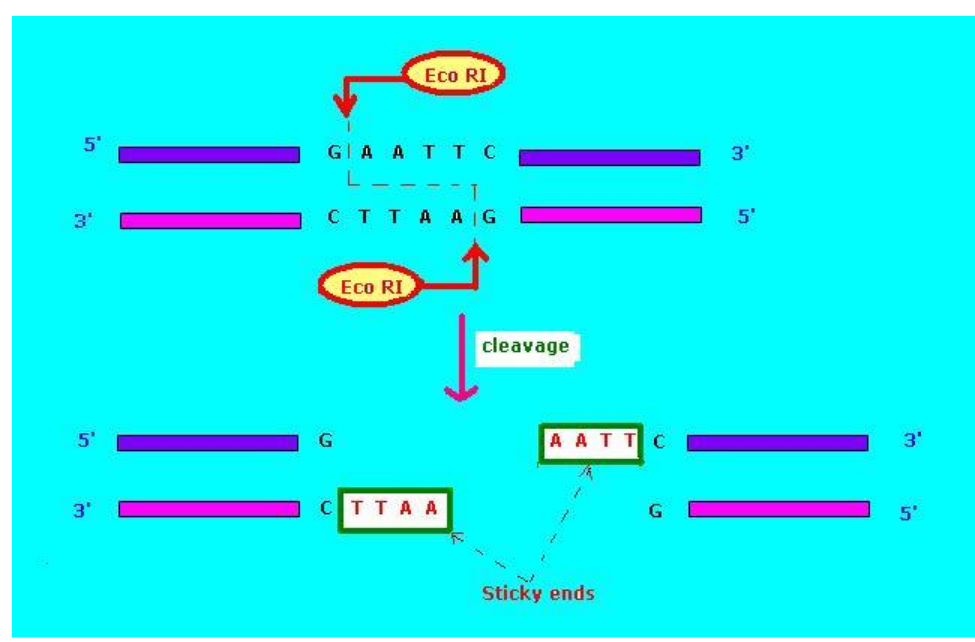
Restriction enzymes and PCR

David Andrés Ayala Usma

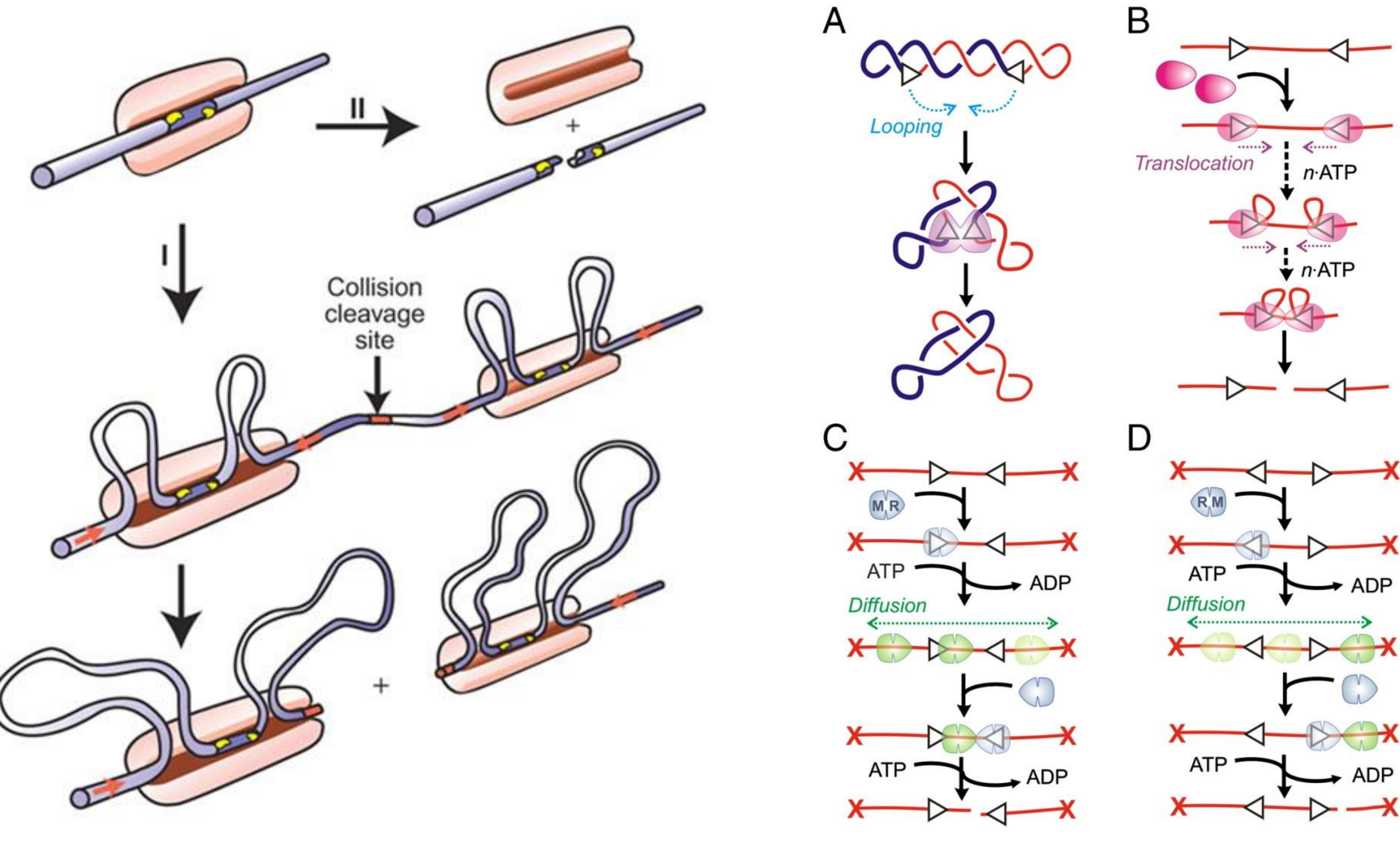
Restriction endonucleases (RE)

What?

- Enzymes or enzymatic complexes which cleave *inside* DNA molecules (Not 5’ or 3’ ends).
- Three types: I, II and III.
- Sequence-specific.
- Blunt or sticky ends



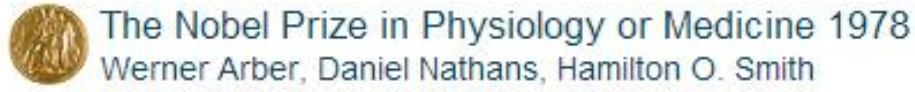
What?



Dryden, 2004

Van Aelst et al., 2010

Who?



The Nobel Prize in Physiology or Medicine 1978

Nobel Prize Award Ceremony

Werner Arber

Daniel Nathans

Hamilton O. Smith

Daniel Nathans

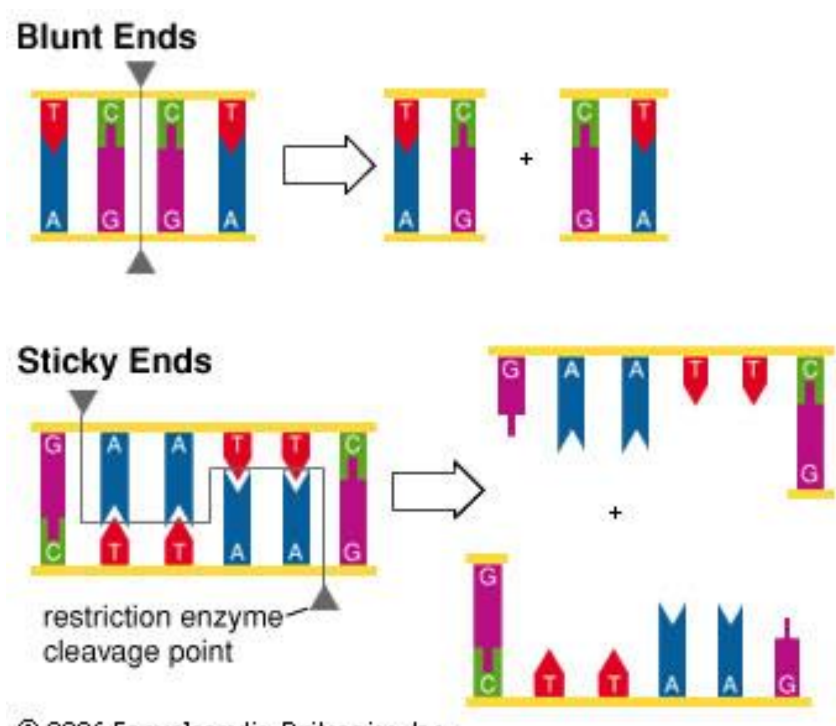
Hamilton O. Smith

The Nobel Prize in Physiology or Medicine 1978 was awarded jointly to Werner Arber, Daniel Nathans and Hamilton O. Smith *"for the discovery of restriction enzymes and their application to problems of molecular genetics"*.

Photos: Copyright © The Nobel Foundation

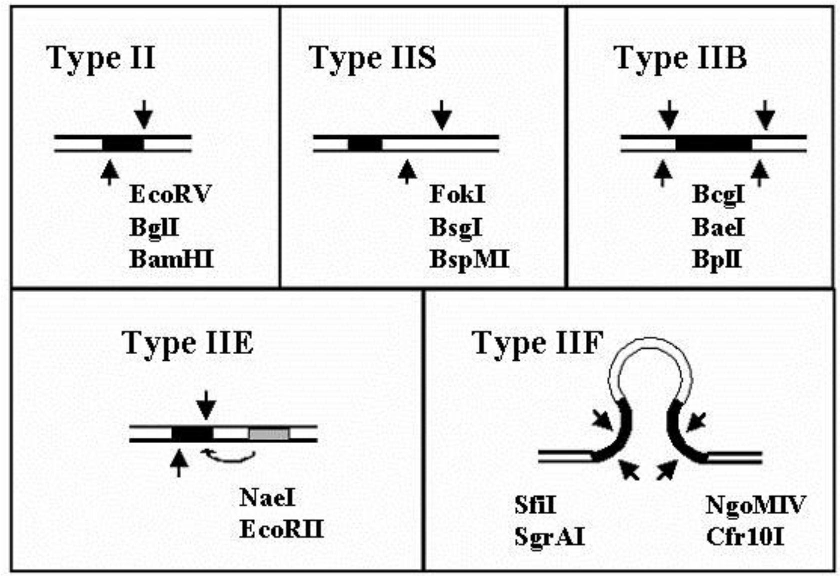
Type II RE

- TIIRE recognize a specific sequence in the DNA and cleave within.
- Hydrolysis of the phosphodiester backbone.
- They only recognize double stranded DNA (dsDNA).
- Blunt or sticky ends.



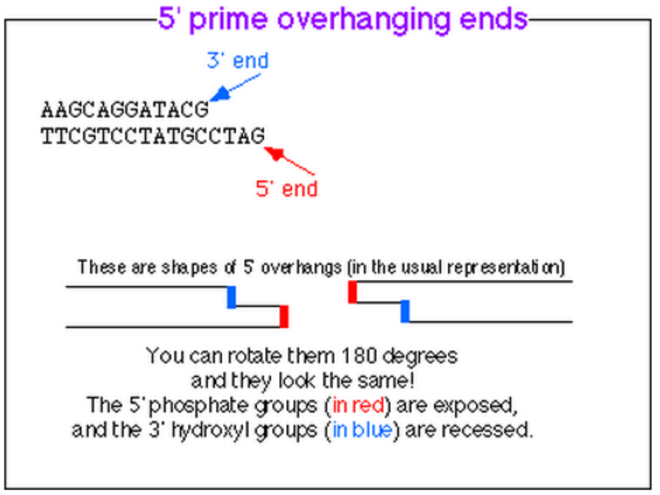
Type II RE subtypes

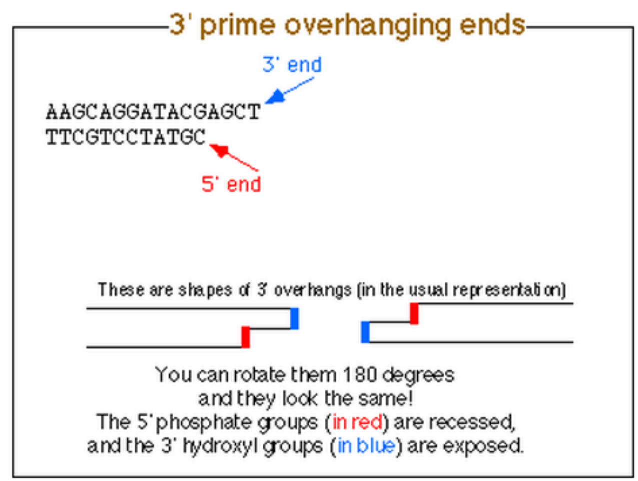
- IIS: Asymmetric recognition of a site. Cleavage in the neighboring 20 bp of the specific sequence.
- IIB: Cleaves DNA on both strands at the ends of the specific sequence.
- IIE: Requires two sites for recognition but cleaves only one of them.
- IIF: Requires two copies of the recognition sequence, and cleaves within both sites.

