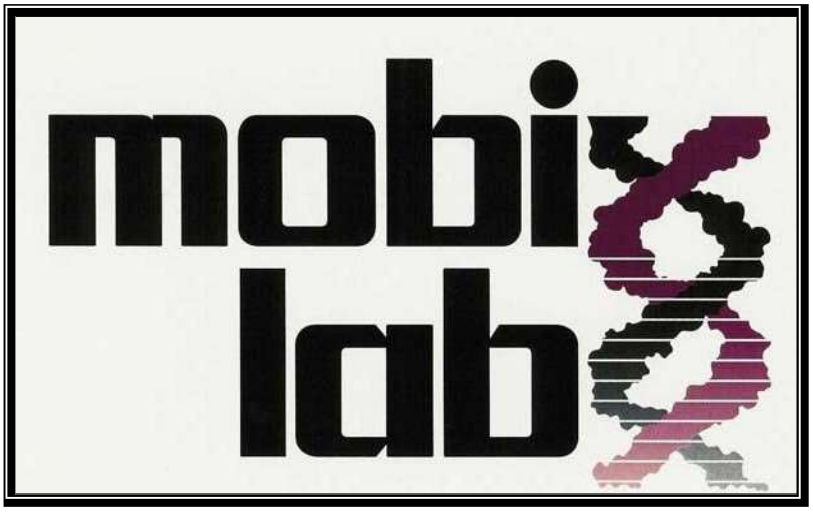


Welcome to the Mobix Lab
McMaster University, HSC 3N4

A Useful Guide to Oligo Synthesis and Automated DNA Sequencing

Personnel

Christine Mader— Facility Manager
Liliana De Sousa—Technician
Leanne Blanchard—Technician
Emily Jardine—Technician
Angela De Sousa—Technician

Services Available

- **Automated DNA sequencing**
  We have an ABI 3730 DNA Analyzer and a SeqStudio Flex Genetic Analyzer. The average read length is 900 bases with good quality DNA.

- **Fragment Analysis & SNP Analysis**
  Performed on the 3730 or the SeqStudio DNA Analyzer.
  Contact us for more information.

- **Oligonucleotide synthesis service**
  Oligos are synthesized by Integrated DNA Technologies (IDT)
  Regular or Modified oligos can be ordered at different scales:
  25 nM, **50** nM, **100** nM, **200** nM, **1** uM, and **Ultramers**.
  Custom Gene synthesis and **gBlocks** (Gene fragments blocks) also available.

IDT offers an **online Oligo ordering portal**. The features include:

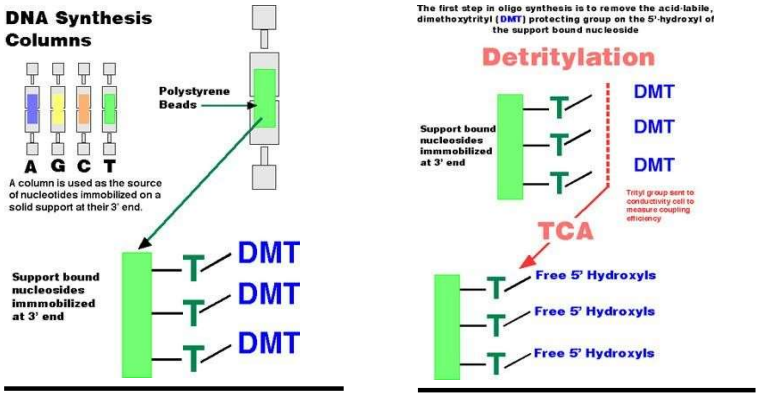
- cheaper per base cost for oligos
- **free shipping, with pick up at HSC-3N4**
- access to ordering history
- evening and weekend ordering
- Sign up today at https://www.idtdna.com/mobix

