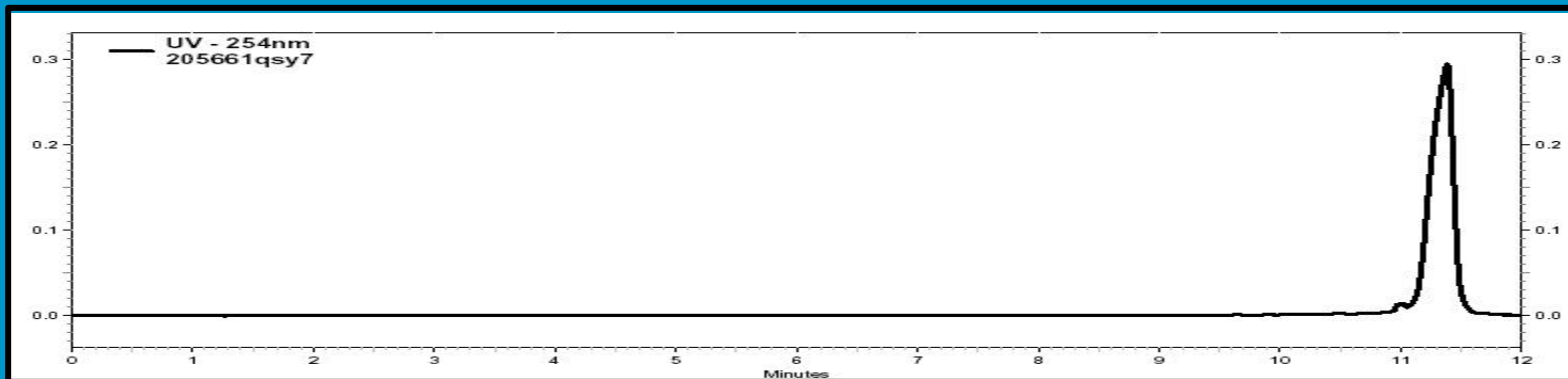
Buffer A: 0.1 M TEAA, pH 7.0; Buffer B: 100% ACN; flow rate = 1.0 ml/min



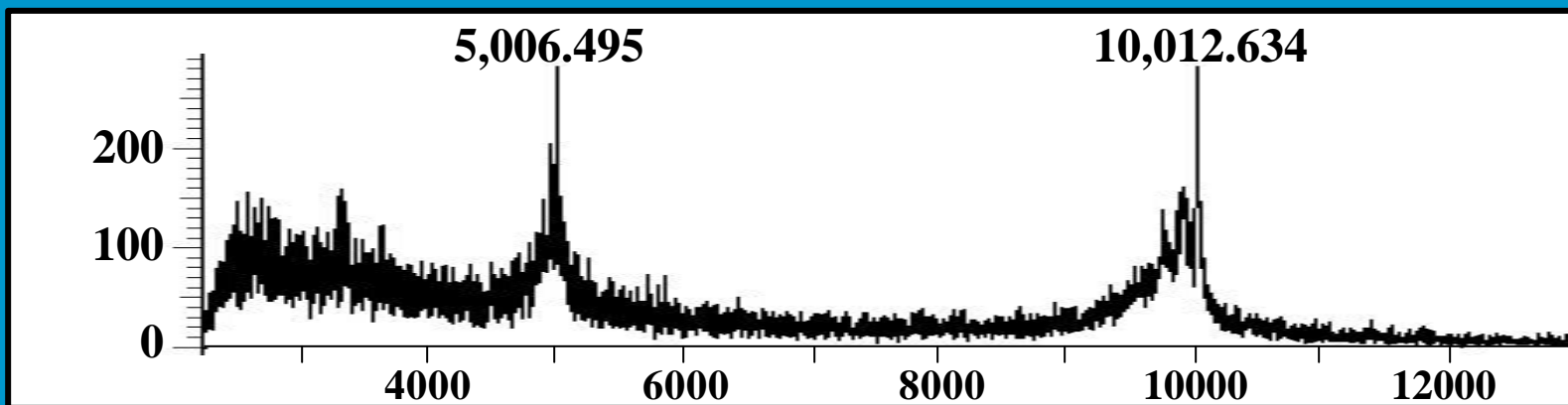
FRET Probe - Post Synthesis

CE vs MALDI-TOF MS for QSY-7 probe

Capillary Electrophoresis (CE)