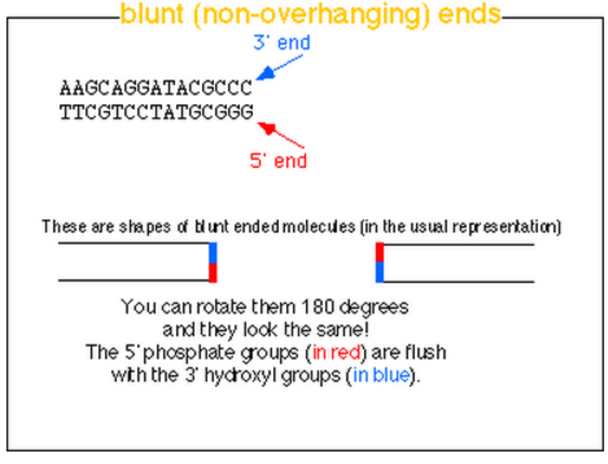


http://www.bris.ac.uk/biochemistry/halford/research.html

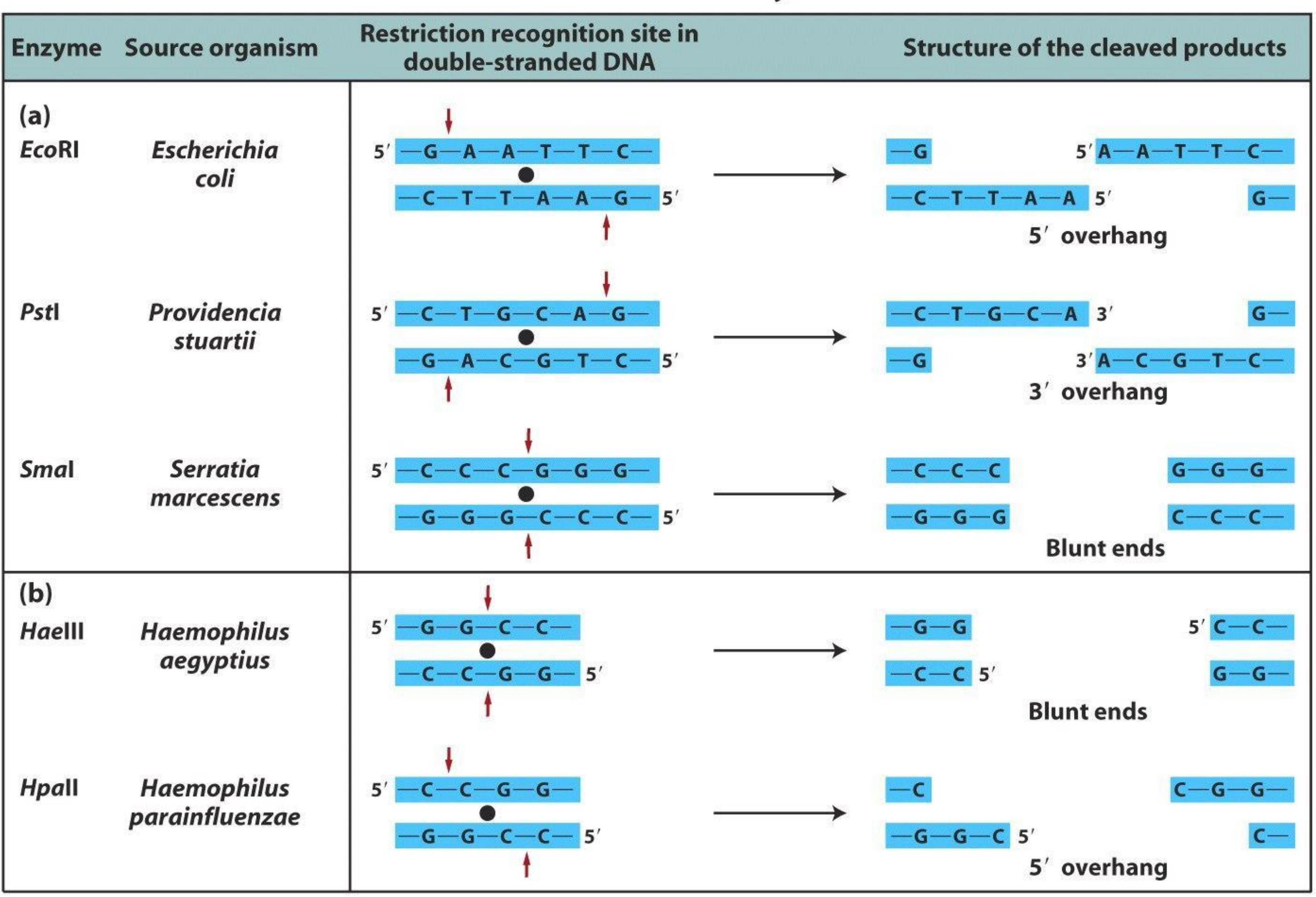
Cleavage



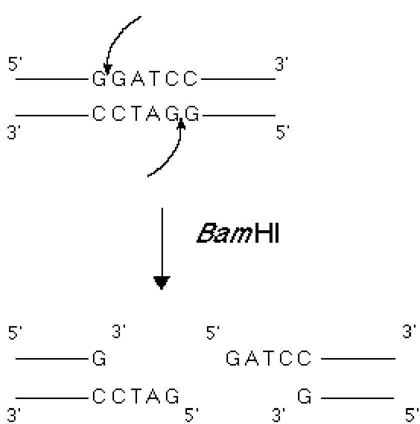




Famous enzymes



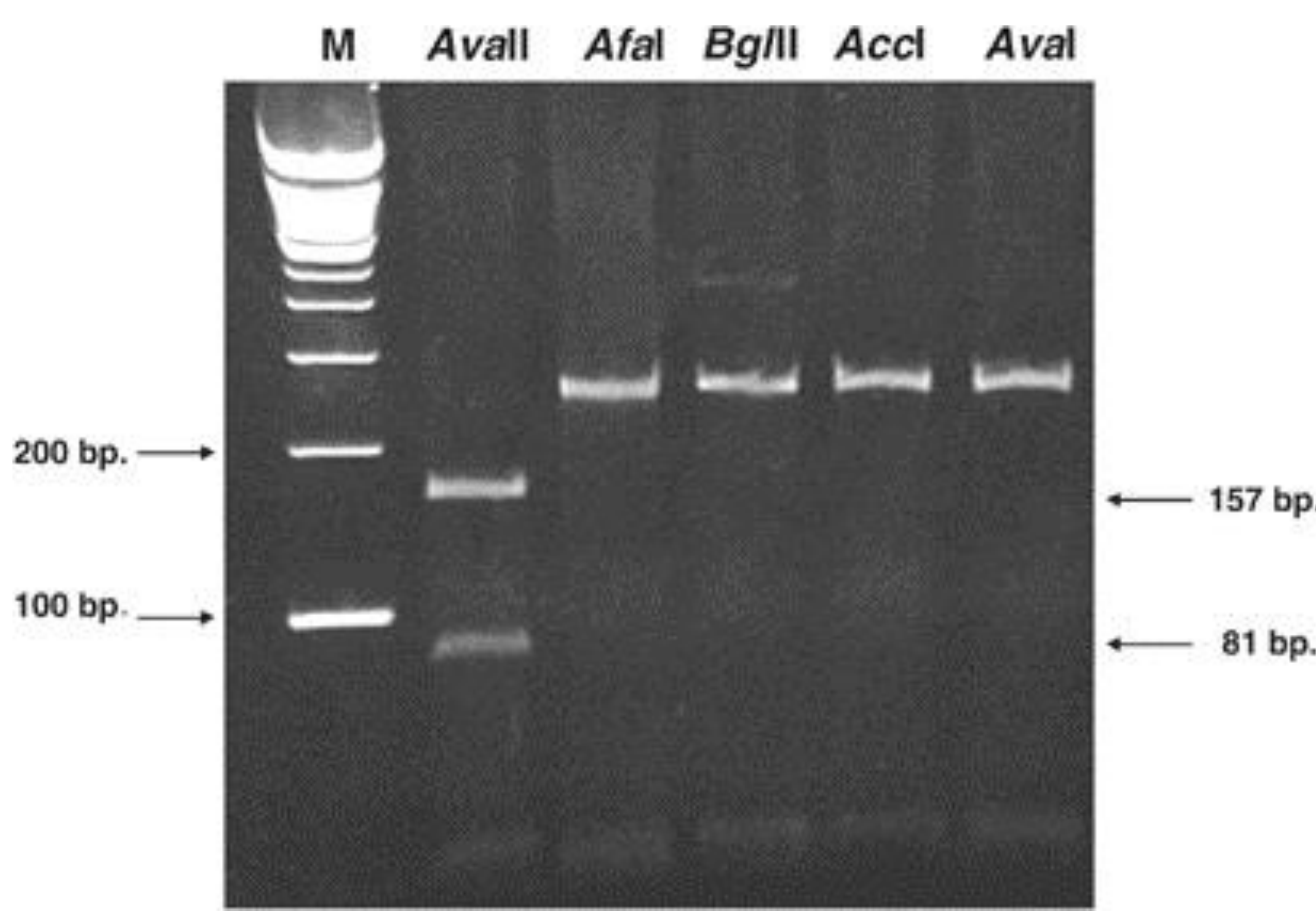
Practice!



GAGGATCCCACCAGGGTTACAGGATAGGAGTCAGGATCCAGAGGACCTAGGATACCTC
CTCCTAGGGTGGTCCCAATGTCCTATCCTCAGTCCTAGGTCTCCTGGATCCTATGGAG

How many fragments are produced?
Which length does it (do they) have?

Practice!



Digestion

A "Typical" Restriction Digest

Restriction Enzyme	10 units is sufficient, generally 1μl is used
DNA	1 μg
10X NEBuffer	5 μl (1X)
BSA	Add to a final concentration of 100 μg/ml (1X) if necessary
Total Reaction Volume	50 μl
Incubation Time	1 hour*
Incubation Temperature	Enzyme dependent

* Can be decreased by using a Time-Saver Qualified enzyme.

Star activity in some cases!

Buffer

- Use at a 1X concentration
- If required, add BSA to a final concentration of 100 μg/ml (1:100 dilution)
- Restriction enzymes that do not require BSA for optimal activity are not adversely affected if BSA is present in the reaction

Digestion

Restriction enzyme	Buffer L	Buffer M	Buffer H	Buffer A	Buffer B	Buffer S
Acc I	50	75	<5	**100**	<5	–
Acc II	5★	**100**	10	100	50	–
Acc III	<5	10	50	5★	**75**	–
Acy I	<5	10	25	<5	**100**	–
Afl II	50	50	5	**100**	25	–
Age I	75★	**100**	10	75	25	–
Alu I	100	**100**	25	150	25	–
Alw44 I	**75**	50	<5	50	25	–
Apa I	**100**	10	<5	50	<5	–
Ase I	10★	50★	**100**	25★	100	–
Ava I *Ava* I (HC)	10	**100**	10	25	50	
Ava II	75★	**100**	5	75★	50	–
Axy II	100★	**100**	50	100★	50	–
Bal I	25	10	<5	25	<5	**100**
BamH I *BamH* I (HC)	75★	100★	75	75★	**100**	–
Bcl I	100★	200★	**100**	100★	200	–
Bgl I	10★	50★	**100**	10★	25	–
Bgl II *Bgl* II (HC)	10★	75★	**100**	50★	150★	–
Bsm I	25★	75★	50	75★	**100**	–
Bsp1286 I	75	50	10	**100**	10	–
BssH II *BssH* II (HC)	50★	50	75	50★	**100**	–
BstE II *BstE* II (HC)	25★	100★	**100**	75★	100	–
BstX I	<5	75	**100**	25	100	–
Dra I *Dra* I (HC)	75	**100**	10	50	75	–
Eco0109 I	100★	**100**	10	100	50	–
EcoR I *EcoR* I (HC)	–	–	**100**	–	150★	–
EcoR II	<5	**100**	75	75	100	–
EcoR V *EcoR* V (HC)	10★	75	**100**	25	150	–
EcoT38 I *EcoT38* I (HC)	150	**100**	5	150	75	–
Fok I	200★	**100**	<5	200★	100	–
Fsp I	25	100	5	**100**	50	–
Hae II	100	**100**	25	75	50	–
Hae III *Hae* III (HC)	75	**100**	100	100	100	–

So many enzymes and buffers!

What if I want to cut with 2 (or more) enzymes?

Double digestion

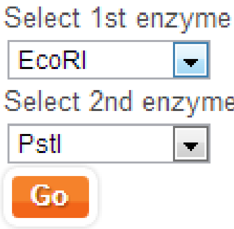
Suggested NEBuffers for Double Digestion

Enzyme		AatII	AvrII	BamHI	BglII	BsgI	EagI	EcoRI	EcoRV	HindIII	KpnI	MseI	NcoI	NdeI	NheI	NotI	PstI	PvuI	SacI	SacII	SalI	SmaI	SpeI	SphI	XbaI	XhoI	XmaI
	–	4	4	3	3	4	3	NEBuffer EcoRI	3	2	1	4	3	4	2	3	3	3	1	4	3	4	4	2	4	4	4
AatII	4	–	4	seq	seq	4	seq	seq	4	4	4	4	4	4	4	seq	4	seq	4	4	seq	4	4	4	4	4	4
AvrII	4	4	–	3	2	4	3	EcoRI	2	2	1	4	4	4	2	3	3	2	1	4	3	4	4	2	4	4	4
BamHI	3	seq	3	–	3	3	3	EcoRI	3	seq	seq	3	3	3	seq	3	3	3	seq	seq	3	seq	seq	3	3	3	seq
BglII	3	seq	2	3	–	3	3	EcoRI	3	2	2	2	3	3	2	3	3	3	2	2	3	seq	2	2	2	3	2
BsgI	4	4	4	3	3	–	seq	seq	4	2	seq	4	4	4	4	3	3	3	4	4	3	4	4	4	4	4	4
EagI	3	seq	3	3	3	seq	–	EcoRI	3	seq	seq	3	3	3	seq	3	3	3	seq	seq	3	seq	seq	3	3	3	seq
EcoRI	NEBuffer EcoRI	seq	EcoRI	EcoRI	EcoRI	seq	EcoRI	–	EcoRI	seq	1	EcoRI	EcoRI	EcoRI	1	EcoRI	EcoRI	EcoRI	1	EcoRI	EcoRI	seq	EcoRI	EcoRI	seq	EcoRI	seq
EcoRV	3	4	2	3	3	4	3	EcoRI	–	2	2	2	3	2	2	3	3	3	2	2	3	4	2	2	2	3	4
HindIII	2	4	2	seq	2	2	seq	seq	2	–	2	2	2	2	2	2	2	2	2	2	seq	4	2	2	2	2	seq
KpnI	1	4	1	seq	2	seq	seq	1	2	2	–	1	1	1	1	2	1	2	1	4	seq	seq	1	1	2	1	4
MseI	4	4	4	3	2	4	3	EcoRI	2	2	1	–	4	4	2	2	3	3	4	4	3	4	4	2	4	4	4
NcoI	3	4	4	3	3	4	3	EcoRI	3	2	1	4	–	4	2	3	3	3	1	4	3	4	4	2	4	4	4
NdeI	4	4	4	3	3	4	3	EcoRI	2	2	1	4	4	–	4	3	3	3	4	4	3	4	4	2	4	4	4
NheI	2	4	2	seq	2	4	seq	1	2	2	1	2	2	4	–	2	2	2	1	4	seq	4	2	2	2	2	4
NotI	3	seq	3	3	3	3	3	EcoRI	3	2	2	2	3	3	2	–	3	3	2	2	3	seq	2	2	3	3	2
PstI	3	4	3	3	3	3	3	EcoRI	3	2	1	3	3	3	2	3	–	3	1	2	3	4	2	2	3	3	4
PvuI	3	seq	2	3	3	3	3	EcoRI	3	2	2	3	3	3	2	3	3	–	2	2	3	seq	2	2	3	3	2
SacI	1	4	1	seq	2	4	seq	1	2	2	1	4	1	4	1	2	1	2	–	4	seq	4	1	1	4	1	4
SacII	4	4	4	seq	2	4	seq	EcoRI	2	2	4	4	4	4	4	2	2	2	4	–	seq	4	4	4	4	4	4
SalI	3	seq	3	3	3	3	3	EcoRI	3	seq	seq	3	3	3	seq	3	3	3	seq	seq	–	seq	seq	3	3	3	seq
SmaI	4	4	4	seq	seq	4	seq	seq	4	4	seq	4	4	4	4	seq	4	seq	4	4	seq	–	4	4	4	4	4
SpeI	4	4	4	seq	2	4	seq	EcoRI	2	2	1	4	4	4	2	2	2	2	1	4	seq	4	–	2	4	4	4
SphI	2	4	2	3	2	4	3	EcoRI	2	2	1	2	2	2	2	2	2	2	1	4	3	4	2	–	2	2	4
XbaI	4	4	4	3	2	4	3	seq	2	2	2	4	4	4	2	3	3	3	4	4	3	4	4	2	–	4	4
XhoI	4	4	4	3	3	4	3	EcoRI	3	2	1	4	4	4	2	3	3	3	1	4	3	4	4	2	4	–	4
XmaI	4	4	4	seq	2	4	seq	seq	4	seq	4	4	4	4	4	2	4	2	4	4	seq	4	4	4	4	4	–