For more information check out our web site at

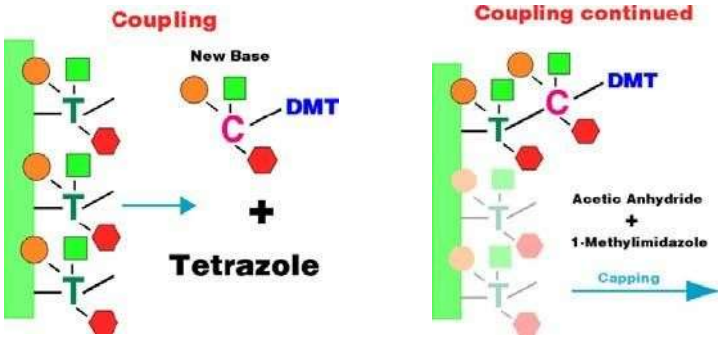
https://healthsci.mcmaster.ca/mobix

Quick Guide to Oligonucleotide Synthesis

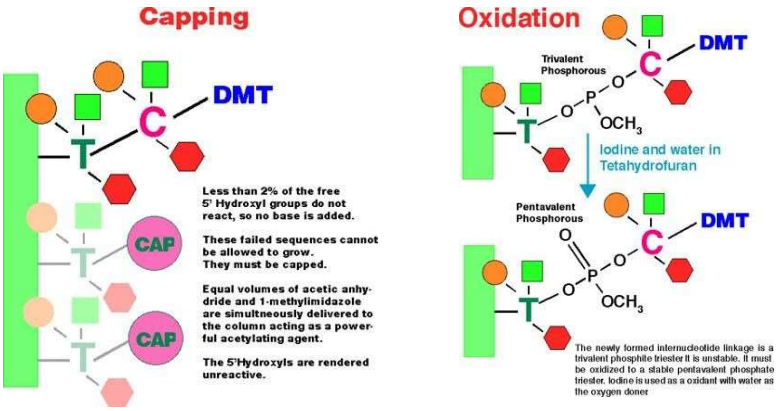
DNA synthesis proceeds in the 3' to 5' direction. Synthesis starts with a column with the 3' base attached. The reactive OH group is —protected by dimethoxytrityl group (DMT or trityl)



First the —trityl protecting group must be removed to generate a free 5' hydroxyl. The next base is added together with the coupling reagent.



Some bases may not have coupled (about 1 - 2%) so any unreacted bases are —capped to block any further reaction. However, a very small number of molecules may continue to elongate and contribute to the n-1 products in the final preparation, even after purification.



Coupling produces a trivalent phosphite bond which must be oxidized to the phosphate. Using a sulphurizing agent at this step will generate a phosphorothioate backbone (often required if the oligos are to be used *in vivo*). Cycles are repeated to add each base of the sequence. The finished oligo is cleaved from the column and incubated in ammonium hydroxide to remove all the protecting groups, ethanol precipitated to remove impurities and the OD260 measured. The whole process takes 36 – 48 hours.

Common Primers

We carry a few **Common Primers** in stock for use in your sequencing reactions at no extra cost to you. We also have them available for purchase.

PRIMERS	SEQUENCE
M13Forward	5'-GTAAAACGACGGCCAGT-3'
M13F(-40)	5'-GTTTTCCCAGTCACGAC-3'
M13Reverse	5'-CAGGAAACAGCTATGAC-3'
M13R(-48)	5'-AGCGGATAACAATTTCACACAGGA-3'
T7 promoter	5'-TAATACGACTCACTATAGGG-3'
T7 terminator	5'-GCTAGTTATTGCTCAGCGG-3'
T3 promoter	5'-ATTAACCCTCACTAAAGGGA-3'
SP6 promoter	5'-GATTTAGGTGACACTATAG-3'
BGH Reverse	5'-TAGAAGGCACAGTCGAGG-3'
pGEX 5'	5'-GGGCTGGCAAGCCACGTTTGGTG-3'
pGEX 3'	5'-CCGGGAGCTGCATGTGTCAGAGG-3'
λgt11 Forward	5'-GGTGGCGACGACTCCTGGAGCCCG-3'
λgt11 Reverse	5'-TTGACACCAGACCAACTGGTAATG-3'
SK primer	5'-TCTAGAACTAGTGGATC-3'
KS primer	5'-CGAGGTCGACGGTATCG-3'
pDONR 201F	5'-TTAACGCTAGCATGGATCT-3'
pDONR 201R	5'-AACATCAGAGATTTTGAGACAC-3'
pBR322 Bam	5'-ATGCGTCCGGCGTAGA-3'
-96 gIII	5'-CCCTCATAGTTAGCGTAACG-3'
oligo dT(18)	5'- TTTTTTTTTTTTTTTTTT-3'
TnN	mix of: T(20)A, T(20)C, T(20)G
Pentadecamers	5'-NNNNNNNNNNNNNNN-3'
Hexamers	5'-NNNNNN-3'

