



**Welcome to the Mobix Lab
Located in LSB B123**

**A Useful Guide
to
Oligo Synthesis
and
Automated DNA Sequencing**

Personnel

Galina Kataeva, PhD— Manager

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Services Available

- **Automated DNA sequencing**

We have a 3730 DNA Analyzer capable of processing 96 samples in 5hrs, Average read length is 900 bases with good quality DNA.

- **Fragment Analysis & SNP Analysis**

Performed on the 3730 DNA Analyzer, call for more information.

- **Oligonucleotide synthesis**

The ABI 3900 DNA Synthesizer can make up to 48 oligos at once, it is usually used for shorter oligos: 15 to 40 size range

We have one ABI 394 and one ABI 3400 DNA Synthesizer which can make 4 oligos each up to 160 bases long. We also use these for specialty oligos and 5' end-labelling

- **Kodak Image station – LSB B123**

An imaging system for chemiluminescence detection

- **Alpha Imager – LSB 412**

An imaging system for UV and VIS visualization of gels

- **Phosphorimager – LSB 314**

Storm 820 phosphorimager is available for detection and quantification of radioactive gels

For more information check out our web site at
<http://www.science.mcmaster.ca/mobixlab/index.html>

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 applications requiring Oligonucleotides

- Polymerase Chain Reaction (PCR) primers
- Sequencing primers: we carry a few common primers in stock for use in your sequencing reactions (no extra cost) or available for purchase. Common primers are all OPC purified.

| PRIMERS | SEQUENCE |
|---------------|--------------------------------|
| M13Forward | 5'-GTAAAACGACGGCCAGT-3' |
| M13F(-40) | 5'-GTTTTCCAGTCACGAC-3' |
| M13Reverse | 5'-CAGGAAACAGCTATGAC-3' |
| M13R(-48) | 5'-AGCGGATAACAATTTACACAGGA-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'-TCTAGAAGTAGTGGATC-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'-TTTTTTTTTTTTTTTTTTT-3' |
| TnN | mix of: T(20)A, T(20)C, T(20)G |
| Pentadecamers | 5'-NNNNNNNNNNNNNNN-3' |
| Hexamers | 5'-NNNNNN-3' |

- Site-directed mutagenesis
- Cloning small regions of a gene

How to order Oligos

Oligos can be ordered on-line from our web site:

<http://www.science.mcmaster.ca/mobixlab/oligo-synthesis/basics.html>

You can also fill out a downloadable form available on the website, then FAX it to (905)526-1427 or email us with all the relevant information to mobixlab@mcmaster.ca

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

2. You need to know the prep size you need:

0.04 μ M yields 5 – 10 OD* (150 – 300 μ g)

0.20 μ M yields 20 – 30 OD*

1.00 μ M yields 100+ OD*

*If OPC purified, yield will be lower.

3. You must specify the clean-up procedure for the oligo, see table below.

a) Desalting is an ethanol precipitation of the whole synthesis prep.

It is usually sufficient for PCR and sequencing primers but the resulting oligo has all of the synthesis products including, 5' truncated sequences.

b) OPC purification, or Trityl-on purification. OPC columns are used to purify the products based on the presence of the DMT group, which must be left ON if the oligo is to be purified. (See page 3).

Recommended for long oligos (>50), and oligos to be cloned directly.

| Purification | Size | % full length (at 98% coupling efficiency) | Applications |
|--------------|----------------|--|---|
| Desalting | 20mer | 65 | Sequencing PCR >50 desalt not recommended unless you intend to purify yourself |
| | 30mer | 53 | |
| | 40mer | 43 | |
| | 50mer | 35 | |
| | 60mer | 30 | |
| | 70mer | 24 | |
| | 80mer | 20 | |
| | 90mer | 16 | |
| 100mer | 13 | | |
| OPC | 20 – 110mer | >85 | Mutagenesis, cloning, any other applications |

Only PAGE purification can guarantee 100% full-length product. We do offer this as a service, but it is very expensive, and yield will be low. You can also do this yourself, methods can be found in “Current Protocols in Molecular Biology” or “Molecular Cloning – a Laboratory Manual”

4. Extras:

- Degenerate base – equimolar amounts of 2 or more bases
- Addition of 5' end phosphate
- Addition of 5' end labels, eg. fluorescent dyes, biotin
- Phosphorothioate backbone

Hints on Primer design

For sequencing:

- usually 18 – 20 bases long at least 40 bases ahead of the area of interest
- $T_m \sim 50^\circ\text{C}$, ~ 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
- T_m more flexible, but both primers should be about the same
- Check both for dimer formation
- Most PCR primers will work for sequencing (<30 bases)
- Primer Designer program available in the Facility (see Galina)

Calculating the amount of oligo present

Oligos are supplied to you lyophilized, with an OD_{260} value.

