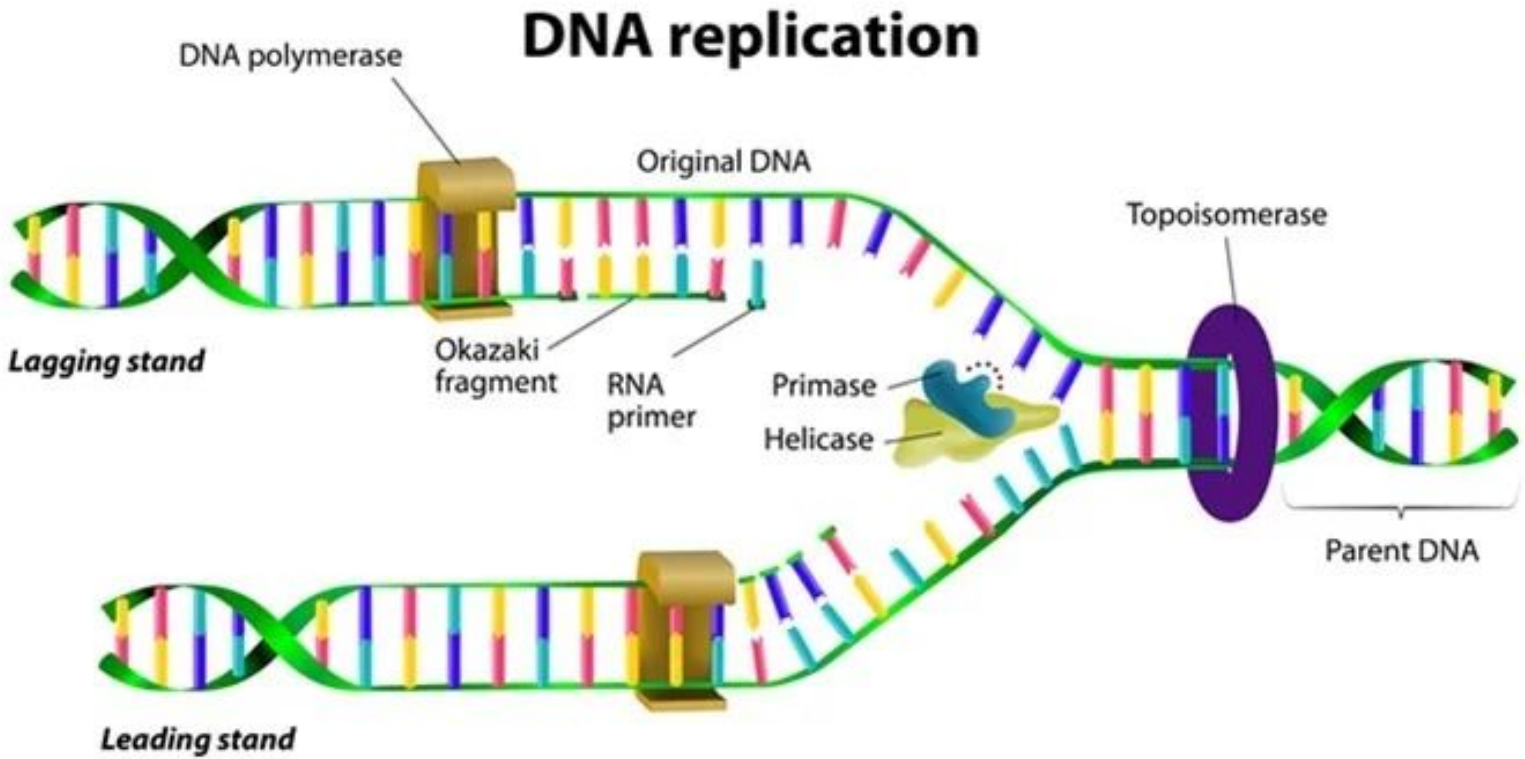
PCR overview

6/18/2024

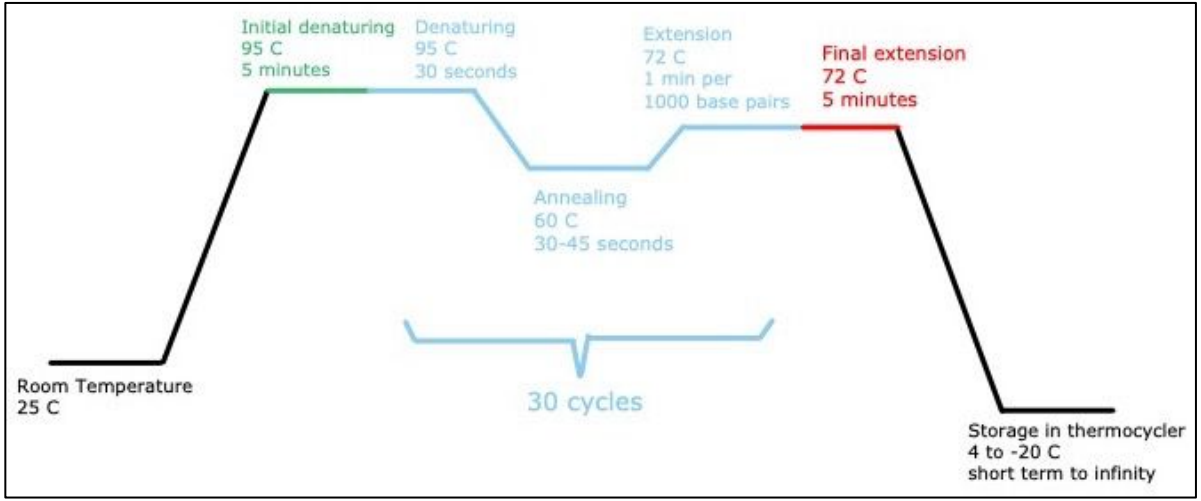


LINK to image

Polymerase Chain Reaction (PCR)

