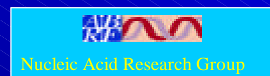
ABRF
NARG 2000-2001 DNA Synthesis Study

Part II. Evaluation of Oligonucleotide Synthesis Utilizing Homopolymers of A(20), G(20), C(20) and T(20), and one heteropolymer (30 mer).

Martha Gunthorpe (1), Jay Fox (2), Brian Holloway (3), Katheryn Lilley (4), Kathy Mills (5), Stephen Scaringe (6), Anthony Yeung (7).

(1) Howard Hughes Medical Institute/University of California, San Francisco 94143, (2) University of Virginia, Charlottesville, VA 22908, (3) CDC (4) University of Cambridge, UK, (5) Millennium, (6) Dharmacon Research, Inc., (7) Fox Chase Cancer Center, Philadelphia, PA 19111.


Nucleic Acid Research Group

We thank the 20 labs that sent in samples to facilitate this study

PART II

Quality Control Is Important!

DNA synthesis chemistry and automated DNA synthesizers can reliably deliver high purity oligonucleotides when they are properly maintained. However, this study finds that a CORE facility should practice thorough quality control to assure the products are always of the highest quality.

We shall see that primers are easy, but longer oligos require well tuned synthesizers if you want a good yield

Research Participants:

20 DNA synthesis cores:

- 16 non-profit
- 4 commercial

30 DNA synthesizers tested:

- ABI 392
- ABI 394
- ABI 3948
- ABI Expedite
- Beckman Oligo 1000

Each core provided five samples, and their synthesis conditions:

- Homopolymer A 20 mer
- Homopolymer G 20 mer
- Homopolymer C 20 mer
- Homopolymer T 20 mer
- Mixed base 30mer

Ref:

Yeung, A. T. and Miller, C. G. A general method of optimizing automated DNA synthesis to decrease chemical consumption to less than half. *Anal. Biochem.* **187**:66- 75, 1990.

The synthesis chemistry differs for each base, so are the problems.

T has no protection groups and harder to detritylate. T20 tells coupling efficiency.

C20 tells deprotection problems and C deamination.

A20 tells of side reactions and depurination

G20 are harder to make or QC, and a real test of your machine.

Mixed 30 shows how a primer will look like in real life, even with machines less than perfect

Each of the 150 oligonucleotides were analyzed by the following QC methods:

- Anion-exchange HPLC at neutral pH and 5M urea.
- Anion-exchange FPLC at pH 12.5.
- Capillary electrophoresis (CE)
- Denaturing HPLC (WAVE)
- ESI mass spectrometry
- MALDI-TOF mass spectrometry
- UV absorbance

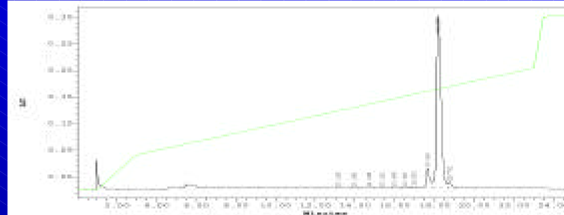
The 30 mers are also tested as DNA sequencing primers for pGEM control template.

Focus is on the real life situation of QC of crude oligos in a good volume core lab, not on which method is most powerful with pure oligos.

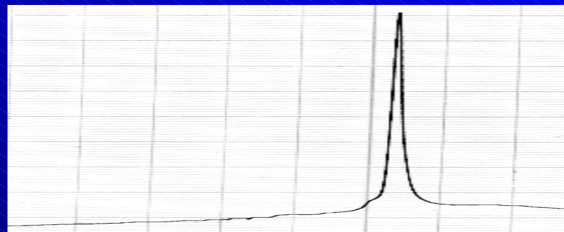
Chromatograms of gel purified oligonucleotides

Illustrating five chromatographic methods

A20 mer, anion-exchange pH 7, 5M urea

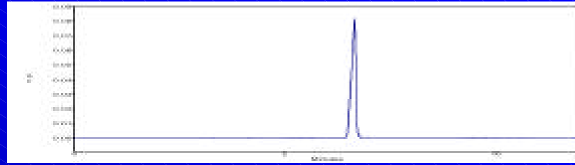


G20 mer, anion-exchange pH 12.5

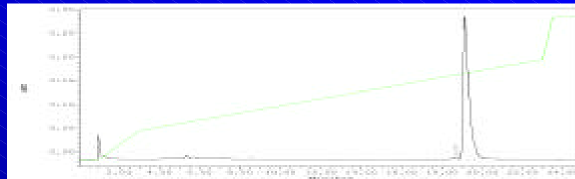


Perhaps ion exchange in HPLC at pH12.5 will combine the best of both worlds. Easy automation.

C20 mer, capillary electrophoresis

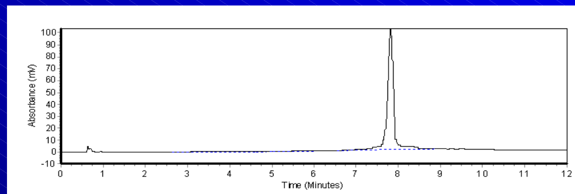


T20 mer, anion-exchange pH 7 5M urea

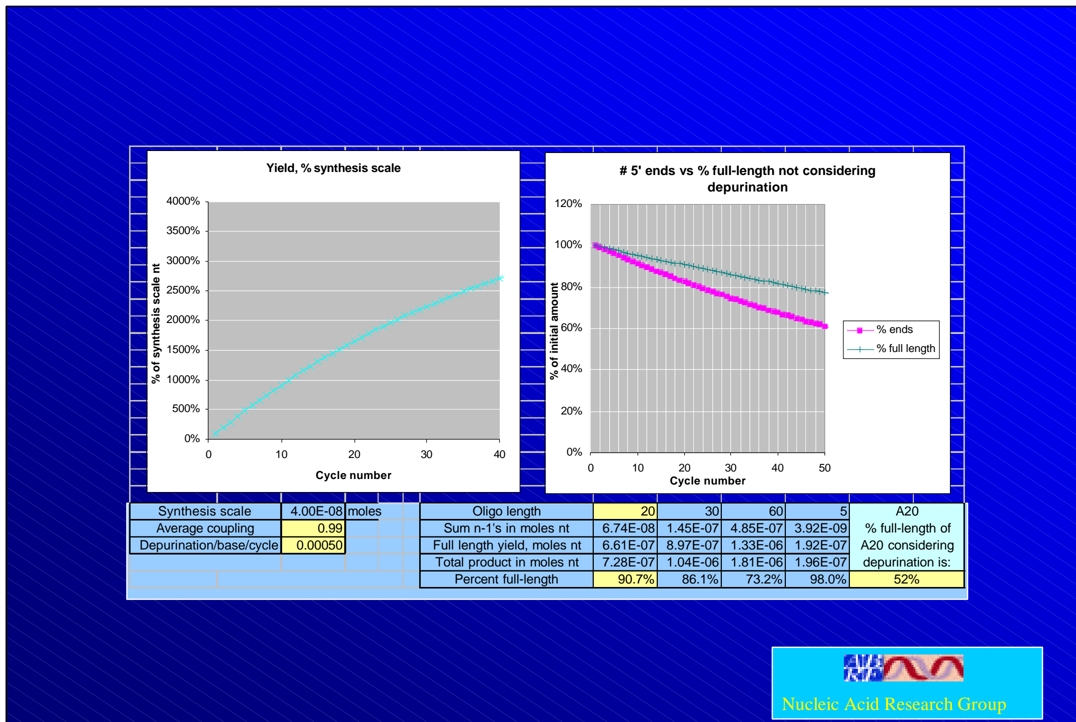


Mixed-base 30mer,

DHPLC



CE is easy too. Notice the sharp front and back you expect of a gel purified oligo.