Converting OD units into something else:

- $1\text{ }OD_{260}\text{ unit} \approx 33\mu\text{g Oligonucleotide}$
- $\mu\text{moles} \cong \frac{\text{total } OD_{260}}{10 \times \text{length of oligo}}$
- For a 100 μM Stock, volume of water to resuspend oligo

$$\text{Volume}_{[100\mu\text{M}]} = \frac{OD_{260}}{1.5N_A + 0.71N_C + 1.20N_G + 0.84N_T} \text{ ml,}$$

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

d) Use the following Excel spreadsheet.

<http://www.science.mcmaster.ca/mobixlab/files/oligo/Primers.xls>

If you fill out the yellow highlighted areas (desired concentration, number of each base) it will figure out what volume to add to your lyophilized oligo in order to get your desired molarity (highlighted in green) using the above equations. The value is given in milliliters.

e) Use this “Oligo Calculator”

http://www.biophp.org/minitools/melting_temperature/demo.php

Troubleshooting primer problems

Storage

Dissolve oligos in TE (pH7.5) or ddH₂O. Store reconstituted oligos in small aliquots at high concentration, e.g. 100μM, at -20°C and they should be good for years. Avoid repeated freezing and thawing. Make lower dilutions as required and store at 4°C for 4 – 6 weeks or -20°C for up to 6 months.

Primer doesn't work

If a primer fails to work in PCR or sequencing it is most likely a problem with the sequence you have chosen to have synthesized, not with the actual oligo itself.

Mutations in primer sequence discovered after PCR and cloning

Oligos are synthesized one base at a time and after each base addition, any molecules that did not get a base added are “capped” to prevent further reaction. 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 could be errors introduced during PCR or recombination/repair events in the bacteria during cloning. Sequence several different isolates to confirm the problem. There have been rare incidences where a G base may have been 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 in long oligos. 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 in 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 do appear to have any problem.

Missing 5' end bases

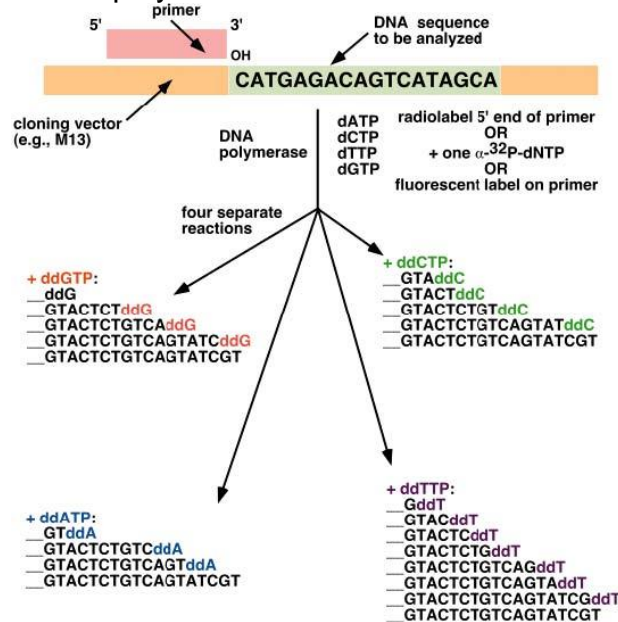
There is a low failure rate of base addition (~2%). If you ordered a primer “desalted”, the final preparation will have a small population of molecules that are all one base shorter than the next from the 5' end (synthesis goes 3' to 5' so the 3' end will be constant). Ordering oligos OPC purified or gel purifying them yourself will help to increase the proportion of full-length product. Incorporating a restriction enzyme site at the 5' end of the oligo, so the final product is digested before cloning, improves cloning efficiency and fidelity, making purification unnecessary.

Problems cloning oligos directly

Synthetic oligos do not have the 5' phosphate group necessary for ligase to work. To clone directly you must add a 5' phosphate using Polynucleotide Kinase (PNK) or order them made with a chemical phosphate added (extra charge).