- Components:

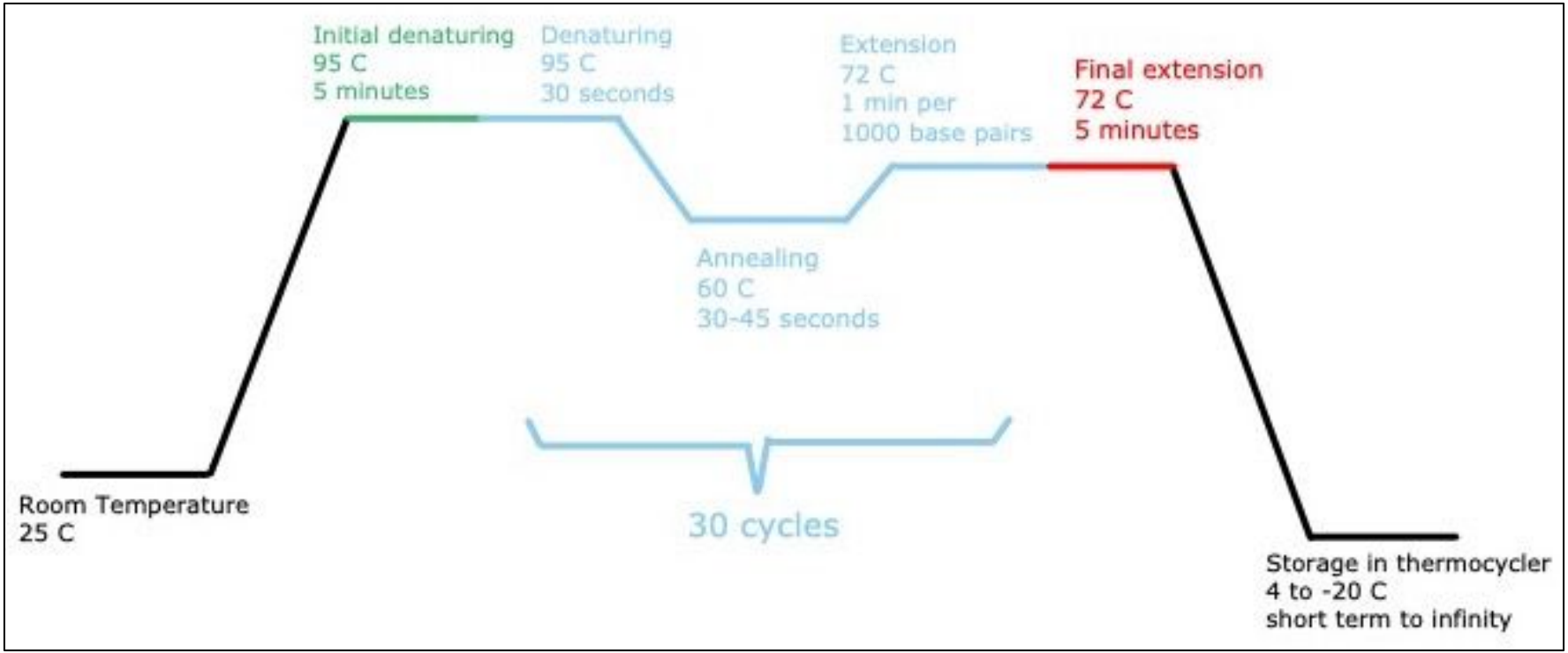
❑ Template DNA
❑ DNA polymerase
❑ Primers
❑ dNTPs
❑ Required cofactor: Mg^{2+}
❑ Buffer