Synthesis scale	4.00E-08	moles
Average coupling	0.99	
Depurination/base/cycle	0.00050	

Oligo length	20	30	60	5
Sum n-1's in moles nt	6.74E-08	1.45E-07	4.85E-07	3.92E-09
Full length yield, moles nt	6.61E-07	8.97E-07	1.33E-06	1.92E-07
Total product in moles nt	7.28E-07	1.04E-06	1.81E-06	1.96E-07
Percent full-length	90.7%	86.1%	73.2%	98.0%

A20 % full-length of A20 considering depurination is:
52%

This is a spreadsheet I set up to simulate DNA synthesis and QC. As synthesis proceeds, yield increase with # of couplings in good yield synthesis. This is not true with DNA synthesizers that are not well tuned as will be shown later.

On the right, while we are accustomed to calculate exponential decrease of synthesis yield, the percentage full length is different and it decreases much more slowly. So even bad coupling produces significant amounts of full length oligo.

Theoretical chromatogram of a 20mer not considering depurination

A20 profile reflecting coupling efficiency and depurination

Synthesis scale	4.00E-08	moles
Average coupling efficiency	0.99	
Depurination/base/cycle	0.00050	

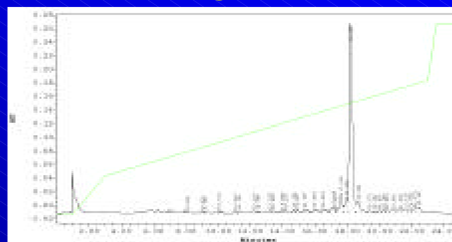
Oligo length	20	30	60	5	A20
Sum n-1's in moles nt	6.74E-08	1.45E-07	4.85E-07	3.92E-09	% full-length of A20 considering depurination is:
Full length yield, moles nt	6.61E-07	8.97E-07	1.33E-06	1.92E-07	
Total product in moles nt	7.28E-07	1.04E-06	1.81E-06	1.96E-07	
Percent full-length	90.7%	86.1%	73.2%	98.0%	52%

Nucleic Acid Research Group

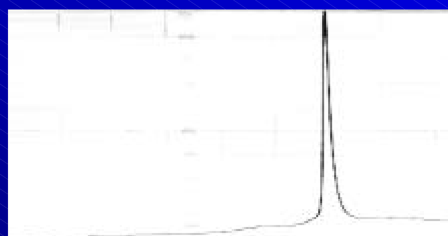
These theoretical chromatograms show how good synthesis should look like. The one including depurination on the right takes into account that the 3' end suffers more synthesis cycles than the 5' end. Notice that a T20 should look glorious, so is the C20, yet A20 and G20 will have lower yield partly because of depurination.

A set of crude oligonucleotides from an instrument with good QC

A20 mer, anion-exchange pH 7, 5M urea

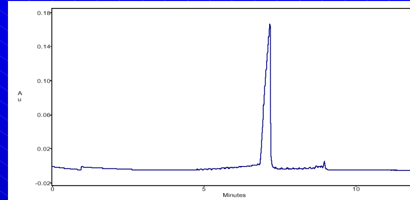


G20 mer, anion-exchange pH 12.5

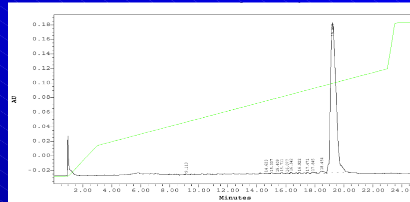


Well kept machines approach theoretical limits of performance. This one uses 2.5X diluted phosphoramidites and primer cycle, with no special synthesis and reagent precautions.

C20 mer,
capillary
electrophoresis

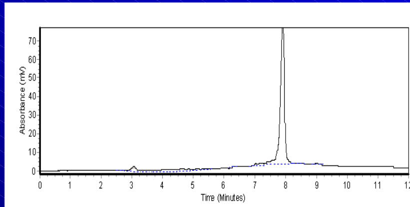


T20 mer, anion-
exchange pH 7
5M urea



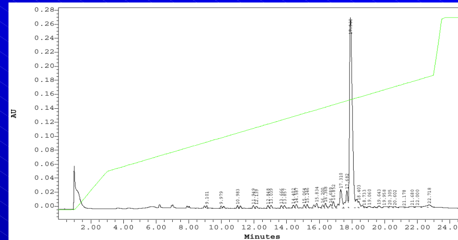
Mixed-base
30mer,

DHPLC

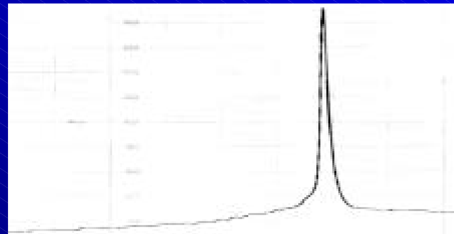


A set of oligonucleotides from another instrument with good QC

A20 mer, anion-exchange pH 7, 5M urea

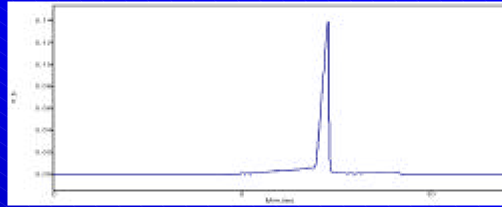


G20 mer, anion-exchange pH 12.5



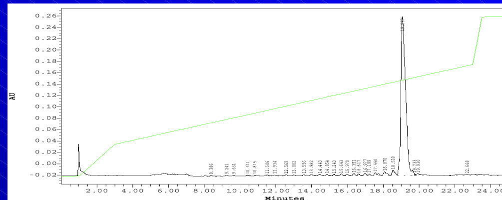
Several DNA synthesizers in this study can make DNA this good. The G here is fast deprotection chemistry dmf.