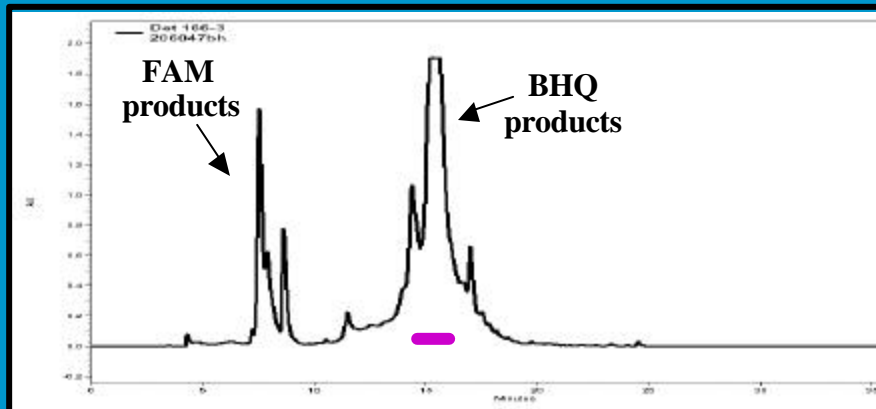
MALDI-TOF Mass Spectrometry



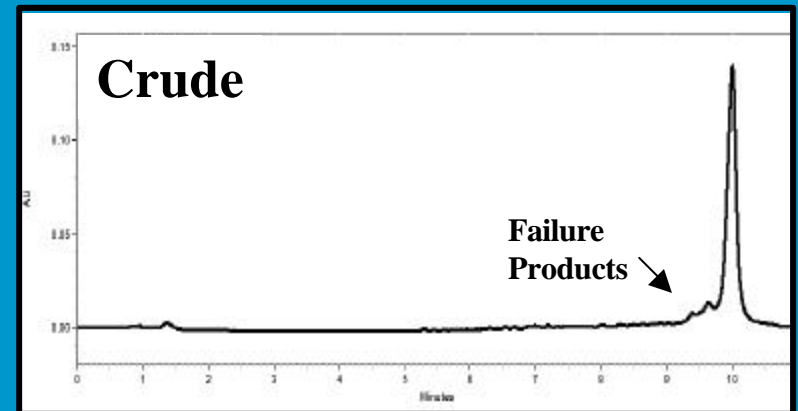
FRET Probe - Post Synthesis

FAM-BHQ1 - efficient labeling

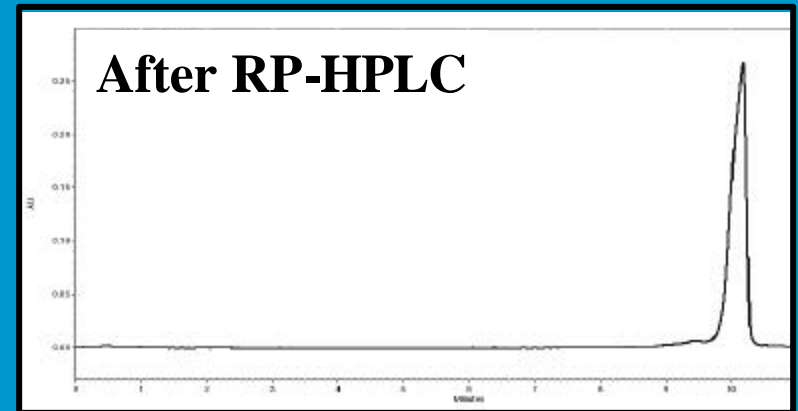
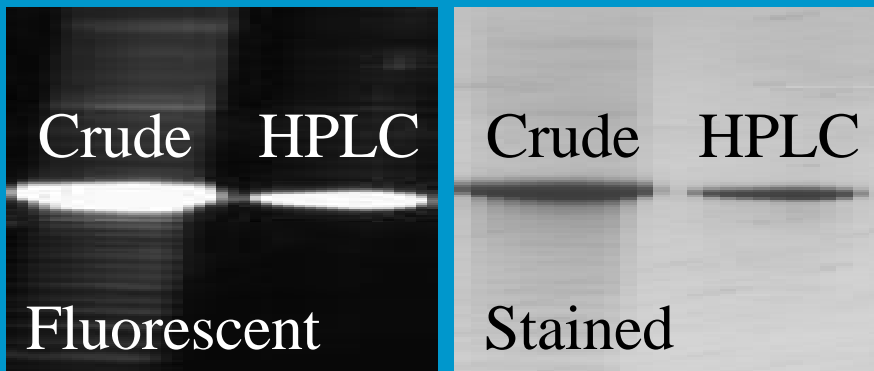
RP-HPLC