Find the best buffer that suits your combination of enzymes with DoubleDigest Finder (NEB) or similars.

https://www.neb.com/tools-and-resources/interactive-tools/double-digest-finder

Double-Digest Finder



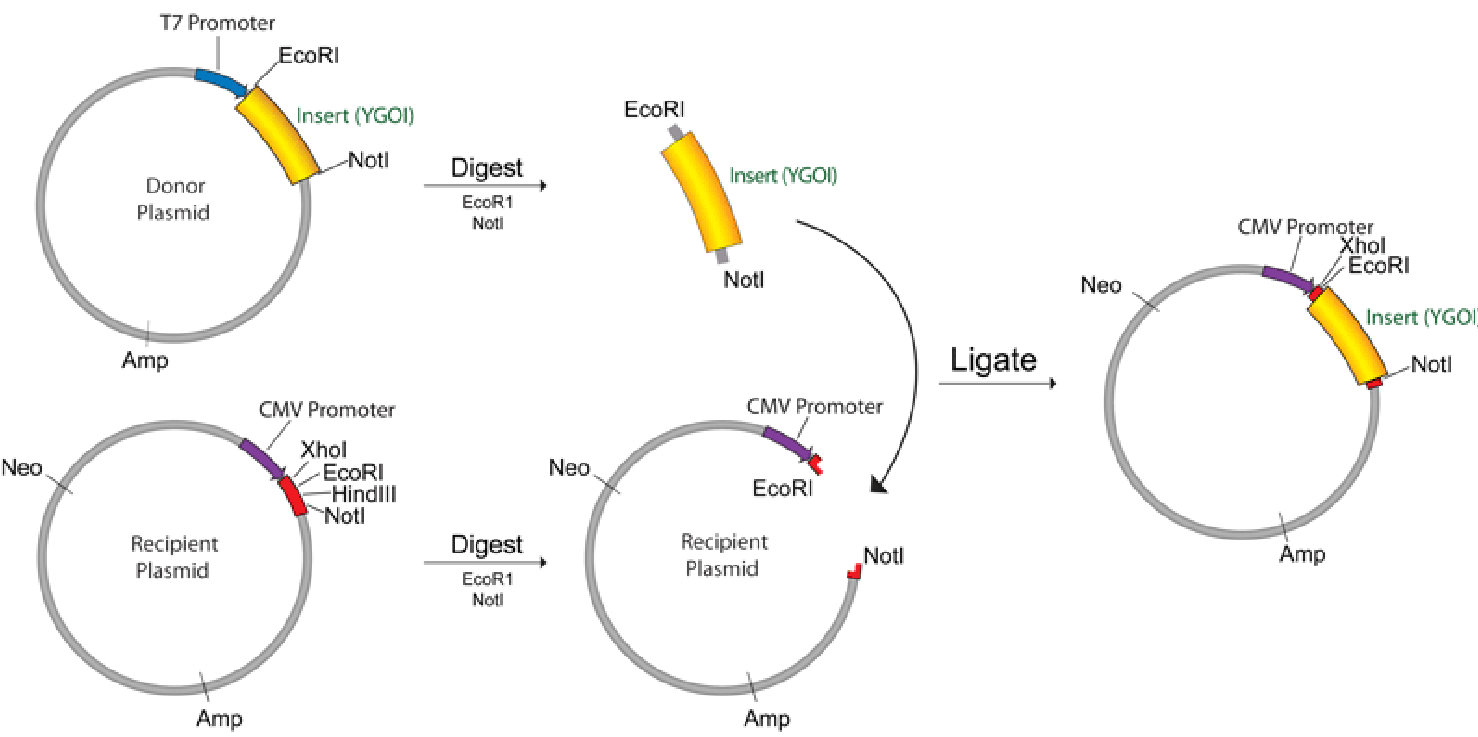
Enzyme	Cat #	Temp	Supplied NEBuffer	Supplements	% Activity in NEBuffer			
				SAM	1.1	2.1	3.1	CutSmart
EcoRI	R0101	37°C	NEBuffer EcoRI	no	25	100*	50	50*
PstI	R0140	37°C	NEBuffer 3.1	no	75	75	100	50*

Double Digest Recommendations for EcoRI + PstI:

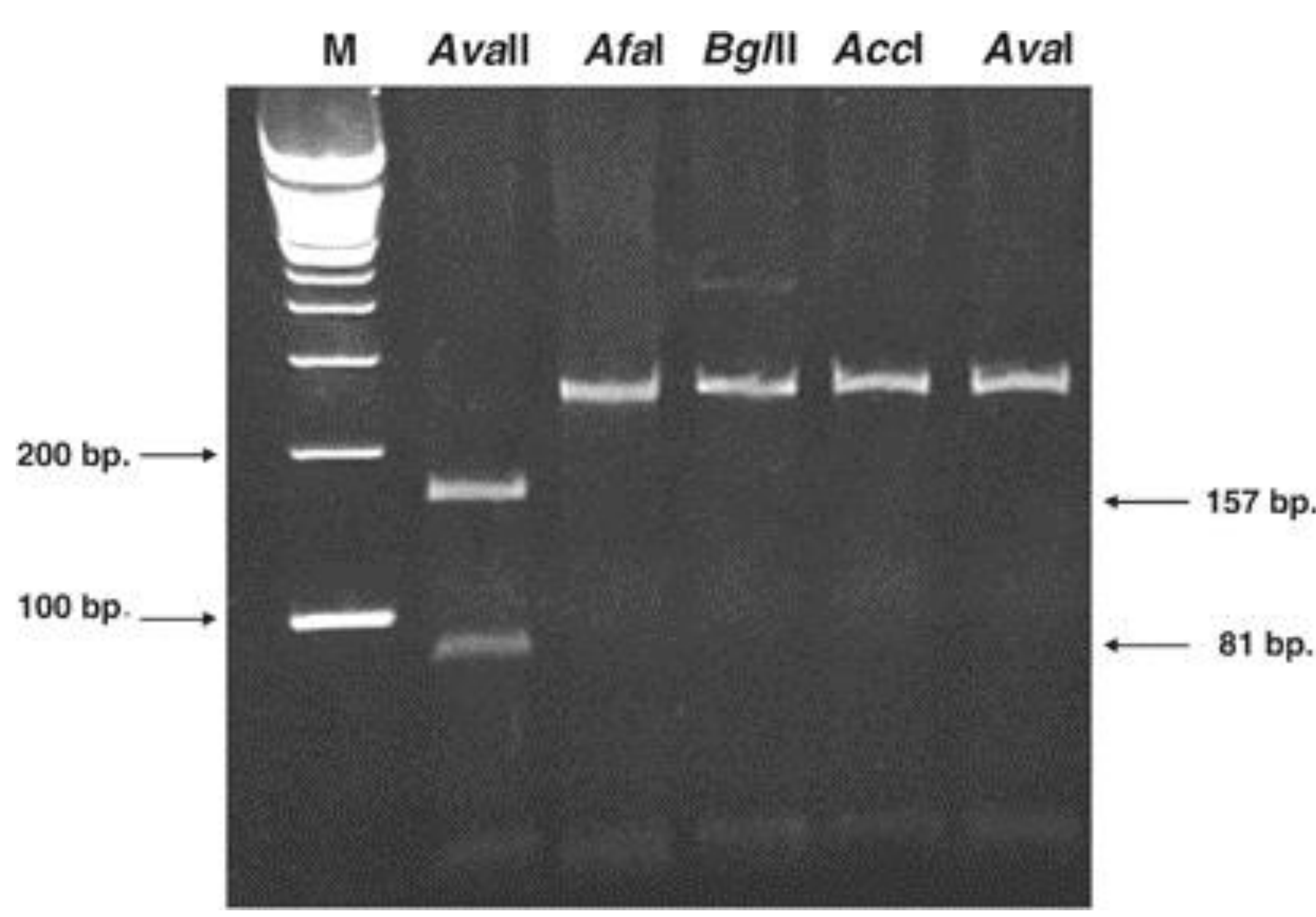
- Digest in NEBuffer 3.1 at 37°C.
- *At least one enzyme has < 100% activity in this buffer, so additional units of enzyme and/or longer incubation time may be necessary.*
- *EcoRI has a High Fidelity version EcoRI-HF™ (R3101)*
  PstI has a High Fidelity version PstI-HF™ (R3140)
  High Fidelity (HF) Restriction Enzymes have been engineered for reduced star activity and have 100% activity in CutSmart Buffer which may simplify your double digest.
- ** May exhibit star activity in this buffer.*

Learn more about improvements to the NEB Restriction Enzyme Buffer System

What is it used for?



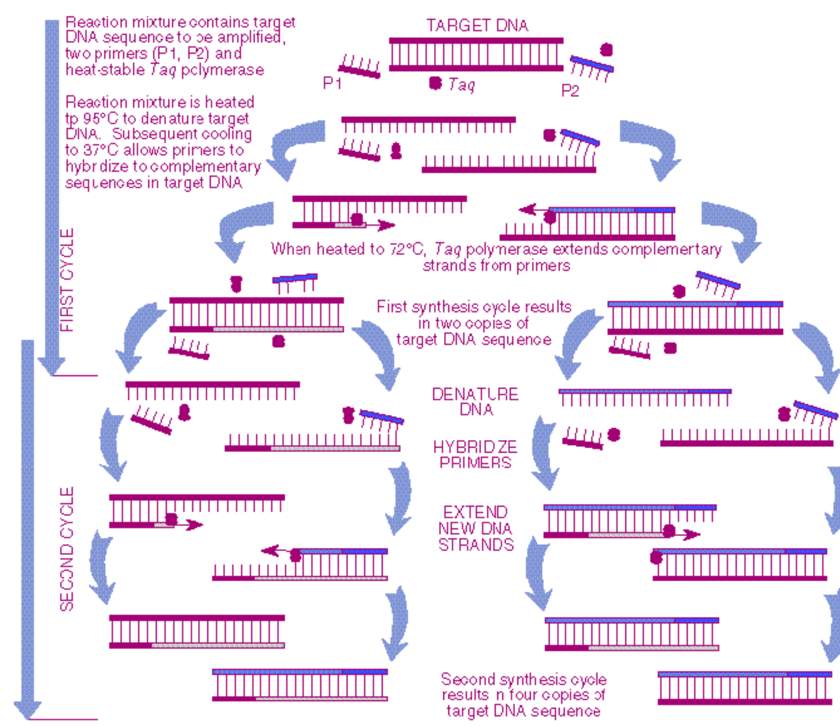
What is it used for?