C20 mer, capillary electrophoresis



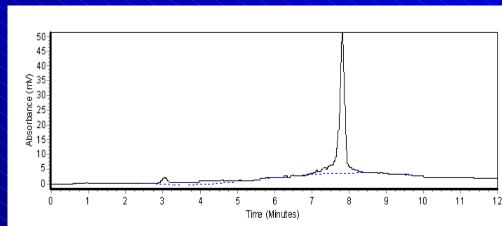
T20 mer, anion-exchange

pH 7, 5M urea



Mixed-base 30mer,

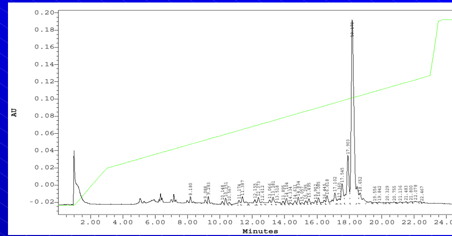
DHPLC



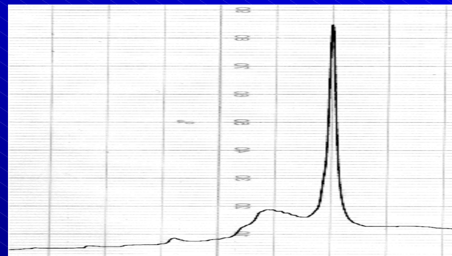
This way, you know you can make oligos that are long, or for special studies.

A set of crude oligonucleotides from an instrument with low QC

A20 mer, anion-exchange pH 7 5M urea

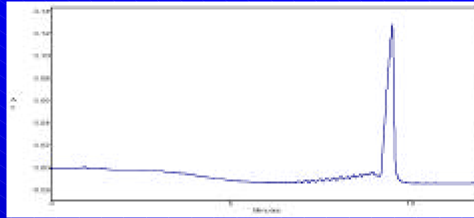


G20 mer, anion-exchange pH 12.5

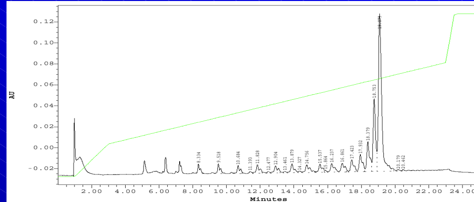


This is not the worst DNA synthesizer in this study. It is in the mid performance range. We can see dupurination, n-1 in A20 and G 20. The sharp gradient of ion exchange at pH12.5 is because FPLC is slow compared with HPLC, so a sharp gradient was used to sum up all the failures for easy visualization.

C20 mer, capillary electrophoresis

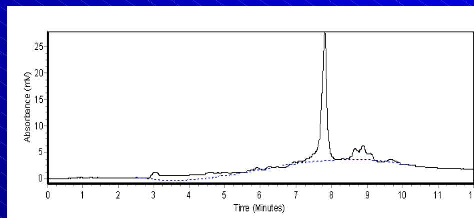


T20 mer, anion-exchange pH 7, 5M urea



Mixed-base 30mer,

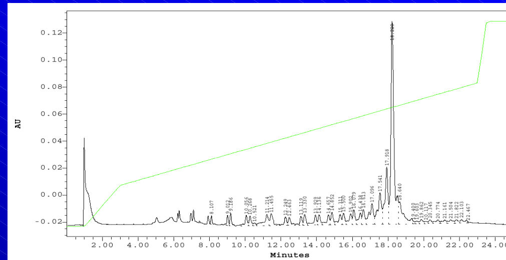
DHPLC



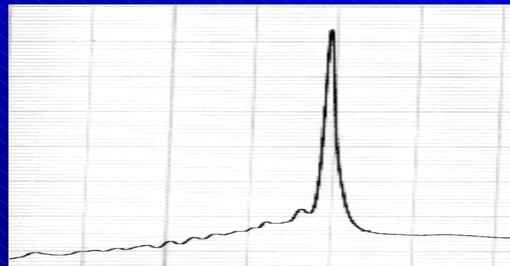
C20 was good, but T20 was not. Deprotection problems or n+1 is evident even in a 30 mer.

Crude oligonucleotides from other instruments compromised in some but not all homopolymers

A20 mer, anion-exchange pH 7, 5M urea

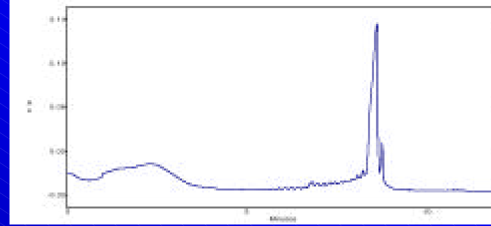


G20 mer, anion-exchange pH 12.5

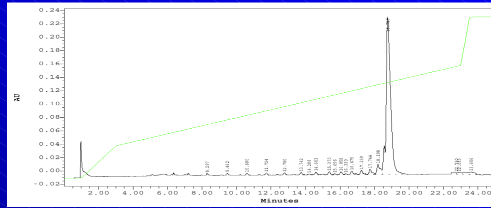


Notice the doublets in A20, sign of depurination. Notice also the high resolution of the anion exchange in 5M urea in HPLC, in just 30 minutes. This was at pH 7. It is to be tested if pH 12.5 is also this good.