How to order Oligos

Oligos can be ordered in two ways:

1. Through the Mobixlab IDT portal: https://www.idtdna.com/mobix
2. Email orders to us by filling out this Excel order template: https://healthsci.mcmaster.ca/mobix/services/oligo-synthesis and email it to mobixlab@mcmaster.ca

1. Always write out the sequence 5' to 3'

2. You need specify the synthesis scale; this is based on how much you will need:

25 nM	3 – 6 OD	
50 nM	7 – 12 OD	(yields based 20-mer, desalted)
200 nM	20 – 30 OD	
1µM	80+ OD	
Utramer	4nM (product is >80%)	

3. You must specify the purification procedure for the oligo:

Desalted oligos will contain the full-length oligo, plus any smaller failed fragments. These oligos are adequate for most applications, such as DNA sequencing, PCR amplification, etc.

HPLC achieves a higher purity level (approximately 85%) but with a significant decrease in yield.

PAGE* purified oligos are the very pure (>90%) but yield is quite low.

(*You can also do this purification yourself with a desalted oligo. Methods can be found in "Current Protocols in Molecular Biology" or "Molecular Cloning – a Laboratory Manual

Expected percentage of full-length product for Oligo Synthesis:

Purification	Size	% full length (at 98% coupling efficiency)	Applications
Desalting	20mer 30mer 40mer 50mer 60mer 70mer 80mer 90mer 100mer	65 53 43 35 30 24 20 16 13	Sequencing/PCR >40 bp desalting is not recommended unless you intend to purify oligo yourself, or have internal restriction enzyme sites on the 5' ends of your oligos (for cloning)
HPLC	20 – 110mer	>85	Any application where accuracy is required

4. Modified Oligos:

Degenerate bases (equimolar)-no additional cost
Degenerate bases (hand mix)-additional cost per mix (inquire)
Many 5' or 3' modifications available (different pricing)
Phosphorothioate backbone (additional cost/base)
RNA or RNA bases available
Gene synthesis or Gene Blocks available

Hints on Primer design

For sequencing: 18 – 20 bases long at least 50 bases upstream of the area of interest

- Tm ~ 50℃
- ~50% GC content
- no long runs of a single base
- no hairpin loops
- single annealing site
- no dimer formation

For PCR: usually 20 – 30 bases long

- Tm more flexible, but both primers should be about the same
- Check for dimer formation
- Most PCR primers will work for sequencing (<30 bases)

Resuspending your Oligos

Spin down the tube containing the dry oligo before opening the tube (microcentrifuge maximum speed for 1 minute)**.** Often dried (lyophilized) oligos become dislodged in the tube during shipping and can be lost if the lid is open before spin down.

Calculate how much TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0) **you will need to add to make a minimum 100 uM stock solution.** If the concentration is less than 100 uMol, primer degradation will increase dramatically even when stored at -20^{o}C.

The amount of oligo present in your tube will be displayed on your specification sheet printout from IDT or Operon. On the IDT sheet, the amount will be displayed as:
X OD_{260} = Y nMoles = Z mg

Where X = OD of the oligo (1 OD ssDNA ≈ 33 ug)
Y = nMoles*
Z = total amount of oligo in mg (or µg on Operon sheets)

*Easiest way to dilute to 100 uMol stock:
Take value of **Y** (nMoles) multiply by 10, this will give you the volume in µl of diluent (1XTE) to add to your tube, in order to get 100 µMol stock.

It is prudent to resuspend your oligos as a molar (µMol) concentration; that way the length or composition of your oligo does not affect the relative concentrations of your oligos. Your stock should be prepared at 100 or 200 µMol concentration. This oligo stock should be aliquoted into smaller volumes. They should be stored at -20℃ and repeat freeze-thawing cycles should be avoided to prevent degradation of your oligo. IDT claims that oligos can be stored this way for around seven months. In their lyophilized state, they can be stored at -20℃ for 2 years.

Your working concentrations should be at 5 to 10 µMol and stored at -20 ℃ in small aliquots of 100 µl or less. They can be kept at 4 ℃ for up to one month, then discarded and a new aliquot of oligo should be used.

Dilute your primers with either TE (pH 8.0 or 7.5) or nuclease-free ddH_20. We do stock IDT products for diluting oligos which can be purchased at our Bio-Bar.