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 labelled ddNTPs. The reaction is carried out in one tube with the use of Taq DNA polymerase. The reaction conditions we use are:

96^o C 10 sec, 50^o C 5 sec, 60^o C 4 min –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 sephadex columns. Samples are suspended in ddH₂O, and are run on the ABI 3730 sequencer.

The ABI 3730 Gene Analyzer uses capillary electrophoresis through a liquid polymer to separate the sequence products. Samples enter the capillaries by electro kinetic injection: only ions enter the capillary. Electrophoresis runs at high voltage – 8.5Kv. Samples travel along the capillary and the fluorescence of each fragment is measured as the samples pass a laser and detector:

The ABI analysis software corrects mobility based on the size of the dyes and produces the Electropherogram (*.ab1 file). Your results will be emailed to you and can include a text file, the .ab1 run file, and a printout of the electropherogram.

How to order sequencing

Preparation of the Template DNA

1. Plasmid DNA

DNA samples to be sequenced must be of high quality. Any kit which mentions that template is suitable for capillary sequencing will work. **DO NOT USE QIAEXII kit - it gives top-heavy data and extremely short reads.** The DNA concentration should be determined accurately. To avoid misleading results due to chromosomal DNA contamination and RNA contamination, we recommend running samples on an agarose gel against a known standard or a size marker such as the MBI MassRuler (#SM0403). As a general rule if you can't see a bright band from 1µl on an ethidium bromide stained agarose gel, then the concentration is too low.

DNA concentration required:

| | | | |
|------------|---|----------------|--------------|
| <10kb | = | 200 ng/µl | 5µl/reaction |
| >10 – 20kb | = | 0.25 – 1 µg/µl | 5µl/reaction |

2. PCR products

Primers must be removed. Any kit which mentions that template is suitable for capillary sequencing will work. **DO NOT USE QIAEXII kit - it gives top-heavy data and extremely short reads.** If more than one product is present you must isolate the band from a gel. If primer-dimers are present it is best to isolate from a gel. Always check recovery and quantify on an agarose gel against a known marker such as the MBI MassRuler (cat # SM0403).

DNA concentration required:

| | | | |
|----------------|---|---------------|--------------|
| 100 – 500 bp | = | 3 – 10 ng/µl | 5µl/reaction |
| 500 – 1000 bp | = | 10 – 20 ng/µl | 5µl/reaction |
| 1000 – 2000 bp | = | 20 – 40 ng/µl | 5µl/reaction |

3. Bacterial genomic DNA

We have sequenced bacterial genomic DNA. It is very difficult and requires special conditions and very accurate quantification of the DNA. We will need 6 µl of 2 µg/µl of very clean DNA. We also have to use more sequencing reaction mix and different sequencing protocol so extra charge will be applied for these reactions. Please come and talk to us if this is something you are considering doing

Preparation of the primer

We require a primer concentration of 1pmole/µl or 1µM; please provide 5µl for each reaction. The T_m should be ~50°C, if it is a bit lower - increase the concentration to 5 or 10µM. Long primers used in PCR (30+) may not work well for sequencing.

Submitting Sequencing Samples

Fill out a submission form. Internal and External forms are available for download on [our web site](#). Please limit your DNA name and primer name to 8 characters, no Greek letters and no punctuation marks. The name on the tubes should be the same as those listed on the order form. Please use 1.5 ml or 0.6 ml microfuge tubes. **Please DO NOT use 0.2 ml tubes.** Please bring your samples to the lab, LSB B123, between 8.30 am to 5

pm or mail them to:

MobixLab, McMaster University, LSB-B123, 1280 Main St. West, Hamilton, ON, L8S 4K1.
Turnaround time is usually 2 working days from the time it arrives at the lab.

Results

If you choose the **WITH EDITING** option, we will look over the results and correct ambiguities in base calling. Options to choose are: text file (bases only); *.ab1 file (bases and electropherogram); printout (electropherogram). Please check your selection on the submission form.

If you choose the **NO EDITING option** we do not correct the sequence. We strongly recommend that you take both the text and *.ab1 file so you can edit the sequence yourself. The freeware from Applied Biosystems will allow you to open this file.

http://marketing.appliedbiosystems.com/mk/get/SSS_login?isource=fr_E_Pg_Prod_AB_Gbl_SeqScan_20050920.
You still can request a printout with this option.