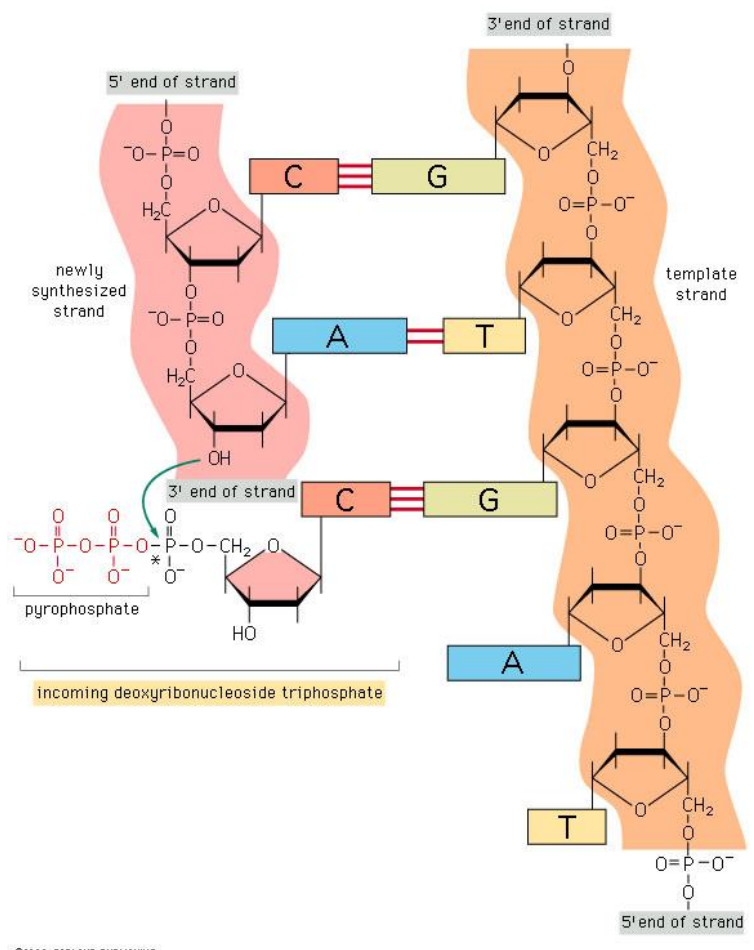
PCR

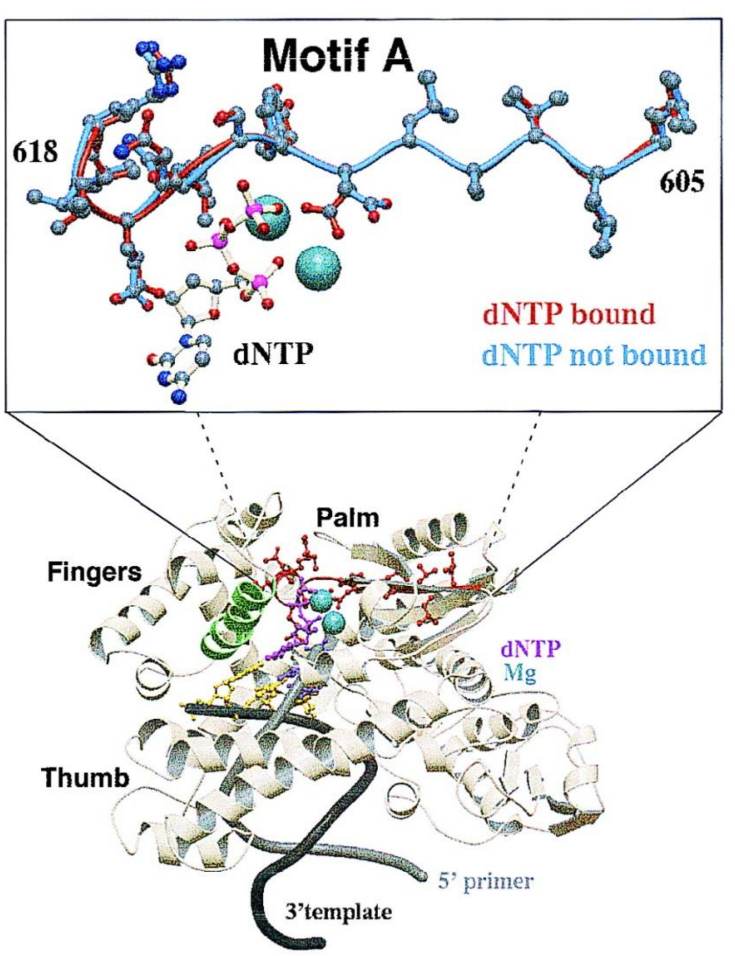
What?

- *In vitro* enzymatic reaction for copying a particular sequence of DNA
- Doesn't need high quantities of DNA to work.
- Lots and lots of applications.
- Detection, cloning, sequencing…

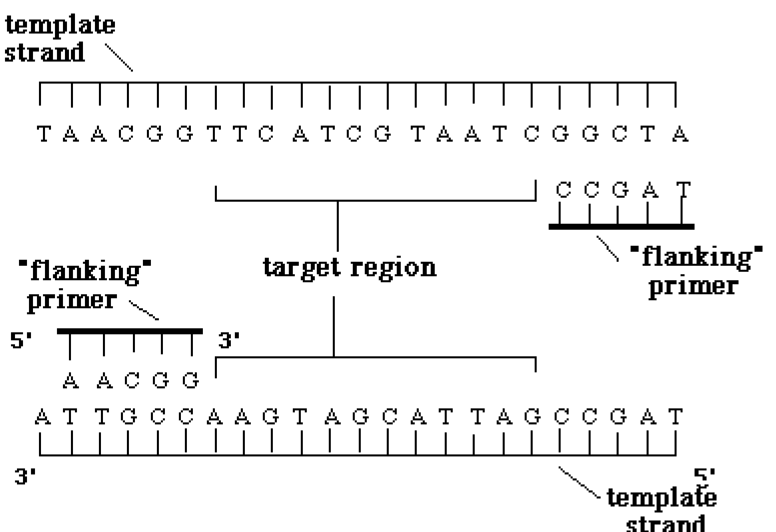


Theory behind





Theory behind



Who?

The Nobel Prize in Chemistry 1993

Nobel Prize Award Ceremony

Kary B. Mullis

Michael Smith

Kary B. Mullis

Michael Smith

The Nobel Prize in Chemistry 1993 was awarded *"for contributions to the developments of methods within DNA-based chemistry"* jointly with one half to Kary B. Mullis *"for his invention of the polymerase chain reaction (PCR) method"* and with one half to Michael Smith *"for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies"*.

Photos: Copyright © The Nobel Foundation

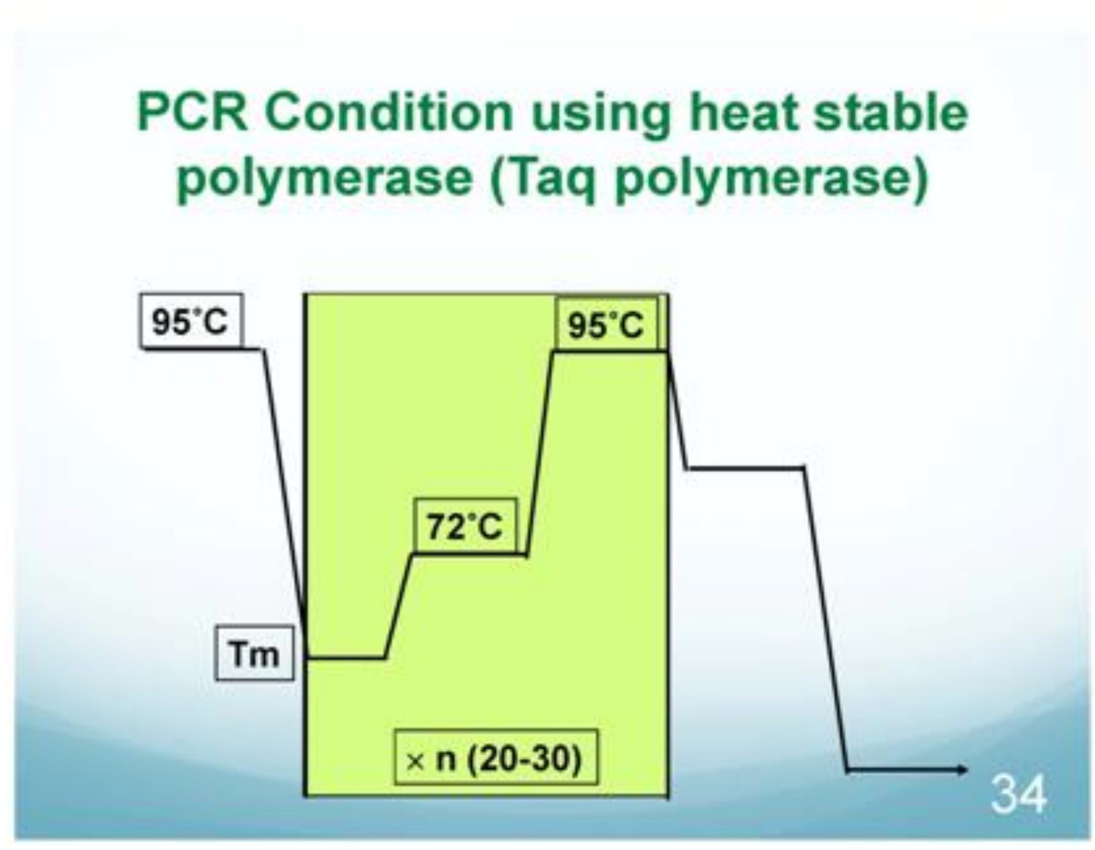
How does it work?

- http://www.youtube.com/watch?v=_YgXcJ4n-kQ

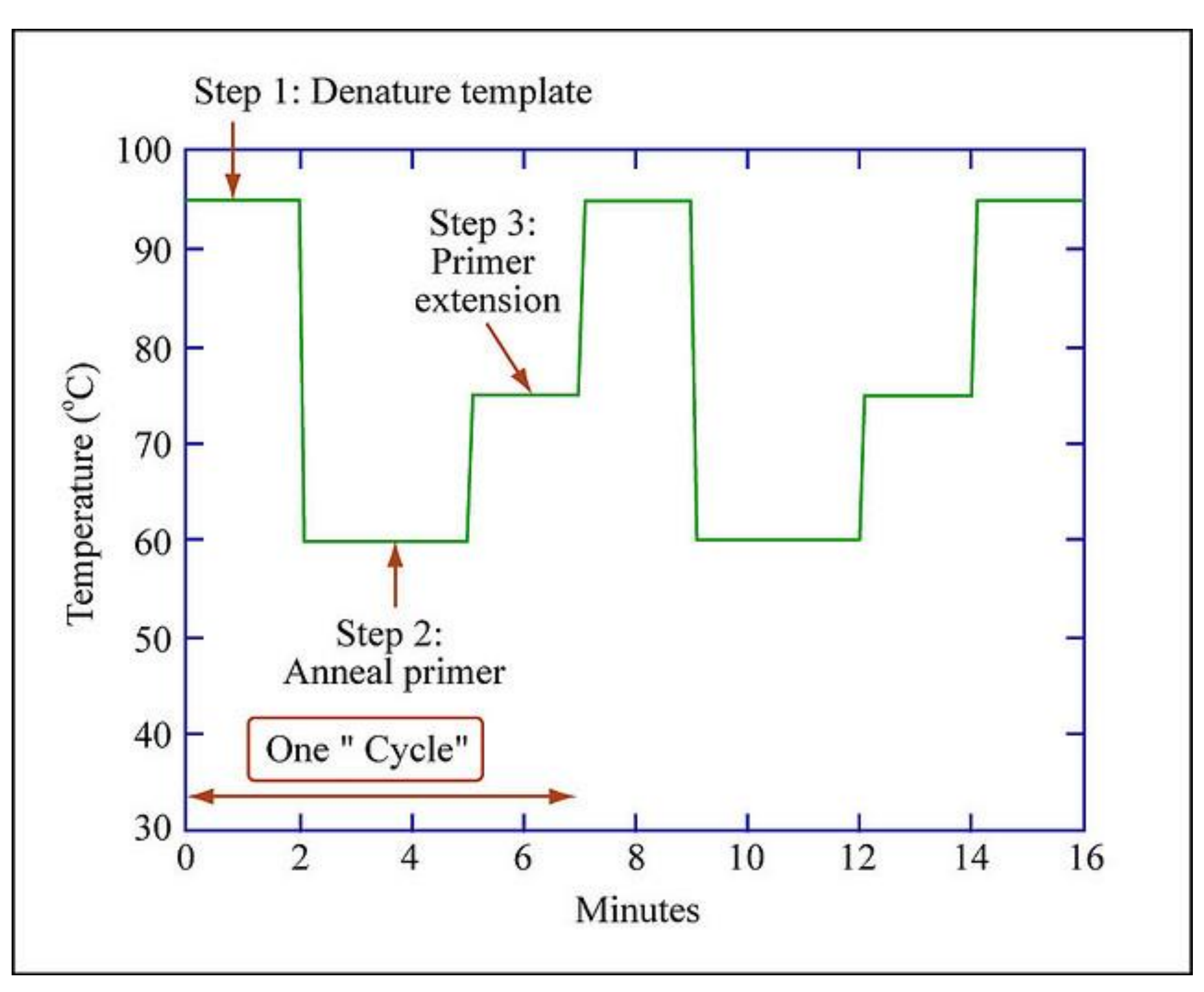
Typical PCR reaction

Reactivo	Concentración del stock	Concentración reacción
Agua		
$MgCl_2$	25 mM	2,5 mM
Buffer	10X	1X
dNTPs	10 mM	0,2 mM
352-F	10 µM	0,3 µM
975-R	10 µM	0,3 µM
Taq polimerasa	5 U/µl	1,5 U
Extracto crudo		

Typical PCR reaction



Typical PCR reaction



Tips and tricks

- Reagent concentrations - "less is usually better" (more specific)
  - primers: final concentration 0.1-1.0 uM
  - $MgCl_2$: final concentration 1.0-4.0 mM (depends on Taq used)
  - dNTPs: final concentration 0.2 mM each dNTP (depends on Taq used)
    - Note: sensitive to repeated freeze/thaws
- Vortex or finger-flick reagents to mix well before use
- Annealing temperature and step times are important
- Hot starts improve reaction efficiency (fewer primer-dimers)
  - Manual: add Taq to tubes in thermalcycler at 94°C (or $MgCl_2$ or dNTPs)
  - TaqStart Antibody (Clontech)
  - FastStart Taq (Roche)