Capillary Electrophoresis (CE)



Gel Electrophoresis



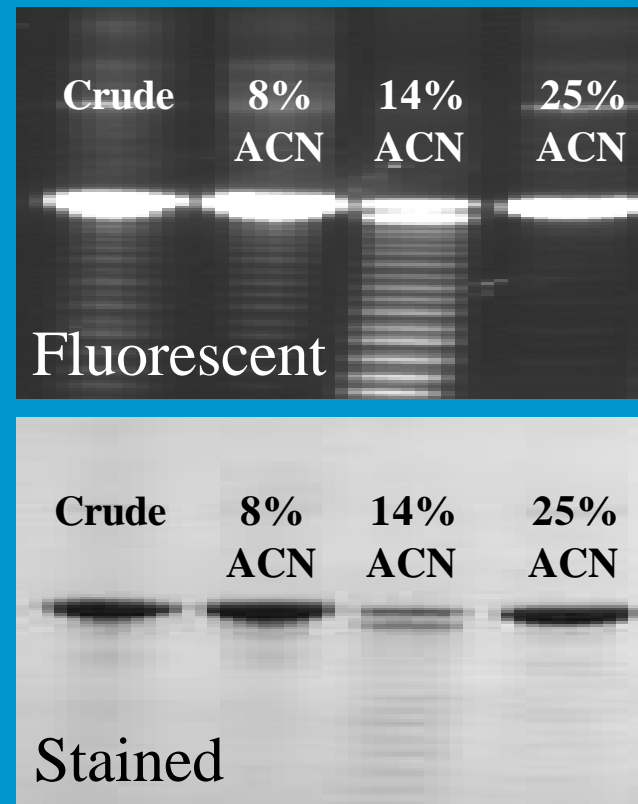
FRET Probe - Post Synthesis

OPC purification of a FAM-BHQ1

Oligo purification cartridge

Protocol

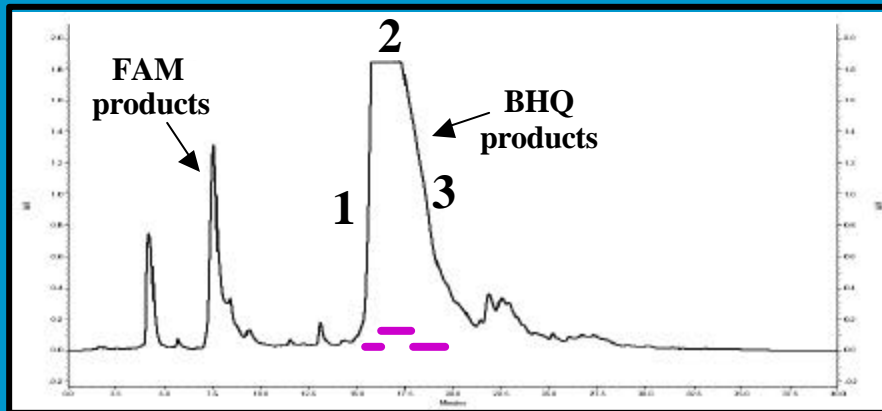
1. Dissolve the FRET probe in 0.1 M TEAA
2. Flush OPC with 100% ACN, followed by 2.0 M TEAA
3. Load FRET probe 3X onto the cartridge
4. Flush with 8% ACN in 0.1 M TEAA
5. Flush with 14% ACN in 0.1 M TEAA
6. Elute purified FRET probe with 25% ACN in 0.1 M TEAA
7. Verify purity on 20% PAGE