Hints on Template preparation

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

GARBAGE IN = GARBAGE OUT

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

- Qiagen column (or similar)
- gel purify if more than one product
- **Always check recovery on a gel afterwards**

Plasmid DNA: must be clean and free of RNA

(RNase does not get rid of RNA it just chews it into little pieces)

- Qiagen column (or similar). Please use water rather than buffer to elute DNA.
- alkaline lysis mini prep method - too much RNA
- alkaline lysis/PEG precipitation to remove RNA – maybe okay but must be free of PEG and salt
- **Always check amount of plasmid on a gel against known standard**

Quantification: OD at 260 nm is only good if there is NO RNA present

Always run 1µl on an ethidium bromide stained agarose gel against mass ruler such as the MBI MassRuler (#SM0403)

If you can't clearly see a thick plasmid band, there isn't enough to sequence!!

Contaminant Limits

The sequencing reaction will fail if there is more than:

- A) 1µg RNA -1ml E.coli culture gives 1 to 5 µg plasmid DNA but 100 to500 µg 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 Sodium Acetate -that's 0.5µl left from a 100µl precipitation
- D) 1.25% ethanol -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 10mM Tris pH8.5 not TE

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 order sheets. Extra charge may apply.

| | |
|------------------------------------|---|
| G/C rich templates | Add DMSO or Betaine, or both. Also, adding more sequencing mix to the reaction sometimes does the trick. |
| Secondary structures | Add DMSO or Betaine to the reaction or Use Hairpin Sequencing Protocol (extra day turnaround time) |
| Large template (larger than 20 Kb) | Use Large Template protocol (extra day turnaround time and extra charge may apply) |
| Bacterial Genomic | Use Genomic Protocol (extra charge) |
| A/T rich sequence | Performing the sequencing reaction at a reduced annealing temperature sometimes improves the results |
| Poly C, C rich | Add 1 M Betaine to reaction mix |
| Poly T | Use the TnN primer |

Troubleshooting your sequence results

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

| | |
|-----------------------|--|
| Reasons for "no data" | Insufficient amount of DNA template (see chart regarding DNA concentrations) |
| | Poor quality of DNA template (see Contaminants affecting DNA sequencing) |
| | Insufficient primer concentration (or no primer added) |
| | Primer T _m is less than 50 °C, the annealing temperature of the sequencing reaction is 50 °C |
| | Template does not have primer site--make sure that the correct primer is chosen for the vector being used. |
| | Primer does not anneal well to priming site. 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, which is linear amplification, unlike PCR, which is exponential amplification. |
| | Technical problem. Sequencing reaction will be done again at no charge |

Short Read – premature termination of the sequence

| | |
|---|---|
| Top-heavy data gradual decrease in signal | Excess DNA template or primer or salt in the sequencing reaction results in top-heavy data because balance of the sequencing reaction is shifted towards generation of the shorter products. DNA purification using QIAEXII kit usually results in top-heavy data. |
|---|---|

| | |
|-------------------------------|--|
| Abrupt decrease in the signal | Region of secondary structure or template sequence idiosyncrasies Repetitive DNA region, GC rich region |
|-------------------------------|--|

Multiple Peaks in the Sequence or Double Sequence

| | |
|--------------|---|
| PCR products | At the beginning of the sequence – primer-dimer sequence. |
| | All the way – PCR product was not purified from primers. |
| | All the way – multiple PCR products being sequenced together. |
| | Frame shift mutation (deletions in one gene copy) |
| Plasmid DNA | After some good sequence – more than 2 colonies picked |
| | Slippage after homopolymer region in template. |
| | Contaminated template (see Contaminants affecting DNA sequencing) |
| | Multiple priming sites or multiple primers |
| Any template | Signal too low, only background peaks are apparent |

“Noisy” Sequence – high background under peaks

| | |
|------------|---|
| Noisy data | The signal is too low (see “no data“) |
| | Signal is too high causing detector saturation; as a result the software can’t call bases properly. |

Other Problems

| | |
|--------------------------|--|
| N-1 signal | Sequencing primer contaminated with smaller length primer (primer was not purified) or primer is degraded due to repeated freeze-thaw cycles of primer |
| | Deletion or insertion of base(s) in DNA template – frameshift mutation |
| | Slippage after homopolymer region such as poly A region. |
| "Spikes" in the sequence | Low DNA concentration or degraded DNA due to contamination with nucleases, or use of Gene Clean to clean up DNA sample |
| | Problem with the polymer on 3730 DNA Analyzer. Contact Mobix lab within 48 hours from receiving this kind of result and we will repeat run free of charge. |

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

The Staff of the MobixLab