http://genome-lab.ucdavis.edu/Protocols/pcr_tips/pcr_tips_and_tricks.htm

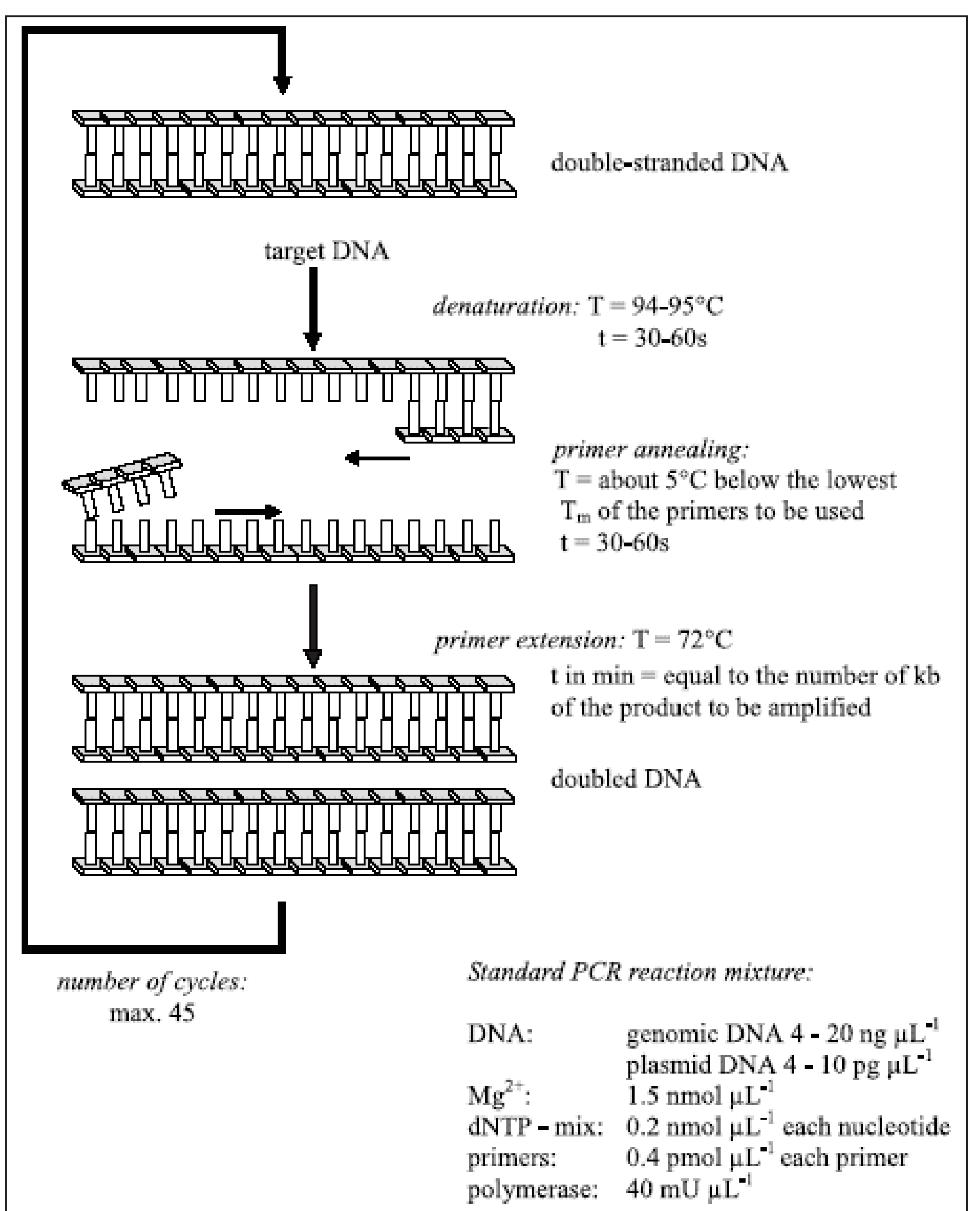
Tips and tricks

- Always check program on Thermal Cycler
- Run negative control(s) to check for contamination
- Make a flow chart of what tried and in what order
- Run a positive control (a sample known to amplify well)
- Always run a ladder on gel (will indicate whether failed PCR or failed detection system)
- Additives for fragments that are very long, G-C rich or prone to secondary structure
  - Glycerol, Formamide, NMP : Allow lower denaturing and annealing temperatures by a few degrees.
  - DMSO decreases incidence of secondary structure

http://genome-lab.ucdavis.edu/Protocols/pcr_tips/pcr_tips_and_tricks.htm

Primers

Primers



Primers

- General Guidelines

http://www.premierbiosoft.com/tech_notes/PCR_Primer_Design.html

- Always write the primer from 5’ to 3’ (Reverse complement on Rv)

Primers

Home | Web Interface | Download | History

Primer3 is a widely used program for designing PCR primers (PCR = "Polymerase Chain Reaction"). PCR is an essential and ubiquitous tool in genetics and molecular biology. Primer3 can also design hybridization probes and sequencing primers.

PCR is used for many different goals. Consequently, primer3 has many different input parameters that you control and that tell primer3 exactly what characteristics make good primers for your goals.

Primer3 -- the C code, the web interface, and the documentation -- are an open source, community-development project hosted by SourceForge.

SOURCEFORGE.NET®

last updated: 10/26/2009 9:46 // xhtml // css

COI – *Homo sapiens*

Primers

- For RT-PCR

In silico PCR amplification

[Input primers in fasta format]

Primer 1[1] 5'- -3' C

Primer 2[1] 5'- -3' C

Migroorganism

Pseudomonas aeruginosa

☐ Include plasmids (if available)

Allow 0 mismatches, but in 1 nucleotides in 3' end

Maximum length of bands

3000 nucleotides

[1] Degenerated nucleotides are allowed; A+T+G+C must be 10 or more.

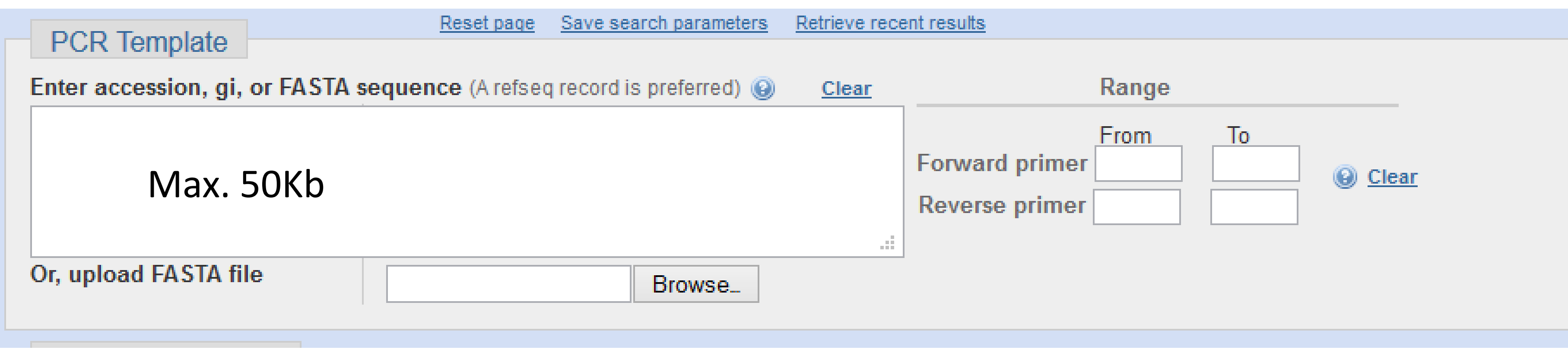
Info

Amplify Reset

Suggestions are welcome

Primer-BLAST

- Two modules
  - Generate candidate primers
    - Primer 3
  - Candidate checking
    - Search against nucleotide databases. Looks for specificity.



Primer-BLAST

Primer Parameters

Use my own forward primer (5'->3' on plus strand) Clear

Use my own reverse primer (5'->3' on minus strand) Clear

	Min	Max
PCR product size	70	1000
# of primers to return	5	

	Min	Opt	Max	Max T_m difference
Primer melting temperatures (T_m)	57.0	60.0	63.0	3

Primer-BLAST

Exon/intron selection

A refseq mRNA sequence as PCR template input is required for options in the section

Exon junction span — No preference

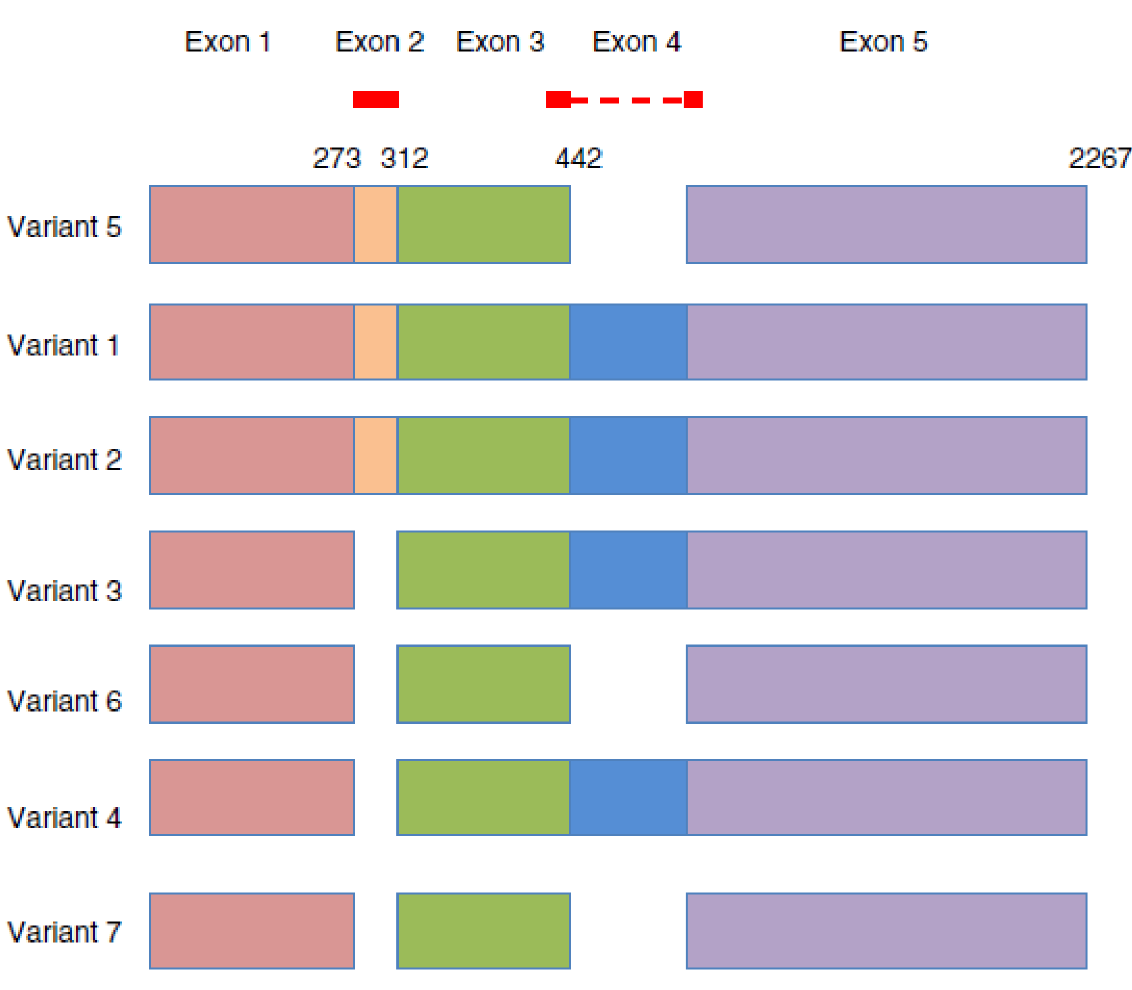
Exon junction match — Exon at 5' side | Exon at 3' side

Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction

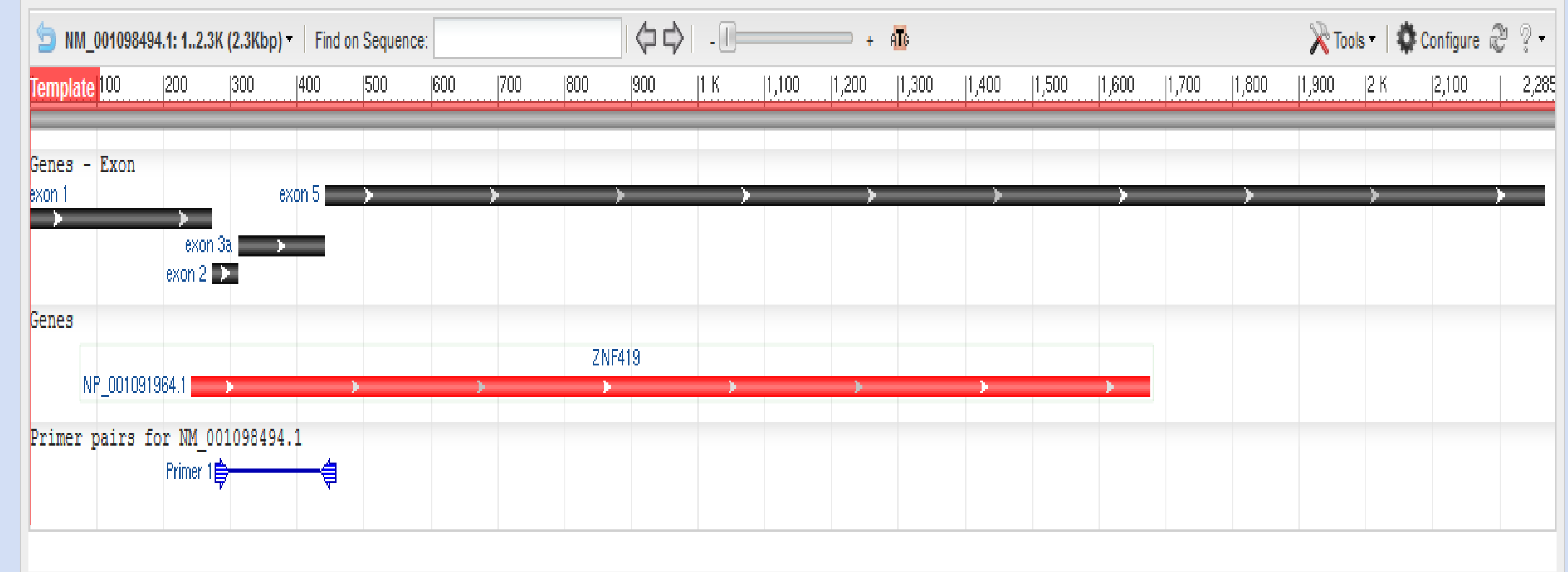
Intron inclusion — Primer pair must be separated by at least one intron on the corresponding genomic DNA