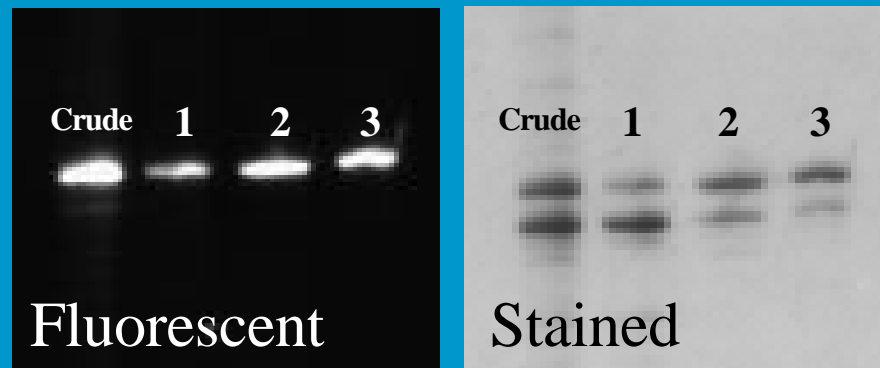
FRET Probe - Post Synthesis

FAM-BHQ1 - inefficient labeling

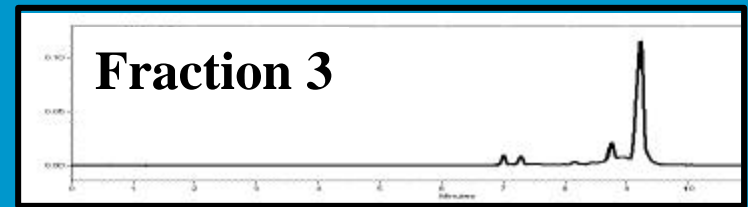
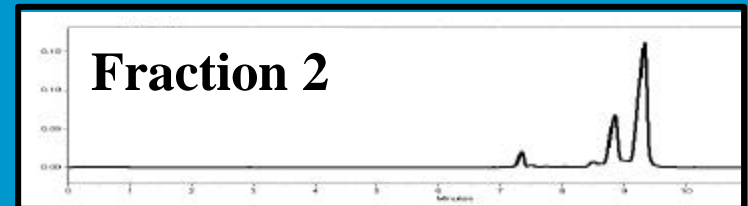
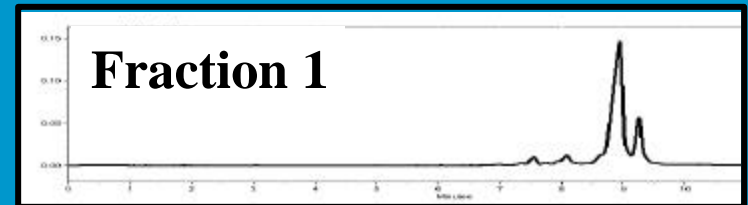
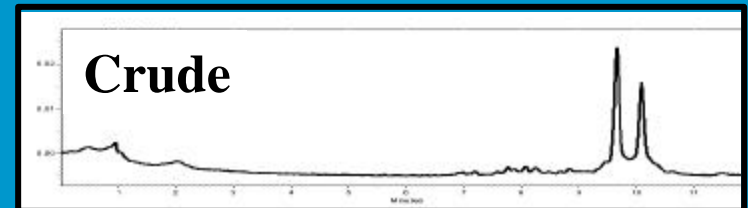
RP-HPLC