Other ways to calculate the amount of oligo based on OD value

If your oligo is supplied to you lyophilized, with just an OD_{260} value:

Converting OD units into something else:

a) 1 OD 260 unit ≈ 33µgs oligonucleotide

b) µmoles≈ total OD 260 /10 x length of oligo

c) For a 100 µM stock, volume of diluent to resuspend oligo:

Volume (ml) = OD 260 /(1.5 nA+0.71 nC+1.2 nG+0.84 nT),

Where **n** is the number of bases A, G, C or T

Troubleshooting primer problems

Storage

Here are the results of IDT oligo stability testing:

Storage Conditions	Degradation begins After
At Room Temperature in water	60 days
At Room Temperature in TE or dry	7 month
At 4℃,-20℃,or-80℃ in TE, Water or dry	7 month
At -20℃ dry	24 month

Primer stopped working

Sometimes a primer that was initially working well stops working. This may be caused by primer degradation. We have seen this with some sequencing primers. We suggest that you take a new aliquot from your stock primer. If the reaction still fails, try having the oligos synthesized again to see if that resolves the issue.

Mutations in primer sequence discovered after PCR and cloning

Oligos are synthesized one base at a time and after each base addition any molecule that did not get a base added is “capped” to prevent further synthesis. The probability that an oligo is synthesized with a base (or bases) missing in the middle is extremely low, but not impossible (see Page 3). If you do get a result of several bases being deleted from within the primer sequence, a more likely explanation would be errors introduced during PCR or recombination/repair events in the bacteria during cloning. Please sequence several different clones to confirm the problem is consistent in all. Rarely, a G base may be converted to the enol tautomer 2,6-diaminopurine, which is recognized as A by DNA polymerase. Thus a G to A transition is observed. Also, during synthesis depurination may occur. These oligos are usually degraded during the deprotection stage, but a small percentage may remain, and these oligos once cloned will appear to have an A or G deletion. Also, failed sequences that do not couple during a subsequent base addition but are not fully capped, may also appear to have a deletion. Lastly, if the base being added couples with another base molecule (very rare) this may cause an insertion. We will remake any oligos that appear to have a problem.

Missing 5’ end bases

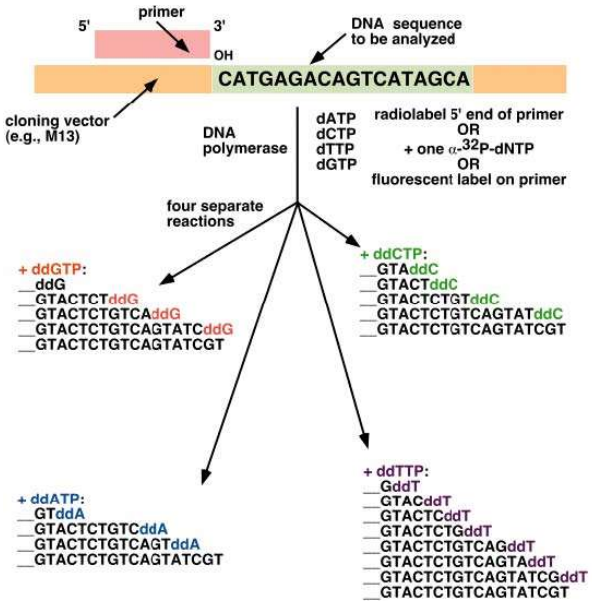
There is a low failure rate per base addition (~2%). If you ordered a primer “desalted” the final preparation will have a small population of molecules all one base shorter than the next from the 5’ end (synthesis goes 3’ to 5’). Ordering oligos HPLC purified will help. Incorporating a restriction enzyme site at the 5’ end of the oligo so that the final product is digested before cloning improves cloning efficiency and fidelity.

Problems cloning oligos directly

Synthetic oligos do not have the 5’ phosphate group necessary for ligation to work. To clone directly you must add a 5’ phosphate using Polynucleotide Kinase or order them made with a chemical 5’phosphate added (extra charge). If cloning into a vector using two RE sites, this is not necessary, since the vector will have the 5’ phosphates on the cut ends.

A Quick Guide to Automated DNA Sequencing

Automated sequencing is based on the Sanger dideoxy sequencing method, which utilizes the fact that DNA polymerase will stop after a dideoxy-nucleotide (ddNTP) is incorporated. Sequencing requires a small primer close to the region to be sequenced, dNTPs, ddNTPs and a DNA polymerase.