Intron length range — Min | Max

Homo sapiens zinc finger protein 419 (ZNF419), transcript variant 5, mRNA



Graphical view of primer pairs



Detailed primer reports

Primer pair 1

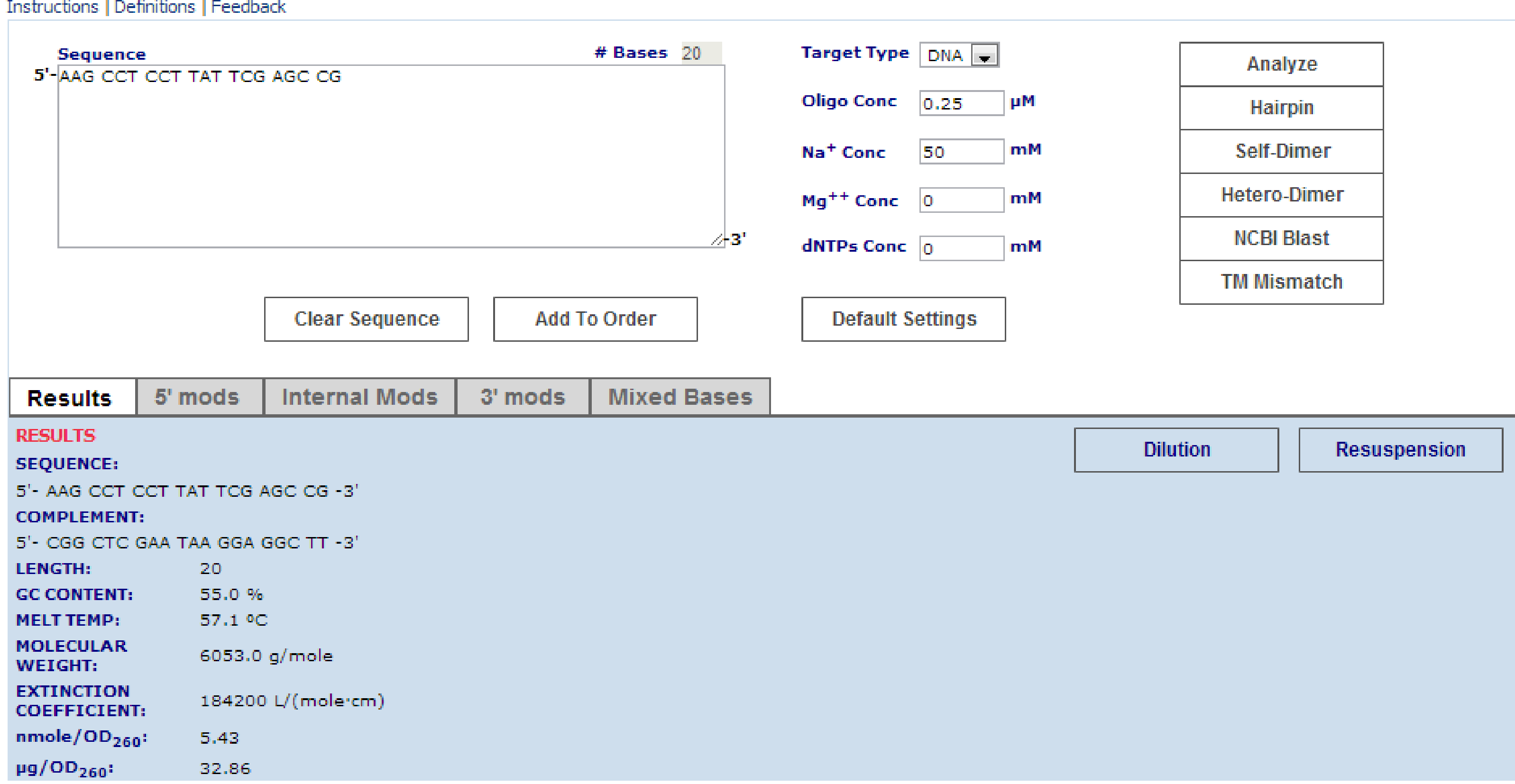
	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	CTGTGGCTGCAGACTTGCTTA	Plus	21	278	298	60.88	52.38	6.00	2.00
Reverse primer	CTCCATGCCAACAACCCAGAGA	Minus	22	457	436	62.53	54.55	4.00	0.00
Product length	180								
Exon junction	442/443 (reverse primer) on template NM_001098494.1								

Products on intended target

>NM_001098494.1 Homo sapiens zinc finger protein 419 (ZNF419), transcript variant 5, mRNA

Search parameters and other details	
Number of Blast hits analyzed	492848
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Misprimed product size deviation	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	1000
Min PCR product size	70
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes

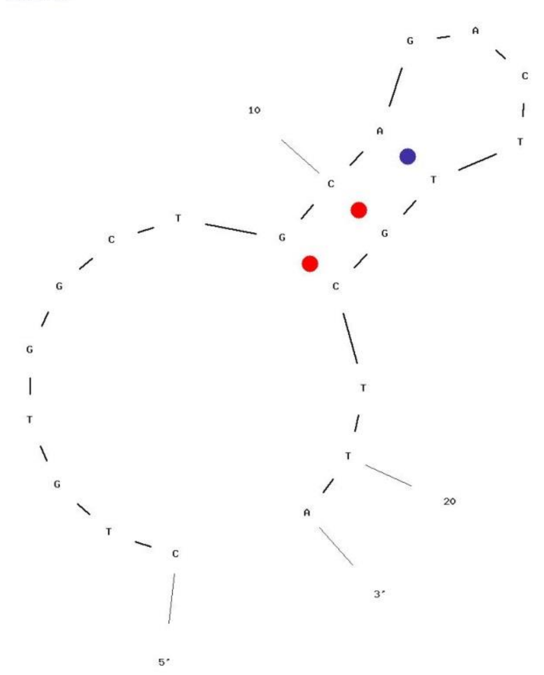
OligoAnalyzer



- http://www.idtdna.com/analyzer/applications/oligoanalyzer/

Output of sir_graph (C)
mfold_util 4.5

Created Fri Feb 15 22:28:22 2013



dG = -1.002 z4odxyyddtl2wi0oogjldlpz_102822P_1

Structure Name	Image	ΔG (kcal.mole^{-1})	Tm (°C)
1		-1.98	45.1
2		-1	37.6

HOMO-DIMER ANALYSIS

Dimer Sequence

```
5'- AAGCCTCCTTATTCGAGCCG -3'
```

Maximum Delta G -42.1 kcal/mole

Delta G -6.76 kcal/mole
Base Pairs 4

```
5' AAGCCTCCTTATTCGAGCCG
              ||||
3'         GCCGAGCTTATTCCTCCGAA
```

HETERO-DIMER ANALYSIS

Primary Sequence

```
5'- AAGCCTCCTTATTCGAGCCG -3'
```

Secondary Sequence

```
5'- AGAATGGGGTCTCCTCCTCC -3'
```

Maximum Delta G -42.1 kcal/mole

Delta G -4.99 kcal/mole
Base Pairs 4

```
5'      AAGCCTCCTTATTCGAGCCG
        : :   ::  ||||
3' CCTCCTCCTCTGGGGTAAGA
```

The mfold Web Server

☑ Pair 1: Primer

Left Primer 1: AAGCCTCCTTATTCGAGCCG

Start: 99 Length: 20 bp Tm: 59.9 C GC: 55.0 % Any: 0.0 End: 0.0 TB: 9.0 HP: 0.0 3' Stab: 5.5 Penalty: 0.105

Structure 1 Folding bases 1 to 20 of 13Apr06-16-38-16-8acbee3e42
dG = 0.19 dH = -16.70 dS = -54.46 T_m = 33.5 ℃

```
----------|   C
          AAG C
          TTC T
GCCGAGCTTA^   C
.        10
```

Structure 2 Folding bases 1 to 20 of 13Apr06-16-38-16-8acbee3e42
dG = 0.47 dH = -22.00 dS = -72.45 T_m = 30.5 ℃

```
        10
AAGC|  CTT
    CTC   \
    GAG   A
GCC-^  CTT
.
```

>gi|251831106:5904-7445 Homo sapiens mitochondrion, complete genome

ATGTTCGCCGACCGTTGACTATTCTCTACAAACCACAAAGACATTGGAACACTATACCTATTA
TTCGGCGCATGAGCTGGAGTCCTAGGCACAGCTCTAAGCCTCCTTATTCGAGCCGAGCTGGGC
CAGCCAGGCAACCTTCTAGGTAACGACCACATCTACAACGTTATCGTCACAGCCCATGCATTT
GTAATAATCTTCTTCATAGTAATACCCATCATAATCGGAGGCTTTGGCAACTGACTAGTTCCC
CTAATAATCGGTGCCCCCGATATGGCGTTTCCCCGCATAAACAACATAAGCTTCTGACTCTTA
CCTCCCTCTCTCCTACTCCTGCTCGCATCTGCTATAGTGGAGGCCGGAGCAGGAACAGGTTGA
ACAGTCTACCCTCCCTTAGCAGGGAACTACTCCCACCCTGGAGCCTCCGTAGACCTAACCATC
TTCTCCTTACACCTAGCAGGTGTCTCCTCTATCTTAGGGGCCATCAATTTCATCACAACAATT
ATCAATATAAAACCCCCTGCCATAACCCAATACCAAACGCCCCTCTTCGTCTGATCCGTCCTA
ATCACAGCAGTCCTACTTCTCCTATCTCTCCCAGTCCTAGCTGCTGGCATCACTATACTACTA
ACAGACCGCAACCTCAACACCACCTTCTTCGACCCCGCCGGAGGAGGAGACCCCATTCTATAC
CAACACCTATTCTGATTTTTCGGTCACCCTGAAGTTTATATTCTTATCCTACCAGGCTTCGGA
ATAATCTCCCATATTGTAACTTACTACTCCGGAAAAAAAGAACCATTTGGATACATAGGTATG
GTCTGAGCTATGATATCAATTGGCTTCCTAGGGTTTATCGTGTGAGCACACCATATATTTACA
GTAGGAATAGACGTAGACACACGAGCATATTTCACCTCCGCTACCATAATCATCGCTATCCCC
ACCGGCGTCAAAGTATTTAGCTGACTCGCCACACTCCACGGAAGCAATATGAAATGATCTGCT
GCAGTGCTCTGAGCCCTAGGATTCATCTTTCTTTTCACCGTAGGTGGCCTGACTGGCATTGTA
TTAGCAAACTCATCACTAGACATCGTACTACACGACACGTACTACGTTGTAGCCCACTTCCAC
TATGTCCTATCAATAGGAGCTGTATTTGCCATCATAGGAGGCTTCATTCACTGATTTCCCCTA
TTCTCAGGCTACACCCTAGACCAAACCTACGCCAAAATCCATTTCACTATCATATTCATCGGC
GTAAATCTAACTTTCTTCCCACAACACTTTCTCGGCCTATCCGGAATGCCCCGACGTTACTCG
GACTACCCCGATGCATACACCACATGAAACATCCTATCATCTGTAGGCTCATTCATTTCTCTA
ACAGCAGTAATATTAATAATTTTCATGATTTGAGAAGCCTTCGCTTCGAAGCGAAAAGTCCTA
ATAGTAGAAGAACCCTCCATAAACCTGGAGTGACTATATGGATGCCCCCCACCCTACCACACA
TTCGAAGAACCCGTATACATAAAATCTAGA

What ifs…

I’m constrained to amplify ends in a sequence?

Want to add restriction sites for cloning?

I need to introduce a mutation on a sequence?

We are in an iGEM project?

Fusion PCR

Theory behind

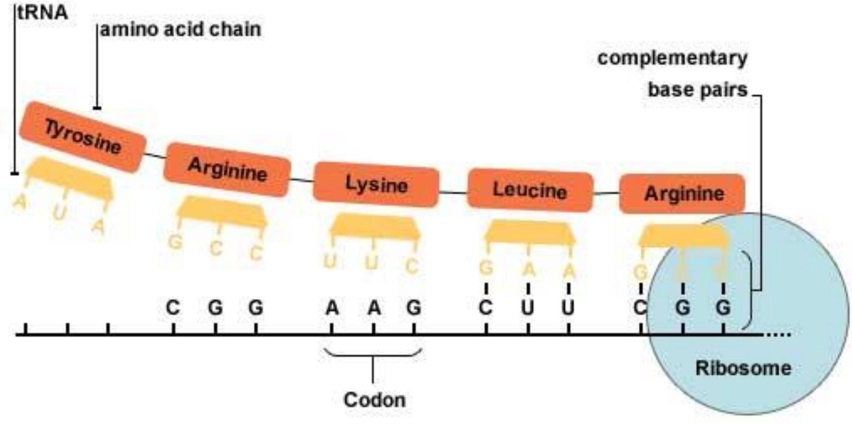
	U	C	A	G	
U	Phe	Ser	Tyr	Cys	U
	Phe	Ser	Tyr	Cys	C
	Leu	Ser	STOP	STOP	A
	Leu	Ser	STOP	Trp	G
C	Leu	Pro	His	Arg	U
	Leu	Pro	His	Arg	C
	Leu	Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ile	Thr	Asn	Ser	U
	Ile	Thr	Asn	Ser	C
	Ile	Thr	Lys	Arg	A
	Met	Thr	Lys	Arg	G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	Glu	Gly	A
	Val	Ala	Glu	Gly	G

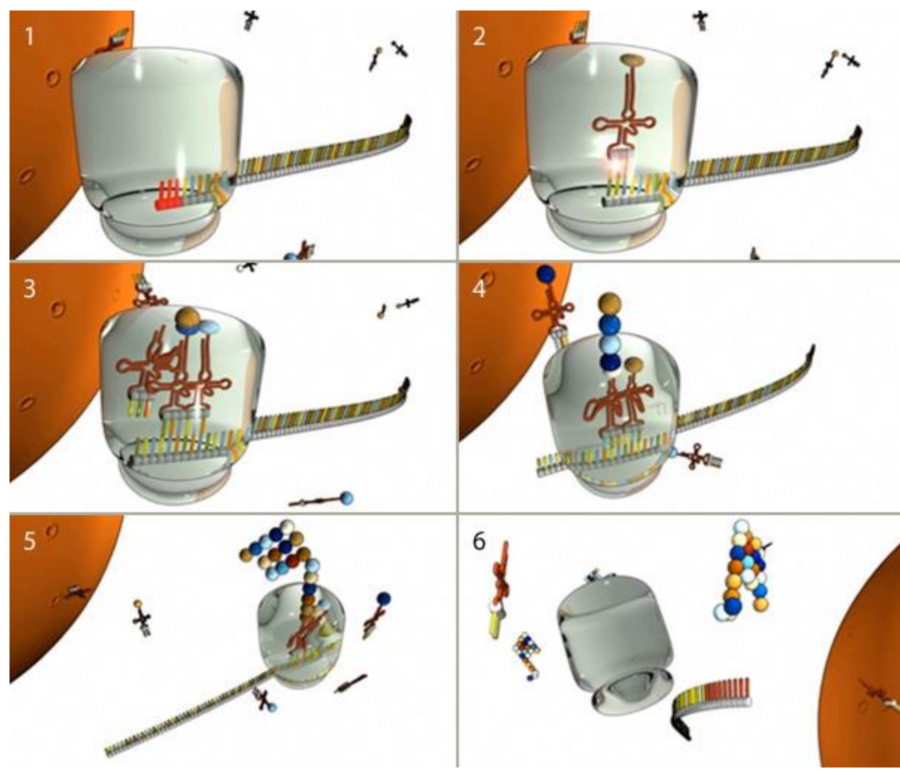
Theory behind

IUPAC for DNA

A	adenosine	S	G C (strong)
C	cytidine	W	A T (weak)
G	guanine	B	C G T (not A)
T	thymidine	D	A G T (not C)
U	uridine	H	A C T (not G)
R	G A (purine)	V	A C G (not T)
Y	T C (pyrimidine)	N	A C G T (any)
K	G T (keto)	–	gap
M	A C (amino)		