C20 mer, capillary electrophoresis

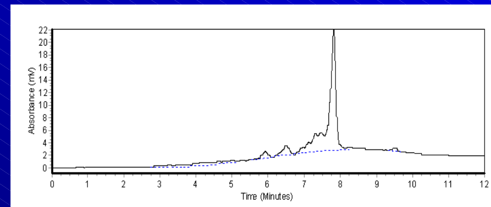


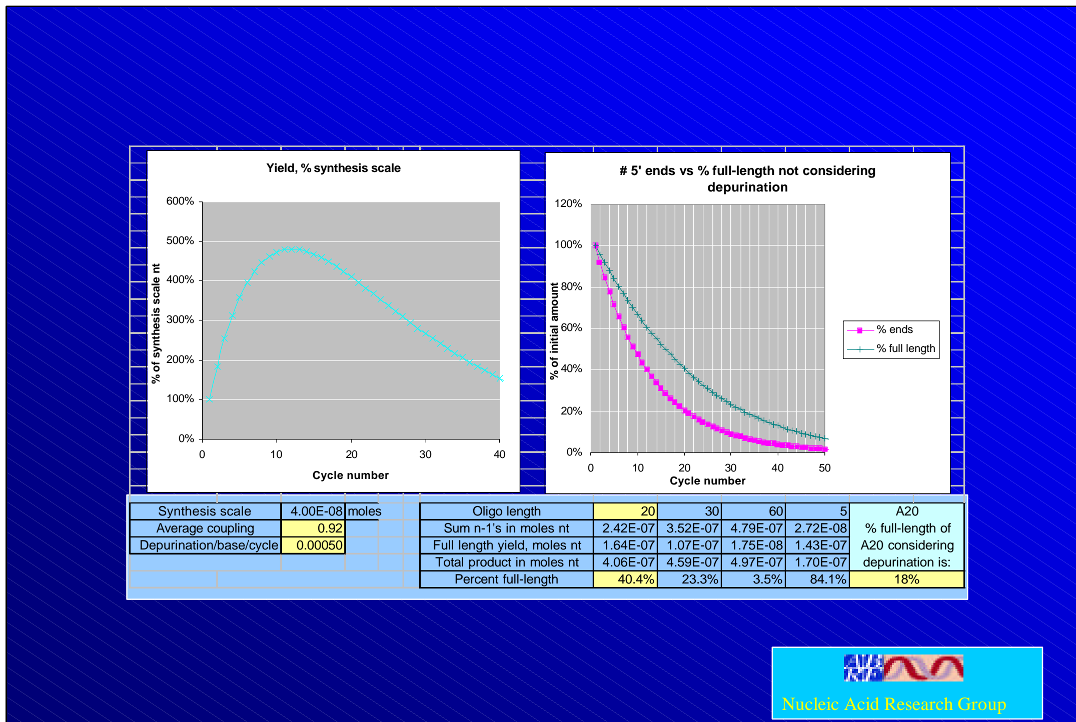
T20 mer, anion-exchange pH 7, 5M urea



Mixed-base 30mer,

DHPLC





With a less optimal machine, yield does not go up with increasing length of the oligo other than the first 8 cycles. This is because of the first few cycles produce doubling, 1/3, 1/4 increases that are significant in spite of low yield. Still, % full-length is significant for primers, but not longer oligos. That is why we need well QC machines.

Theoretical chromatogram of a 20mer not considering depurination

A20 profile reflecting coupling efficiency and depurination

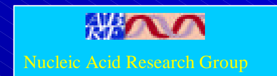
Synthesis scale	4.00E-08	moles
Average coupling efficiency	0.92	
Depurination/base/cycle	0.00050	

Oligo length	20	30	60	5	A20
Sum n-1's in moles nt	2.42E-07	3.52E-07	4.79E-07	2.72E-08	% full-length of
Full length yield, moles nt	1.64E-07	1.07E-07	1.75E-08	1.43E-07	A20 considering
Total product in moles nt	4.06E-07	4.59E-07	4.97E-07	1.70E-07	depurination is:
Percent full-length	40.4%	23.3%	3.5%	84.1%	18%

Nucleic Acid Research Group

If a sharp gradient is used, as for FPLC pH 12.5 anion exchange, you see the hump of n-1's. Notice that with depurination being a factor, the full-length A20 and G20 diminish even when T20 and C20 look good.

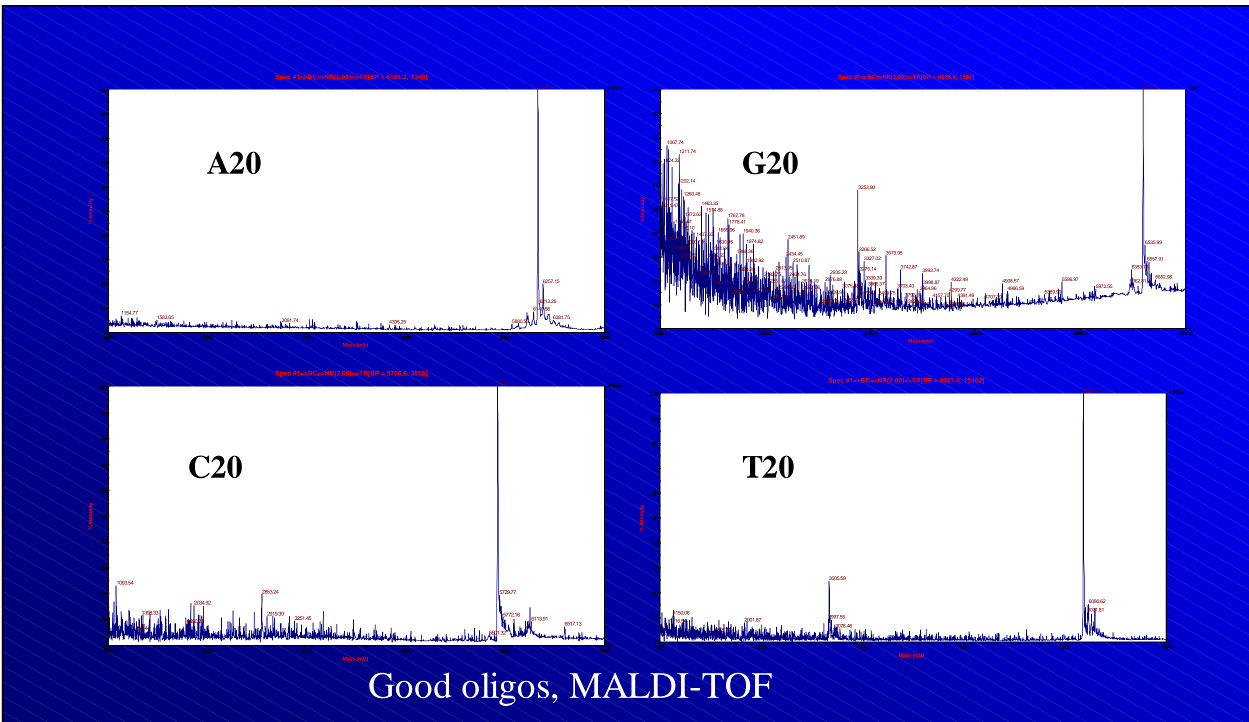
- A DNA synthesizer with marginal performance can still make useful primers.
- Only a DNA synthesizer with good QC can make good long oligonucleotides and those for more demanding applications.



Examples of demanding applications needing good synthesizers are: DNA repair substrates, antisense oligos, fluorescent probes for SNP determination, molecular beacons, RNA-DNA hybrids, and gene synthesis.

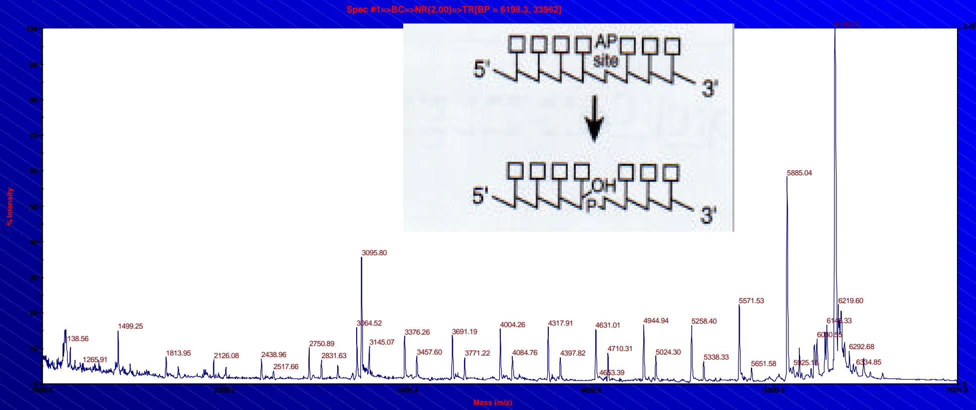
Mass spectrometry is important for analyzing oligonucleotide modifications.