Gel Electrophoresis

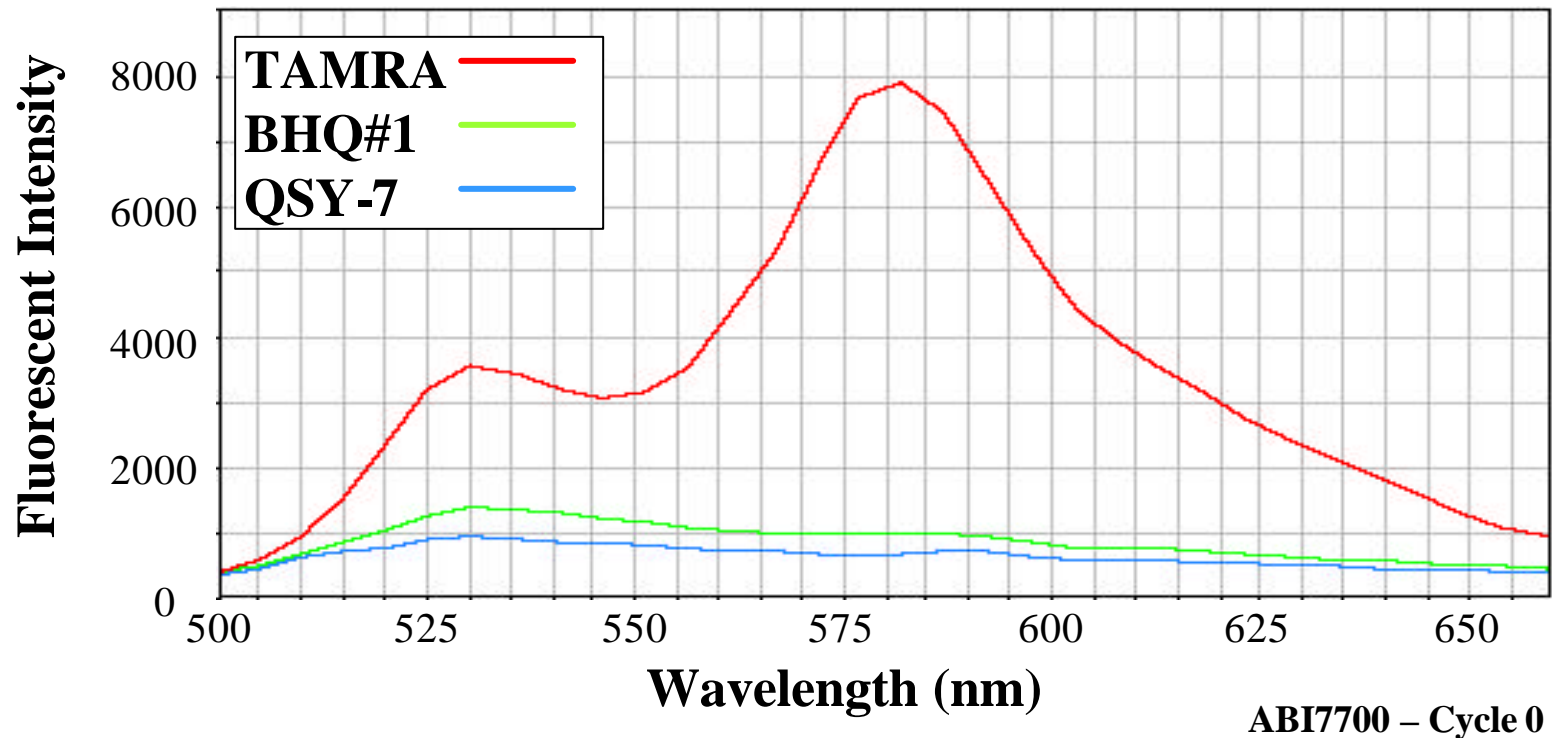


Capillary Electrophoresis (CE)