Modifications for automation include the use of fluorescently labeled ddNTPs so that the reaction is carried out in one tube and the use of Taq DNA polymerase to allow repeated cycles of the reaction. The reaction conditions we use are:

96℃ 10 secs, 50℃ 5 secs, 60℃ 4 mins for 30 cycles

NB. This is not PCR amplification (i.e. exponential), it is linear amplification:

one primer - one direction

Following the reaction unincorporated dyes are removed using clean-up beads, and samples are then resuspended in dilute EDTA and then run on the sequencer.

The ABI 3730 Gene Analyzer uses capillary electrophoresis through a liquid polymer to separate the sequence products. DNA enters the capillaries by electrokinetic injection (only ions enter the capillary). DNA fragments travel along the capillary by high voltage (8.5 KV) electrophoresis and the fluorescence of each band is measured as they pass a laser and detector.

The ABI analysis software corrects mobility based on the size of the dyes and produces the electropherogram (*.ab1 file). We check over the sequence, however, we do not edit ambiguous bases, unless you have requested “with editing” (extra charge).

We suggest that you check the data files (*.ab1) using the freeware FinchTV or SeqScanner in order to correct any ambiguities in your sequencing results. Your results will be emailed to you and can include a text file, the *.ab1 run file, and a printout of the electropherogram (if requested).

Preparation of the Template DNA

We require 5 µl of DNA for **each** sequencing reaction.

1. **Plasmid DNA or PCR reaction products**

DNA samples to be sequenced must be clean and of high quality. For plasmid DNA we recommend using a purification kit that is suitable for capillary sequencing. The DNA concentration should be determined accurately. To avoid misleading results due to chromosomal DNA contamination and RNA contamination, we recommend running 1 to 2 ul of your DNA sample on an agarose gel against a DNA ladder or Mass Ruler with known DNA mass for each band (FroggaBio DNA ladders).

For our capillary sequencing, DNA concentrations work well when they are:

1 ng/µl for every 100 bases of template

So, for a 7 kb plasmid + 1 kb insert, 80 ng/µl is sufficient
For a PCR product that is 400 bp long, 4 ng/µl is sufficient

Too much DNA or too little DNA will sometimes cause the reaction to fail. The reaction can tolerate a range of concentrations, but the best results will be obtained from giving us the desired amount.

2. **Cosmid or Lambda genomic DNA** (<40 kb)

We use different sequencing conditions and a different protocol for sequencing larger templates. We require the DNA to be at 500 ng/µl, and the sequencing primer to be at 10 µM. Since we use more sequencing mix, the cost is slightly higher than normal sequencing. Please inquire.

3. **Bacterial genomic DNA** (<10Mbp)

We are able to sequence bacterial genomic DNA from small bacterial genomes. It is very difficult and requires special conditions and very clean and accurate quantification of the DNA. Please **contact us** if this is something you are considering. The cost for sequencing is much higher than normal sequencing. Please inquire.

4. **Eukaryotic genomic DNA** –this cannot be done.

You have to amplify your gene of interest and submit the PCR product for sequencing.

Preparation of the primer

Primer concentration should be at 1pmole/µl or 1µM. Please provide 5µl for **each** reaction. The T_m of your primer should be ~50℃, if it is a bit lower, please increase the concentration to 5 or 10µM. Long primers used in PCR (30+) may not work well for sequencing. Also, if you have any degenerate bases in your primer, please increase the primer concentration accordingly.

Submitting Sequencing Samples

Please fill out a submission form. Limit your DNA name and primer name to 8 characters (or less), no Greek letters or punctuation marks. Please use 1.5 ml tubes, labeled on the top with the same name as it appears on the order form. Please DO NOT use 0.2 ml tubes.

You can mail your samples or bring your samples to the lab, HSC-3N4, between 9:30 am to 5 pm. Turnaround time is usually 2 working days.

Results

If you choose the **WITH EDITING** option, we will look over the results and correct ambiguous bases (extra charge). If instead you choose the **NO EDITING** option, we will not correct ambiguous bases, but we do suggest that you correct the sequence yourself using the freeware FinchTV or ABI SeqScanner. You will need the *.ab1 file to do this.

When the sequencing is complete, we will email you the results. You can choose to have the text file, the *.ab1 data file, and a printout of the electropherogram by ticking off the boxes on the submission form.