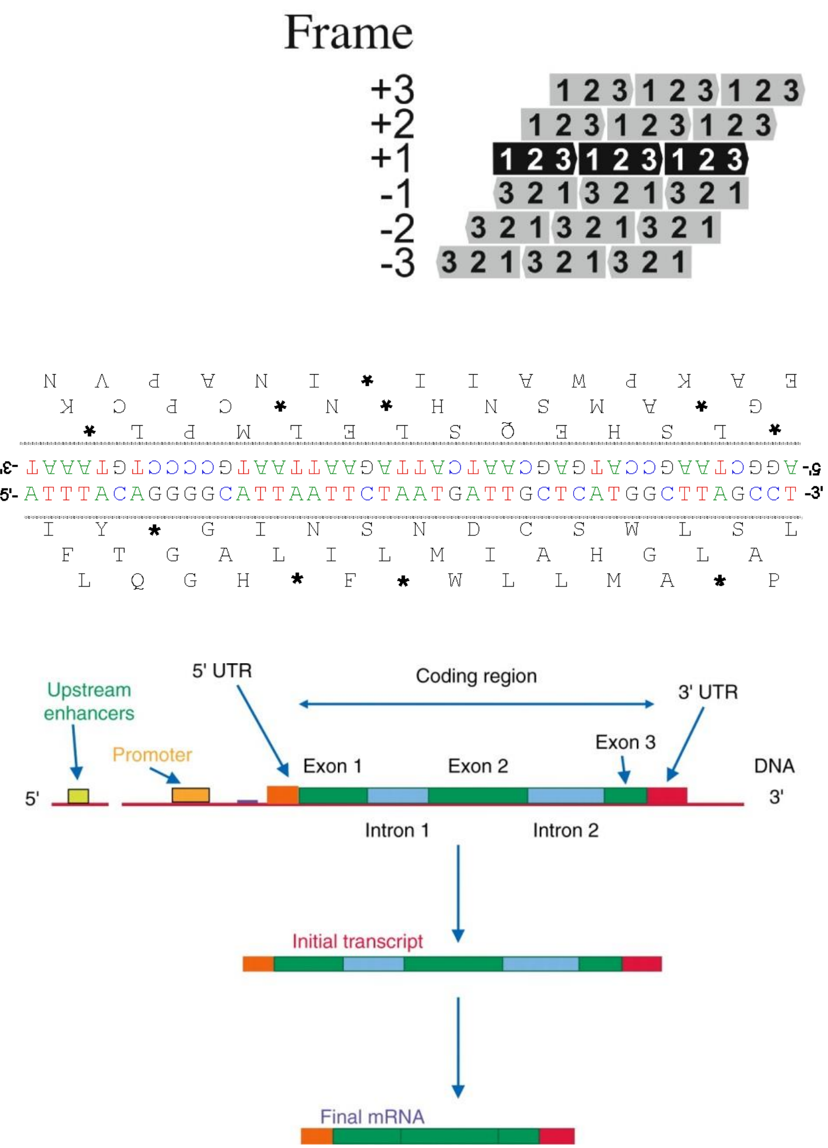
Theory behind





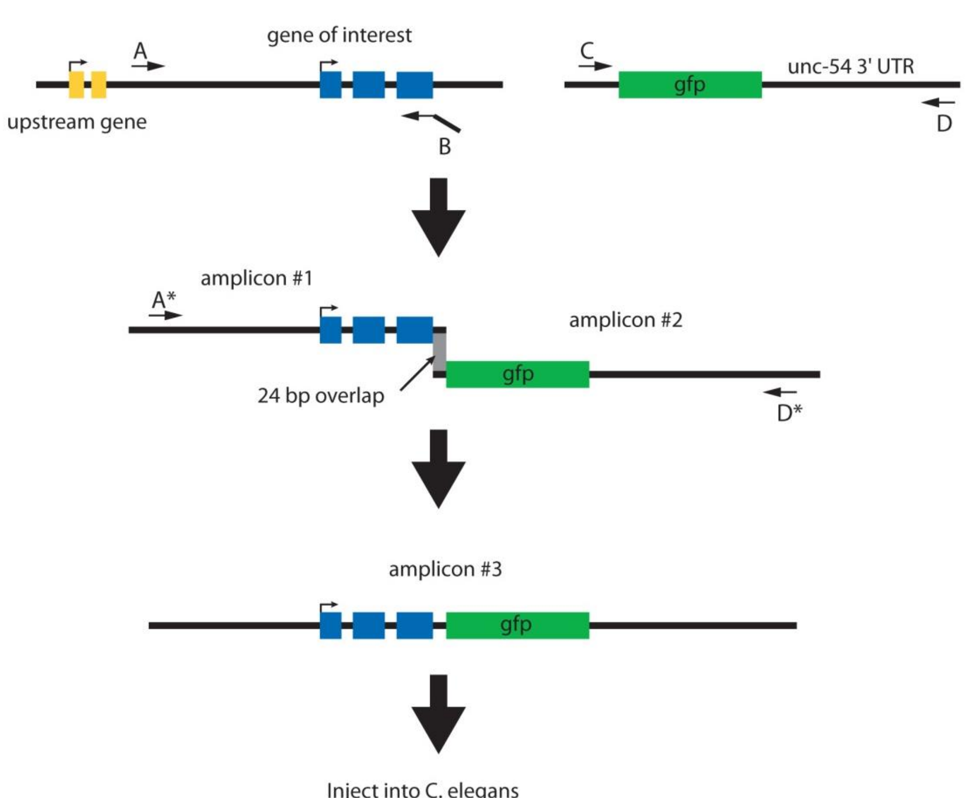
Genes: ORF vs. CDS

- Gene: Molecular unit of heredity… Sequence that origins a coding RNA or ncRNA.
- ORF: Nucleotidic sequence from a start codon to an end codon. Potential CDS.
- CDS: Actual sequence to be translated.



What is it used for?

- Artificial constructs of DNA.
- Directed mutations
- Bind protein domains, peptidic chimaeras… Playing LEGO with CDSs.
- Facilitar procesos de clonación y expresión de proteínas.



What is it used for?

Yeast
Yeast 2002; **19**: 141–149.
DOI: 10.1002/yea.806

Yeast Functional Analysis Report

Marker-fusion PCR for one-step mutagenesis of essential genes in yeast

Ana A. Kitazono[1,2], Brian T. D. Tobe[1,3], Helen Kalton[1], Noam Diamant[1] and Stephen J. Kron[1,2,3*]
[1] *Center for Molecular Oncology, University of Chicago, Chicago, IL 60637, USA*
[2] *Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA*
[3] *Committee on Cancer Biology, University of Chicago, Chicago, IL 60637, USA*

Journal of Virological Methods 108 (2003) 67–74

www.elsevier.com/locate/jviromet

A rapid and convenient variant of fusion-PCR to construct chimeric flaviviruses

Nathalie Charlier[a], Richard Molenkamp[b], Pieter Leyssen[a], Anne-Mieke Vandamme[c], Erik De Clercq[a], Peter Bredenbeek[b], Johan Neyts[a,*]

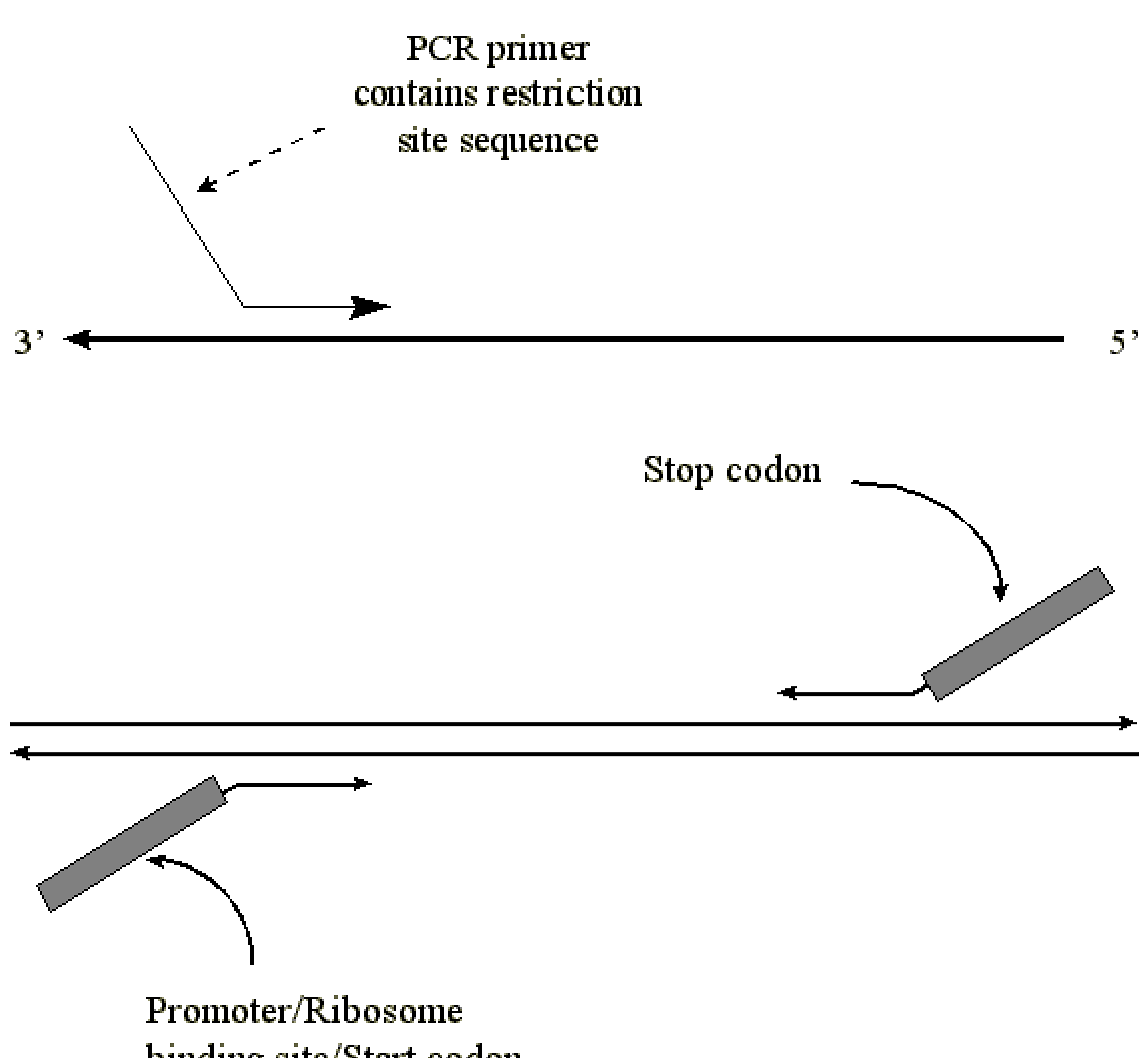
2624–2636 *Nucleic Acids Research, 2010, Vol. 38, No. 8* *Published online 12 April 2010*
doi:10.1093/nar/gkq179

In-Fusion BioBrick assembly and re-engineering

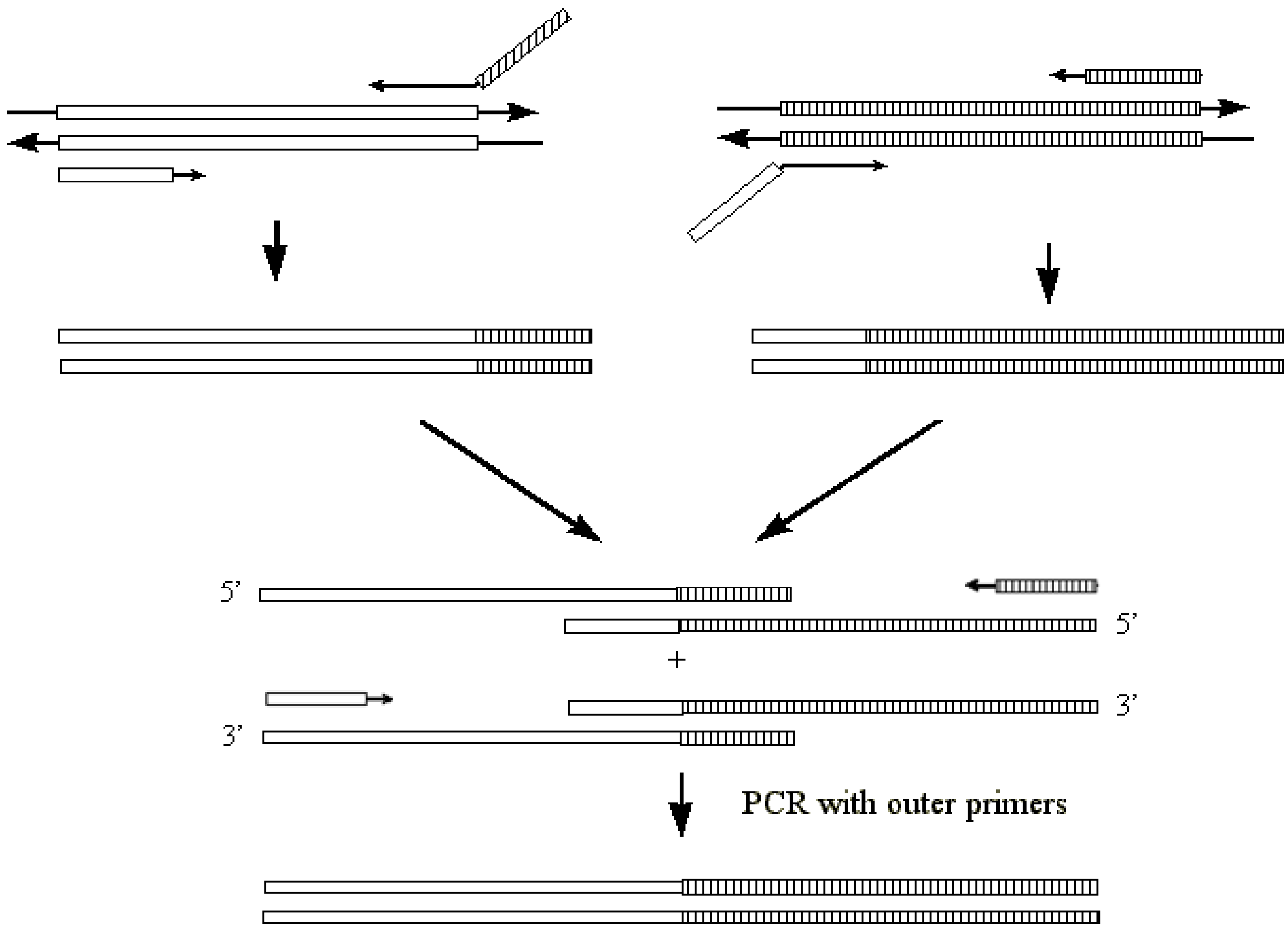
Sean C. Sleight*, Bryan A. Bartley, Jane A. Lieviant and Herbert M. Sauro