- Steps:

❑ Denaturation (95ºC) of the template into single strands.
❑ Annealing (54ºC) of primers to each original strand for new strand synthesis.
❑ Extension (72ºC) of the new DNA strands from the primers.

Thermo Fischer, n.d.
Delidow et al., n.d.
Labster, n.d.
Hernández, n.d.


Initial denaturing
95 C
5 minutes
Denaturing
95 C
30 seconds
Extension
72 C
1 min per
1000 base pairs
Final extension
72 C
5 minutes
Annealing
60 C
30-45 seconds
30 cycles
Room Temperature
25 C
Storage in thermocycler
4 to -20 C
short term to infinity

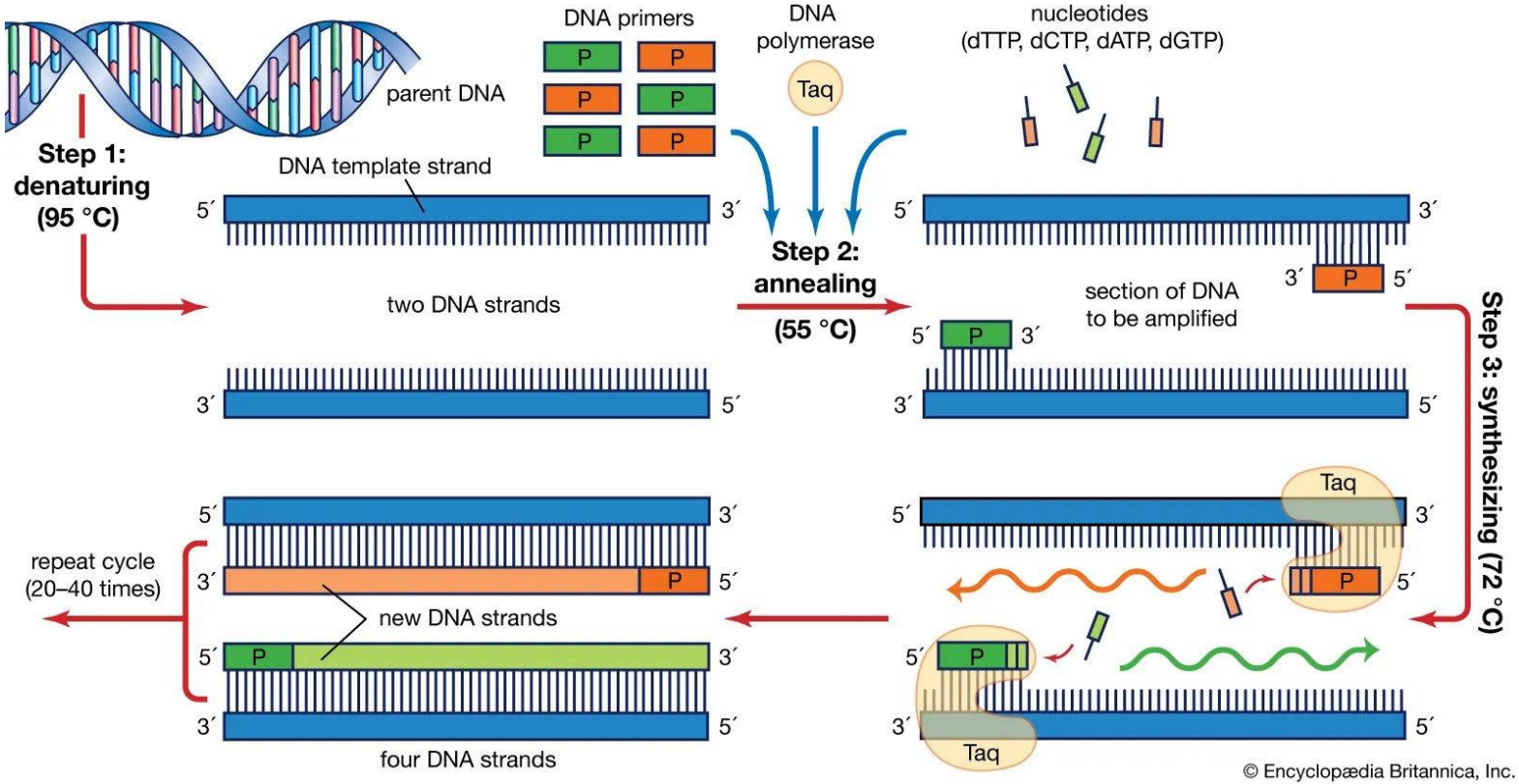
Taq Polymerase

DNA polymerase I from *Thermus aquaticus* (Taq polymerase) is the most famous representative enzyme among the thermostable DNA polymerases. Taq polymerase was identified from *T. aquaticus* isolated from **Yellowstone National Park i**n Montana, USA. The report was published by Chien et al. (1976) as her Master's course study. At that time, nobody foresaw how famous this enzyme would later become. **In 1985, PCR (polymerase chain reaction) technology** using the Klenow fragment of DNA polymerase I from *Escherichia coli* was reported (Saiki et al., 1985). It was easily imagined that a heat-stable DNA polymerase that is not inactivated at the denaturation step from double-stranded to single-stranded DNA would transform this method of gene amplification to a practical technology. Subsequently, a simple and robust PCR method using Taq polymerase was published (Saiki et al., 1988). Due to the heat stability of Taq polymerase, the reaction tube could remain in the incubator after the reaction mixture containing the DNA polymerase was prepared, and only temperature changes were required for PCR.