Hints on Template preparation

Sequencing success depends on the quality of the DNA being sequenced:

GARBAGE IN = GARBAGE OUT

PCR products: must have primers removed and be a single band

- PCR Clean-up kit (many)
- Gel purify (if more than one product, or primer/dimers present)

Plasmid DNA: must be clean and free of RNA

- (RNase-does not get rid of RNA it just chews it into little pieces)
- Use a Plasmid Clean-up kit (many) or Phenol/Chloroform extraction to remove smaller RNA contaminants

Always check amount of DNA on a gel against a known standard

Contaminant Limits

The sequencing reaction will fail if there is more than:

A) 1µg RNA -1ml E.coli culture gives 1 to 5 µgs plasmid DNA but 100 to500 µgs RNA. If you RNase treat you must phenol extract and isopropanol precipitate.
B) 0.3% PEG -that's 2.5µl left from a 100µl precipitation
C) 0.5mM NaAc -that's 0.5µl left from a 100µl precipitation
D) 1.25% EtOH -that's about 2µl left from a 100µl precipitation
E) 0% phenol -in other words not even a trace!
F) 0% chloroform -likewise!
G) 5mM CsCl -that is also not very much from a CsCl gradient
H) 5mM EDTA -dissolve your DNA in water or diluted elution buffer (1:10)

Sequencing Difficult Templates

We have had some success in getting through difficult sequences. You can request the following to help obtain better results. Just write down any special instructions to the submission form. Extra charge may apply.

G/C rich templates	Add DMSO to the reaction
Secondary structures	Hairpin Protocol (extra charge applies)
A/T rich sequence	AT-rich Protocol (extra charge applies)
Poly C, C rich	Add 1 M betaine to reaction mix
Poly T	Use the TnN primer

Troubleshooting your sequence results

A good website for trouble-shooting sequencing results can be found here: http://www.etonbio.com/sequencing/troubleshooting.php

No Data – signal strength is too low to give meaningful sequence

Reasons for "no data"	Insufficient amount of DNA template
	Poor quality of DNA template (see Contaminants affecting sequencing)
	Insufficient primer concentration/no primer added
	Primer T_m is less than 50 °C, so primer is not annealing during the sequencing reaction
	Template does not have priming site – make sure that the correct primer is chosen for the vector being used
	Primer does not anneal well to priming site/priming site has been altered. A set of primers may produce a PCR product, but one primer may not anneal as efficiently and therefore not work well for DNA sequencing.
	Sequencing product lost during clean-up procedure. Sequencing reaction will be done again at no charge.

Short Read – premature termination of the sequence

Top-heavy data	Excess DNA template, primer, or contaminants in the sequencing reaction result in top-heavy data because the balance of the sequencing reaction is shifted towards generation of shorter products.
Gradual decrease in signal	Repetitive DNA region, GC rich region, salt/contaminant in the reaction preventing production of longer sequencing products.
Abrupt decrease in the signal	Region of secondary structure or template sequence idiosyncrasies
	Low concentration of template, leading to top-heavy data and low sequencing signal, no long sequencing products

Multiple Peaks in the Sequence or Double Sequence

PCR products	At the beginning of the sequence – primer/dimer sequence or non-specific PCR products
	All the way – PCR product was not purified from primers.
	All the way – multiple PCR products being sequenced together.
	Frame shift mutation – insertion/deletion (**indel**) in one gene copy or mutation introduced by Taq polymerase during PCR amplification
Plasmid DNA	After some good sequence – more than 1 colony was picked, so you have more than one type of plasmid in your prep
	Contaminated template (see Contaminants affecting DNA sequencing)
Any template	Signal too low, only background peaks are apparent
	Slippage after homopolymer region in template.
	All the way – Multiple priming sites for the sequencing primer

"Noisy" Sequence – high background under peaks

Noisy data	The signal is too low (see "no data")
	Signal is too high causing detector saturation, as result the software can't call bases properly, especially in the T bases.

Other Problems

N-1 signal	Sequencing primer degraded or contaminated with failed shorter synthesis primers
"Spikes" or "Dye Blobs" in the sequence	Problem with the polymer on 3730 Genetic analyzer. Contact Mobix lab within 48 hours from receiving this kind of result and we will repeat run free of charge.
	Low DNA concentration causes "dye blobs" since the leftover ddNTPs fluorescence is higher than the sequencing peaks.

We hope these hints will help you obtain the best results from our facility.
We wish you success in your research.

The Staff of the Mobix