Department of Bioengineering, University of Washington, Seattle, WA 98195, USA

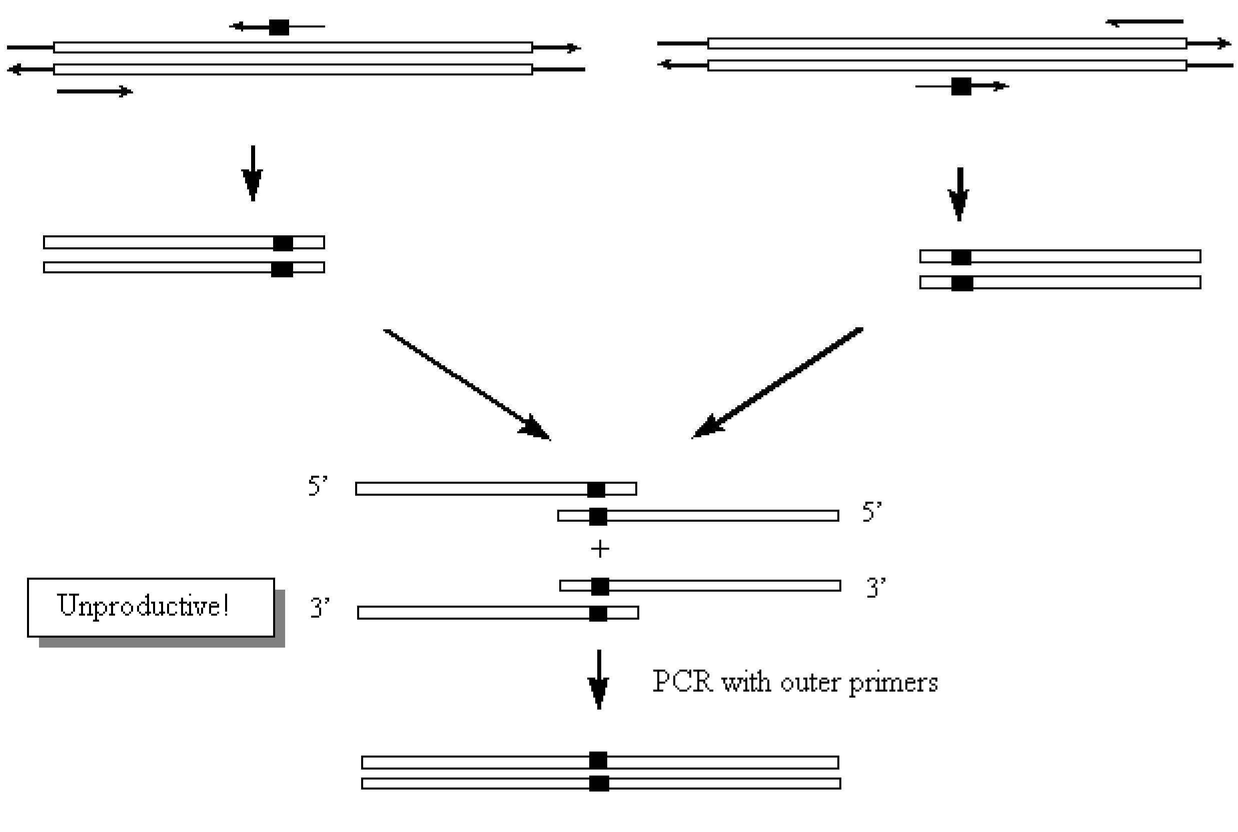
Add sequences at the ends



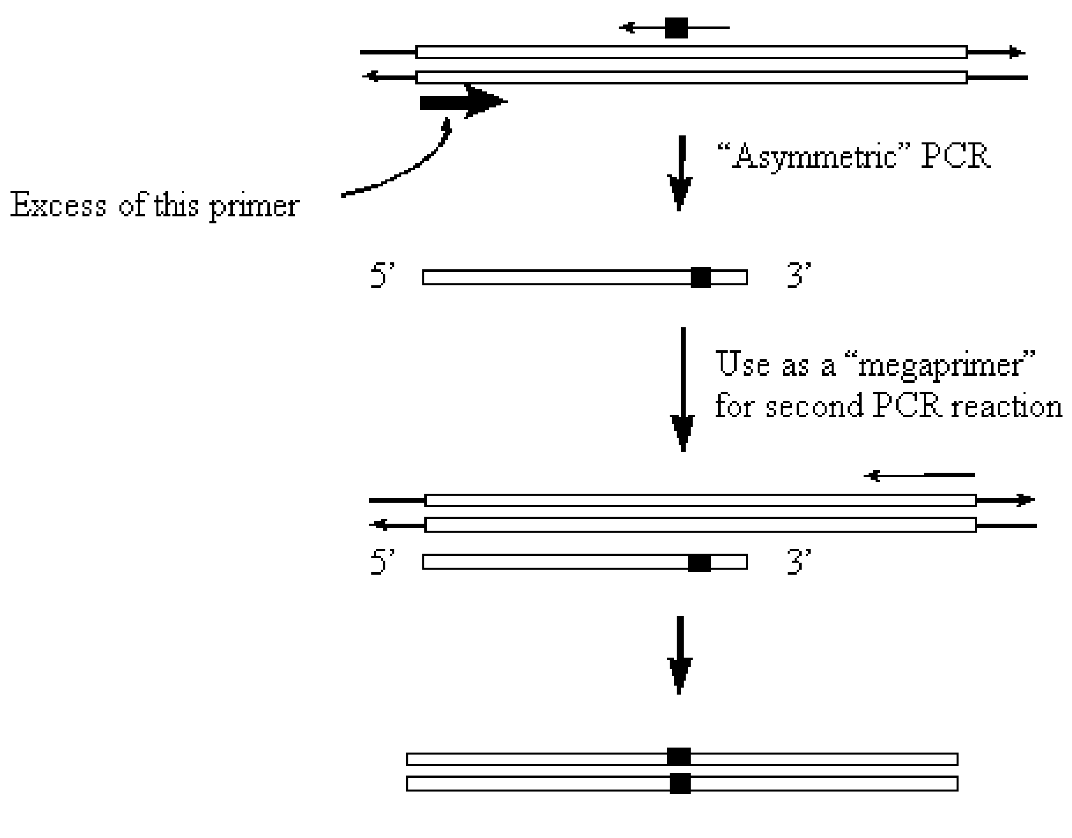
Gene fusion



Mutations



Point mutations



OPEN ACCESS Freely available online

Inverse Fusion PCR Cloning

Markus Spiliotis*

Institut für Parasitologie, Universität Bern, Bern, Switzerland

Abstract

Inverse fusion PCR cloning (IFPC) is an easy, PCR based three-step cloning method that allows the seamless and directional insertion of PCR products into virtually all plasmids, this with a free choice of the insertion site. The PCR-derived inserts contain a vector-complementary 5′-end that allows a fusion with the vector by an overlap extension PCR, and the resulting amplified insert-vector fusions are then circularized by ligation prior transformation. A minimal amount of starting material is needed and experimental steps are reduced. Untreated circular plasmid, or alternatively bacteria containing the plasmid, can be used as templates for the insertion, and clean-up of the insert fragment is not urgently required. The whole cloning procedure can be performed within a minimal hands-on time and results in the generation of hundreds to ten-thousands of positive colonies, with a minimal background.

Citation: Spiliotis M (2012) Inverse Fusion PCR Cloning. PLoS ONE 7(4): e35407. doi:10.1371/journal.pone.0035407

Editor: Eric A. Weaver, Mayo Clinic, United States of America

Received December 2, 2011; **Accepted** March 15, 2012; **Published** April 17, 2012

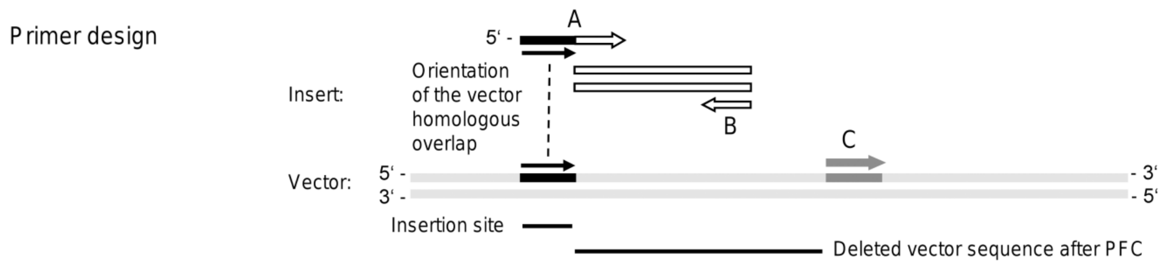
Copyright: © 2012 Markus Spiliotis. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

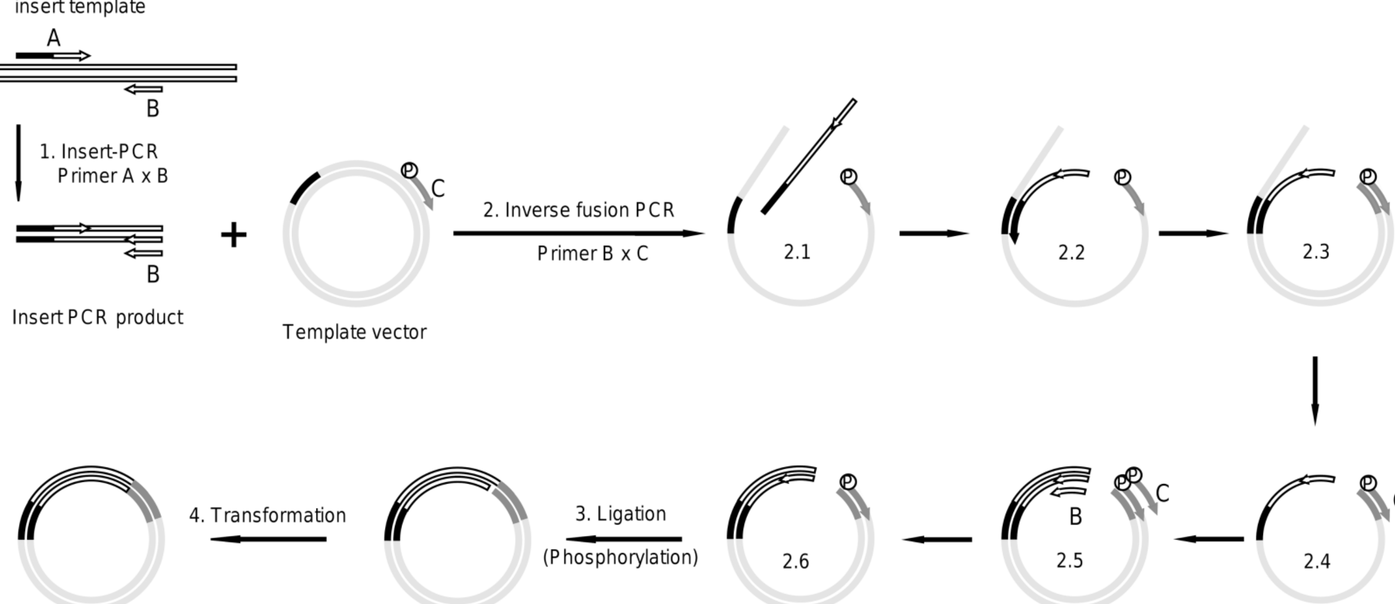
Funding: This work was supported by the Swiss National Science Foundation (grant no. NF1003A-12590-1; URL http://www.snf.ch/E/Pages/default.aspx). The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The author has declared that no competing interests exist.

* E-mail: markus.spiliotis@vetsuisse.unibe.ch

Inverse Fusion PCR cloning





Design tips

- There are lots of programs for primer design, but are very limited for this family of techniques.
- Primers in Fusion PCRs tend to be veeeeery looooong (40-60 pb). Don't panic!
- Again, high complementarity at the 3' end of the primer, and high GC% in the last nucleotides of this end if possible.
- Check carefully the formation of secondary structures and primer dimers (avoid values < -5 Kcal/mol).
- When adding restriction sites at the ends of the sequence, insert at least 4 nucleotides more at the end of the primer, so the RE activity is not impeded. Consultar: http://goo.gl/zu9cz
- Play with temperature gradients, Touchdown PCR, DMSO, betain or Two-Step PCR when it's inevitable to have horrible primers.

Design tips

- If you're intending to fuse protein domains, BE CAREFUL. Frame shifts are an important issue if the primers are not properly designed. Mind the frame!

- Some researchers add spacer residues between domains, to avoid (more) folding errors in the final protein.

- Check first if your sequence has the restriction sites you want to include. If so, change the sites in your primers to avoid innecesary time and money consumption.

- If you have a sequence visualization software, things flow easier: Geneious, CLC Sequence Viewer, Sequencher, UGENE...

- More magic tricks! http://goo.gl/23cPX y http://pcrworld.blogspot.com/