This include modifications that are intended or unintended side reactions.

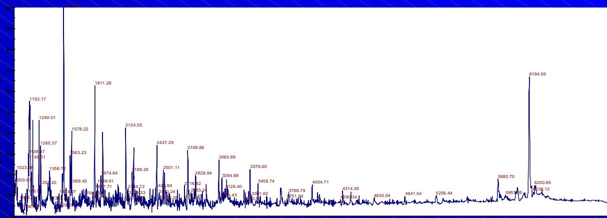


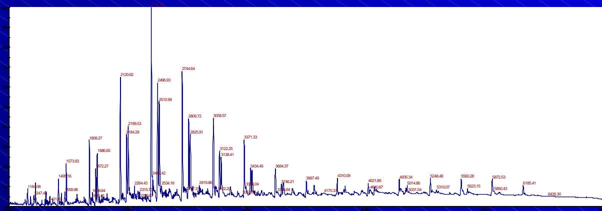
MALDI-TOF is good for looking at the mass, can work for all 4 homopolymers. However, the presence of salt in many samples makes this analysis difficult. Moreover, the distribution of DNA crystals in unpredictable locations in a ring form in maldi-samples make the process difficult to automate.

Homopolymer A-20mer
an example of depurination



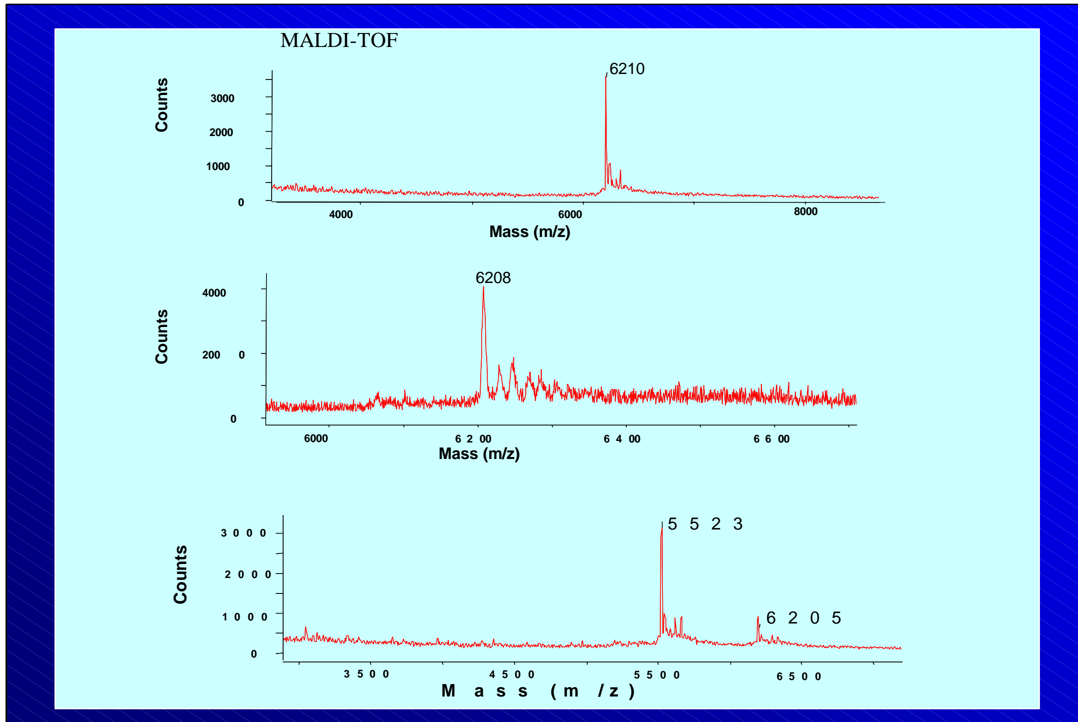
Depurination leads to alkali hydrolysis. The n-1 has a PO4 on the 5' end after beta-elimination, part of the time, and thus th e+80 n-1 peaks.