FRET Probe - Post Synthesis

Spectral Characteristics of Quenchers



FRET Probe - Post Synthesis

Summary of yields & cost

0.2 umole scale		<u>Cost</u> (\$)	<u>Yield</u> (nm)	<u># of rxns</u> *	<u>Cost/rxn</u> (¢)
In-house Probe	FAM-TAMRA CPG	60	47.4	18,970	0.32
	FAM-TAMRA C6dT	100	33.4	13,343	0.75
	FAM-TAMRA 3' NHS	70	16.7	6,680	1.05
	FAM-TAMRA internal NHS	95	15.3	6,120	1.55
	FAM-QSY-7 3' NHS	80	14.9	5,970	1.34
	FAM-QSY-7 internal NHS	105	13.9	5,546	1.89
	FAM-BHQ	60	62.7	25,072	0.24
	Dye NHS-BHQ	85	27.3	10,901	0.77
Commercial	FAM-TAMRA	334	20.0	8,000	4.18
	Dye NHS-TAMRA	437	20.0	8,000	5.47
	FAM-BHQ	292	20.0	8,000	3.66
	Dye NHS-BHQ	432	20.0	8,000	5.40
	MGB - Eclipse	516	20.0	8,000	6.45

* Number of reactions based on a 25 uL PCR reaction using a 100 nM final concentration of probe