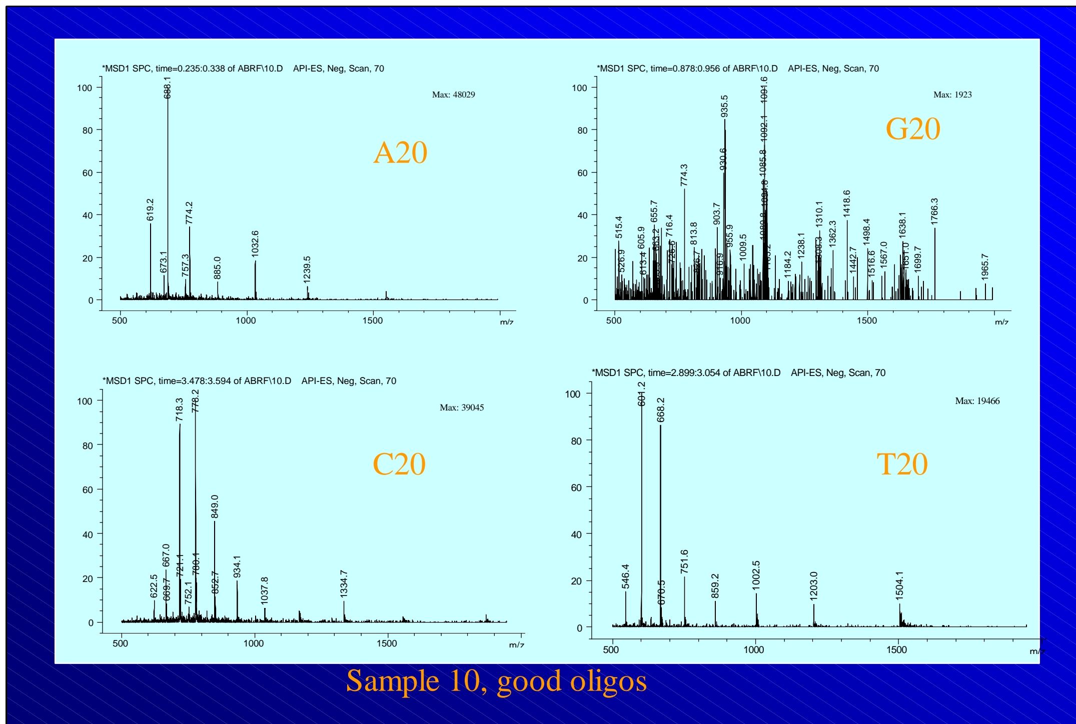


Lower quality A20 mers, MALDI-TOF analysis

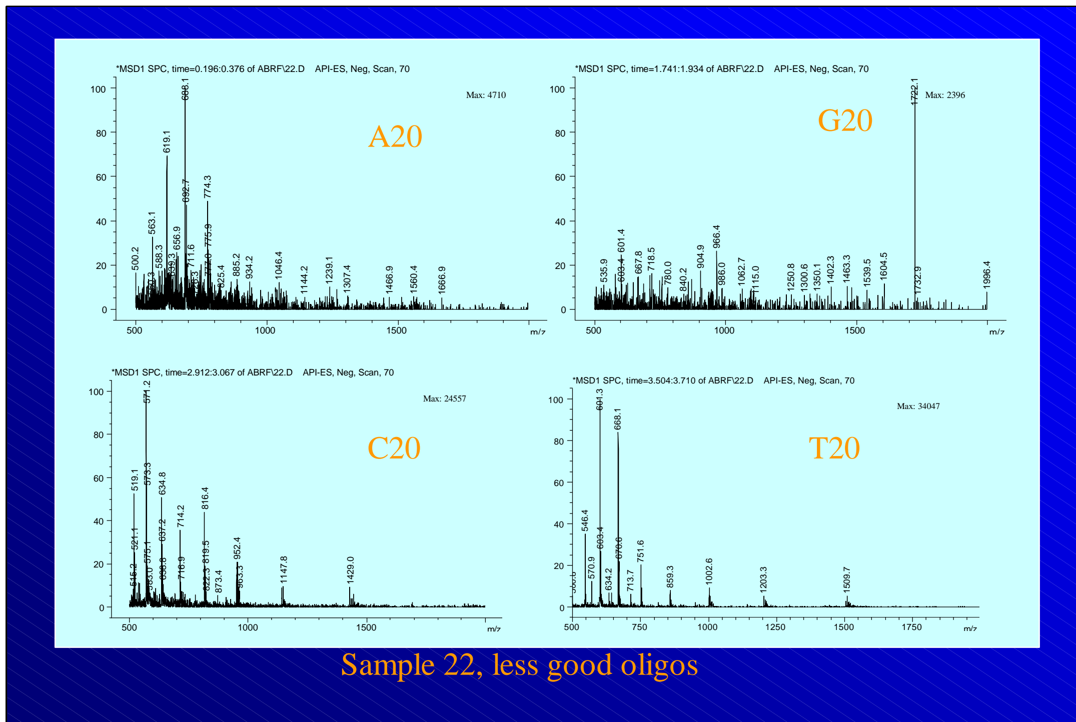
Remember that MALDI-TOF is not quantitative and lower mass flies much better. Still, depurination for these two A20 oligos is obvious.



In the bottom panel, most of this 5’ TET oligo has no TET!!

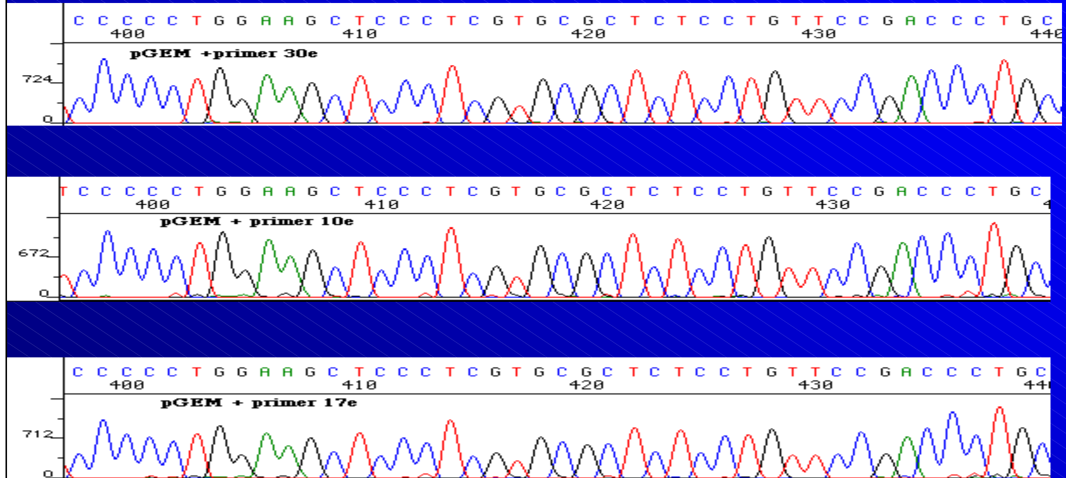


Electrospray ionization (ESI) mass spec can analyze oligo pretty well with quick automation. Salt is a problem, as is G20.

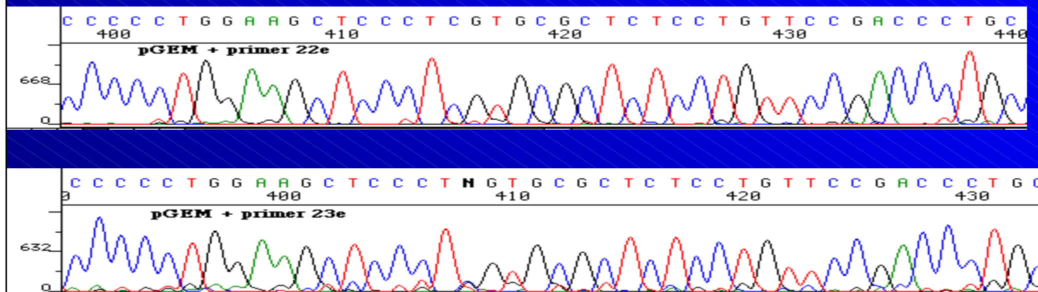


A set of less good oligos by ESI MS analysis

Sequencing pGEM with purified or crude good quality primers



Sequencing with crude moderate to low quality primers



Even these sequencing is nice, though as a researcher, N’s at 410 nucleotide will mess up my day because I will have to sequence again with another new primer.

Retention time of oligonucleotides and signal intensity on DHPLC

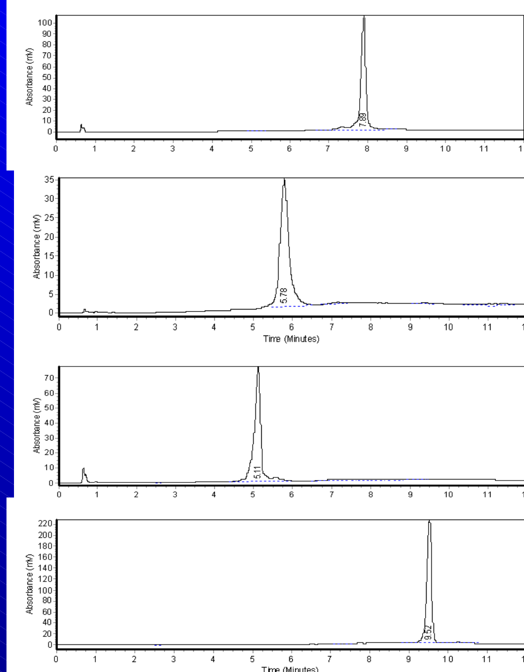
Oligosep Prep HC column, 50 x 7.5 mm.
2.5 ml/min . Column temperature 80 C.

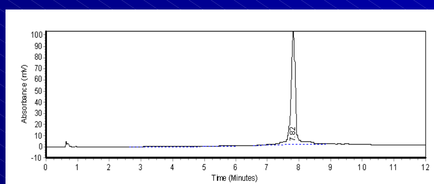
Eluants were as follows:

A= 100mM TEAA, pH 7.0
B= 100 mM TEAA in 25% CH3CN, 75% H2O

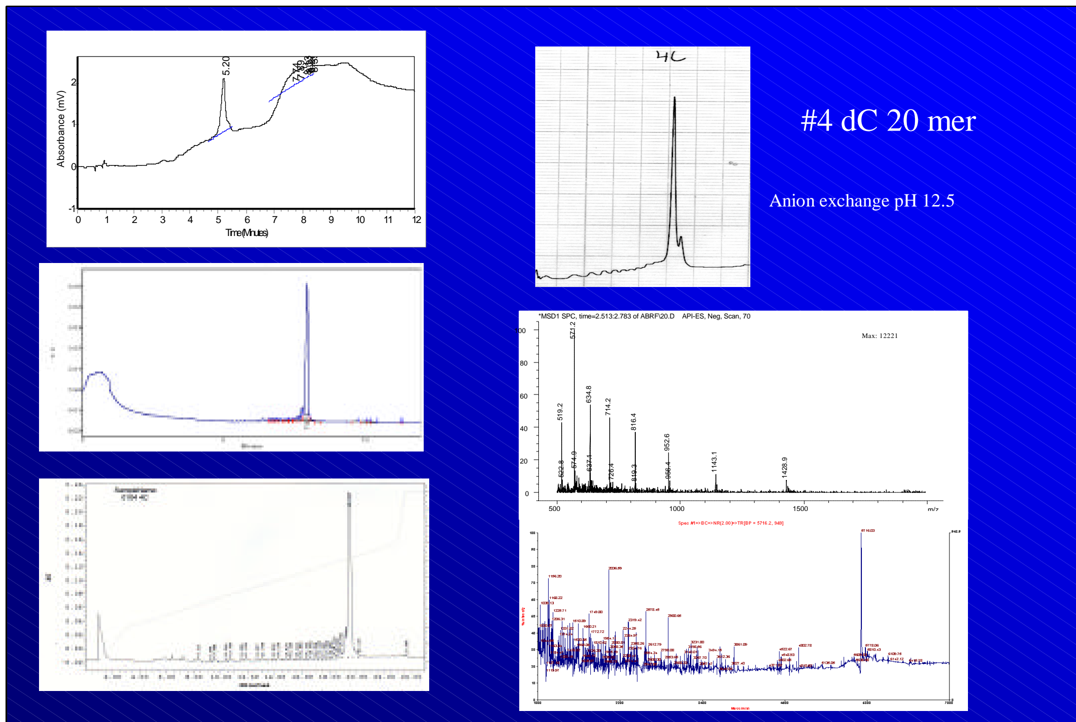
The gradient was 20-62% B in 12 min.

All samples were dissolved in 0.2 ml of water and transferred to 96 well plate. 50 ul of each was injected. The chromatograms were followed at 260 nm.