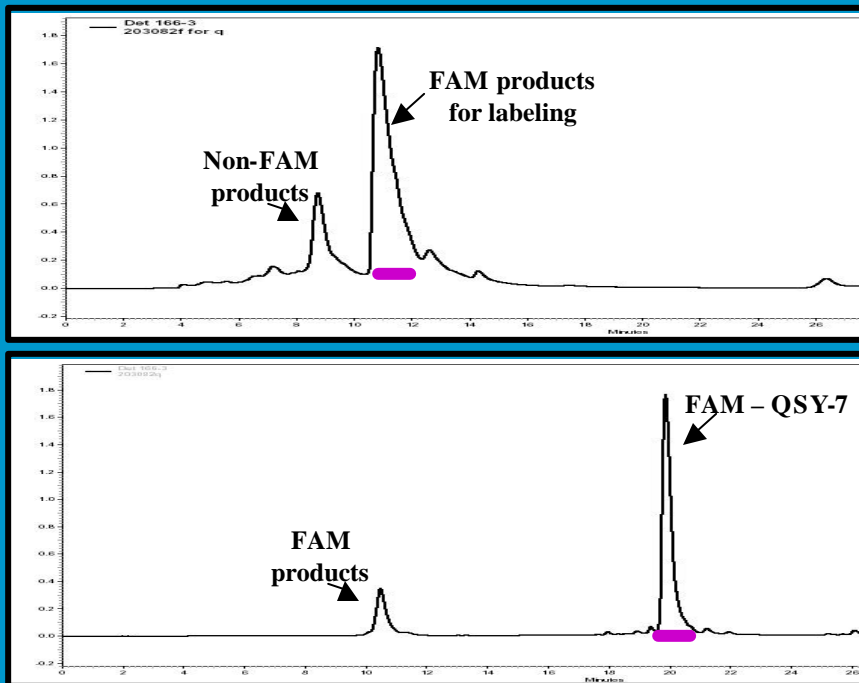


FRET Probe - Post Synthesis

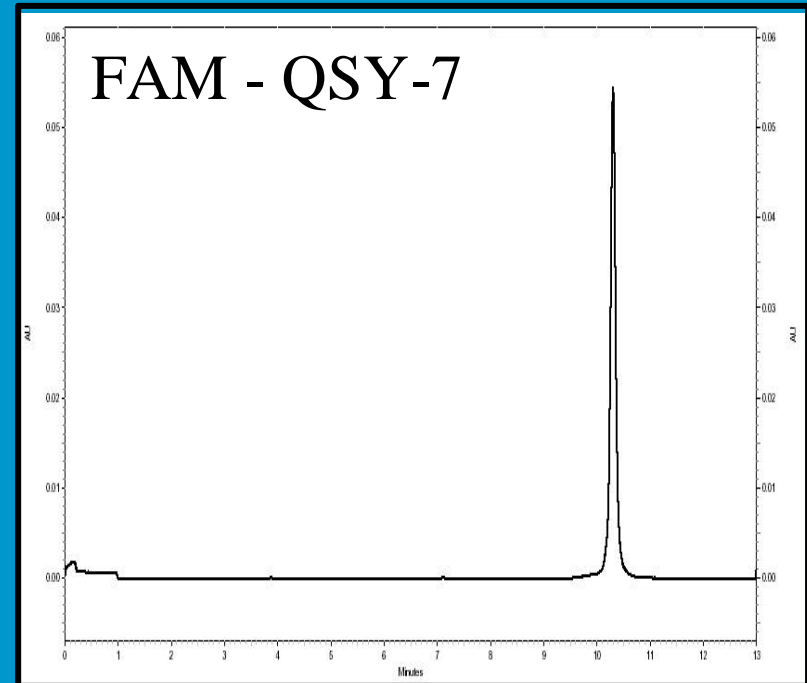
Good synthesis - Poor quencher

An example of an apparently good probe that fails to quench

RP-HPLC



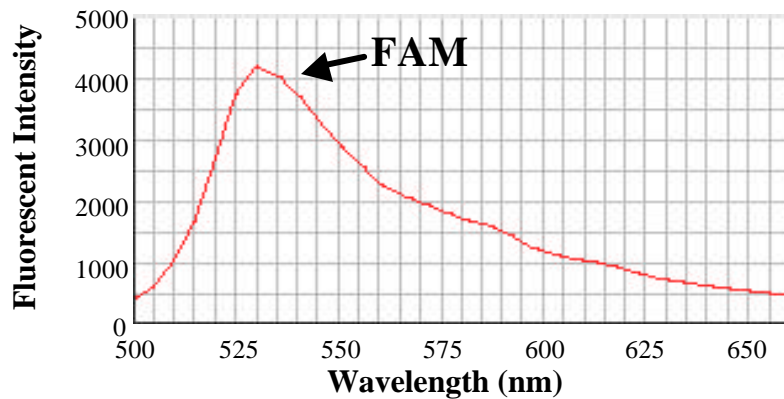
Capillary Electrophoresis (CE)



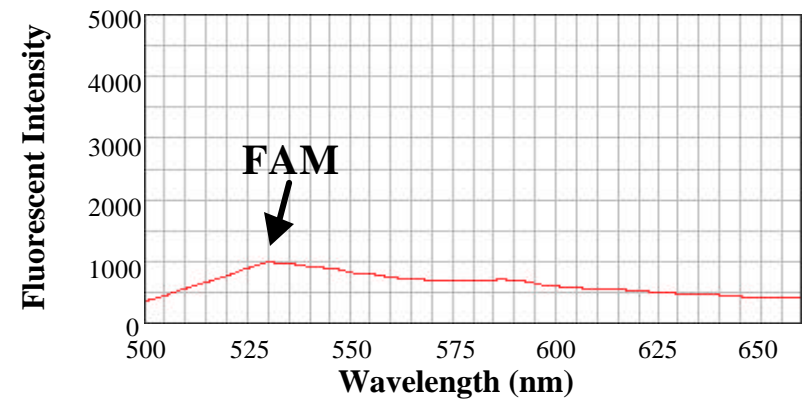
FRET probe - Design

Effect of quencher location on probes over 30 bp

End labeled FRET probe



Internally labeled FRET probe



Summary

- **Optimize DNA synthesizer performance - run homopolymers to test.**
- **Optimization of the 5' reporter coupling is critical to the synthesis of FRET probes**
- **Do not double dilute phosphoramidites**
- **Use dG^{dmf} instead of dG^{ibu}**
- **Dark quenchers appear to be superior to TAMRA quenched probes**
- **For probes over 30 bp in length place quencher internally**

Acknowledgements

Josef Limor

Karen McCaustland

Nicky Sulaiman

Judy Froelich

Reid Horton