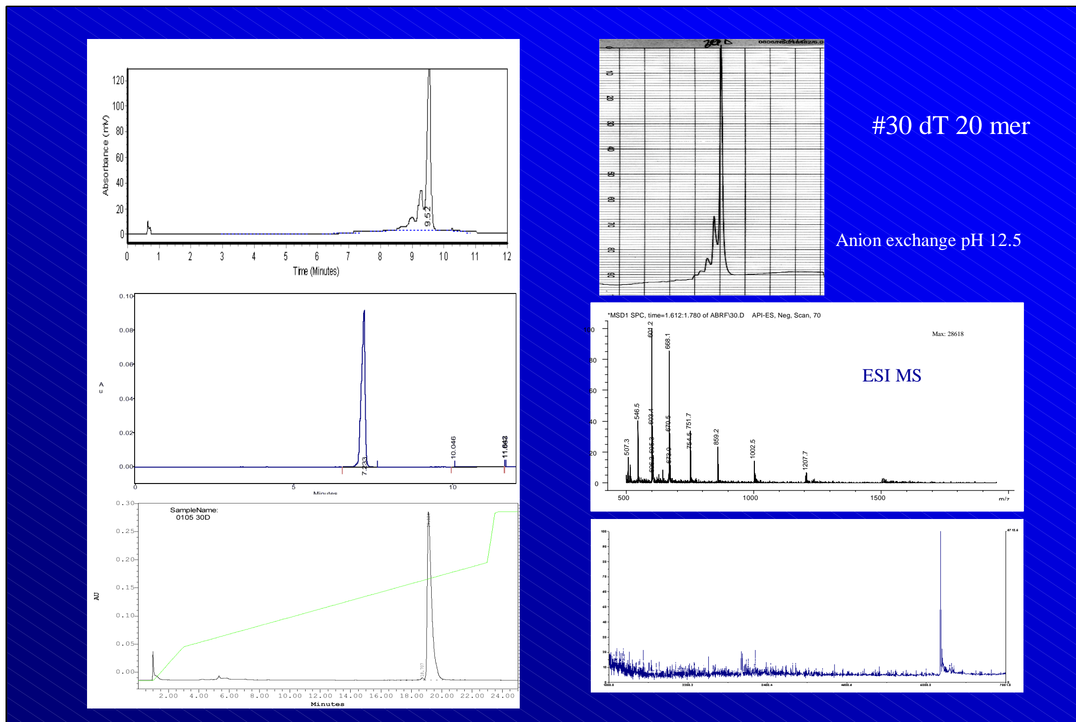




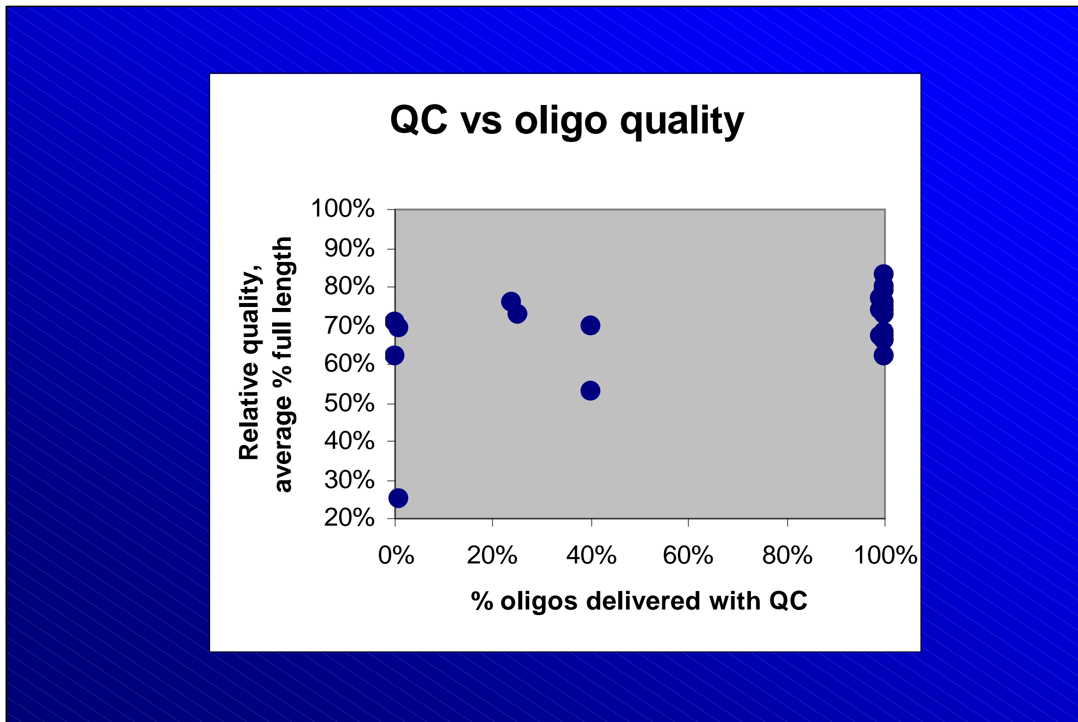
WAVE in this buffer is not length-dependent, however, there is another buffer system that is said to be length-dependent. In this study, WAVE analysis of G20 and C20 are difficult to interpret. We have both problems of low sample recovery from the column, broad humps of unknown nature, or just no chromatogram with some oligo preparations. WAVE may be too sensitive to structure in this buffer system. The heteropolymers were trouble-free.



This slide illustrates a problem to be looked into: is this a deprotection problem, as shown by humps on the right side on the main peak? Not all QC methods can see this. Top left is WAVE. Middle left is CE. Bottom left is anion exchange in %M urea pH 7.



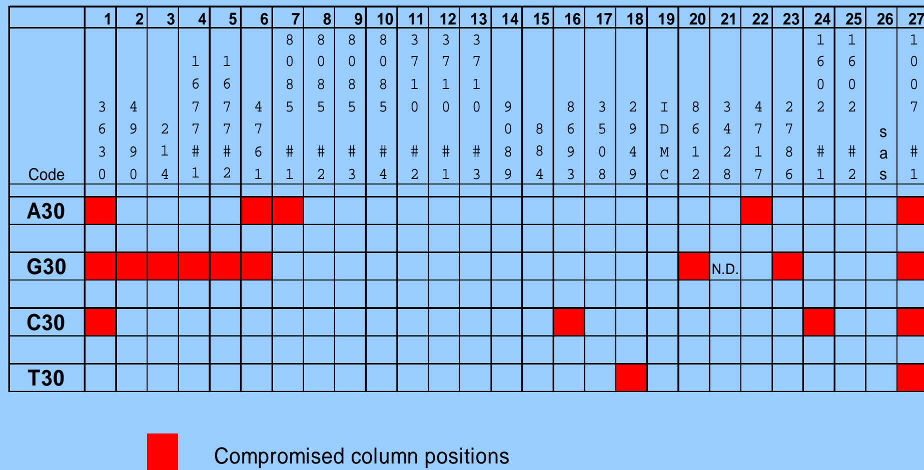
This slide illustrates another problem to be looked into: is this deprotection problem, as shown by humps on the right side on the main peak. Not all QC methods can see this.



Not all machines with no QC are bad. Most DNA synthesizers and DNA synthesis chemistry are pretty good.

Not all DNA synthesizers with 100% QC are great. It depends on how the QC is done, and what one does with the information.

However, the best machines all have great QC!



The 27 DNA synthesizers tested, the sample number code used in our database, and the corresponding 4 letter code given by their submission laboratory are shown. Red squares show the compromised bases in the synthesis of each machine. Some that give good looking primers actually are compromised in one or more bases. In a long oligo, that adds up to a not so great oligo. In a primer, you would not notice the compromise.

Homopolymers for QC, once or twice a year, is the way to go to fine tune a DNA synthesizer. If your QC method cannot handle G20, you may consider a GTGTGTGTGTGTGTGTGTGT instead, although the G problems will be less visible.

Strengths and weakness of QC methods:

- UV absorbance. Useful if column loading were even and if expected yield is known.
- Anion-exchange HPLC at neutral pH and 5M urea. Quantitative. High resolution but sometimes sensitive to structure. Cannot analyze G homopolymer. No mass info. May miss small no-charge modifications.
- Anion-exchange FPLC at pH 12.5. Not structure sensitive but no mass info.
- Capillary electrophoresis (CE) Easily automated. No mass info. Structure sensitive.
- Denaturing HPLC. Automated. G and C structure sensitive. Need more development.
- ESI mass spectrometry. Easily automated. Mass info. Salt sensitive. Problem with G homopolymer.
- MALDI-TOF mass spectrometry. Mass info. Structure sensitive. Salt sensitive. Hard to automate. Non-quantitative.
- DNA sequencing. Not very informative because most primers work well.

PART II-Conclusion:

- A core facility that QC 100% of the oligonucleotides can detect instrument and chemical failures immediately.
- QC of heteropolymer oligonucleotides can show whether the amount of full-length species is sufficient.
- Use of homopolymers in QC can identify situations where the synthesis of some of the four nucleotides are compromised.

PART II-Conclusion:

- The ABI 384 is a great instrument, but other instruments can also make great oligos.
- The double-diluted phosphoramidite primer cycle on the 394 can make great oligos.
- Synthesis scale is not a limiting factor.
- Some fast chemistry can make good oligos.
- More than one company supplies good reagents.
- Synthesis support is not the limiting factor.

QC! QC! QC!

Pay attention to details

You can do it!

Lets stay in touch

We would really like the rest of the 111 DNA synthesis CORES listed in the ABRF directory to get back in touch with us. My email is AT_Yeung@fccc.edu. From this study of 27 DNA synthesizers, we know that there are many more out there that are making great oligos, and some delivering not so great oligos. If you want assistance, to QC, or to improve your instruments' performance, we are able and willing to contribute. Some of us are Principal Investigators in addition to CORE facility directors. We see both perspectives. We believe that providing the best possible oligonucleotides is the only reason, but an indispensable one, that justifies the existence of DNA synthesis core facilities.

Thank you very much!

NARG.