Paper link HERE

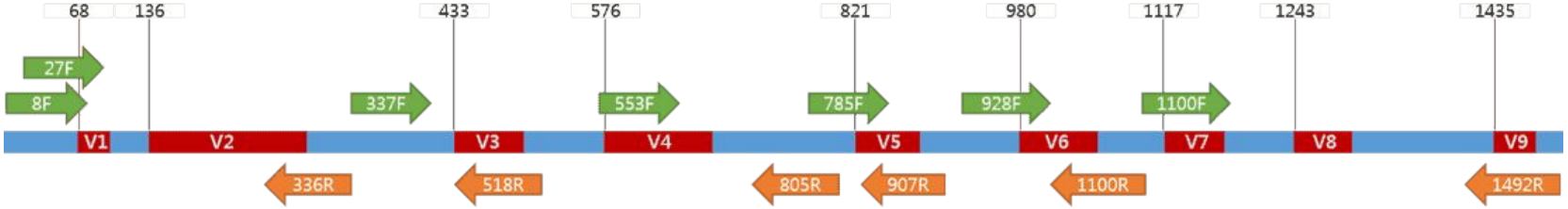
Yellowstone Grand Prismatic Spring

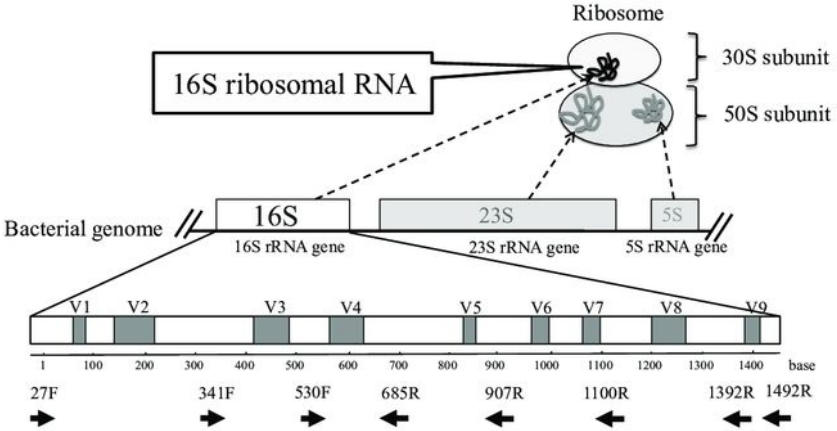
Polymerase Chain Reaction (PCR)



Encyclopaedia Britannica, n.d.

Schematic of the Bacteria 16S rRNA Gene





CJ Bioscience, 2017
Fukuda, 2016

Schematic of the Bacteria 16S rRNA Gene and ISR

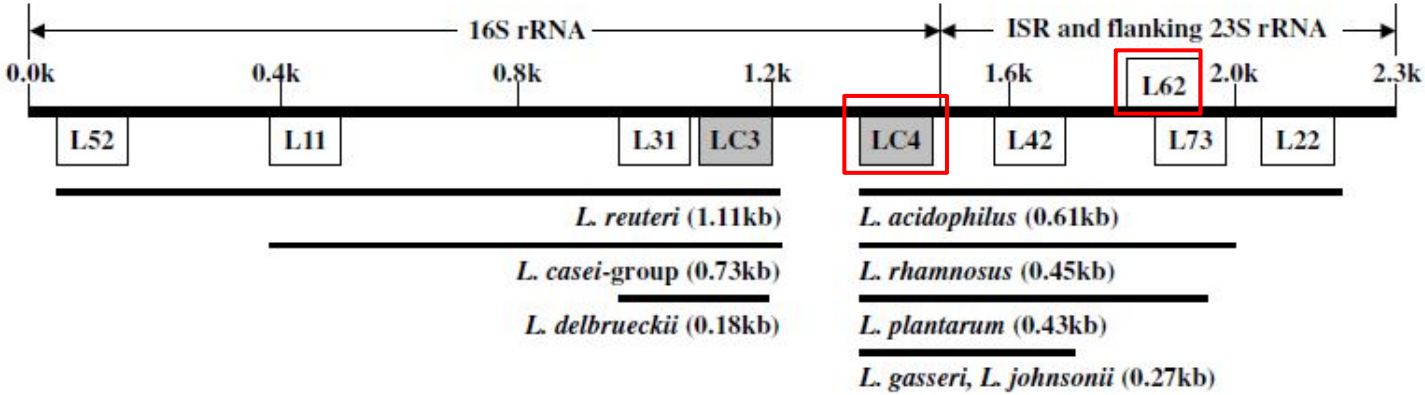


Fig. 2. Alignment of the approximate location of the PCR primers and sizes of each PCR product; PCR primers used were IDLC3R (LC3), IDLC4F (LC4), IDL11F (L11), IDL22R (L22), IDL31F (L31), IDL42R (L42), IDL52F (L52), IDL62R (L62) and IDL73R (L73) (see Table 1). Upper bold lines indicate three rRNA regions. ISR, 16S-23S rRNA intergenic spacer region. Tetragonal boxes indicate annealing position of primers on genomic DNA sequences of *Lactobacillus* species, respectively. Lower lines indicate expected sizes of the amplicon produced from each *Lactobacillus* species.

Kwon et al., 2004

Schematic of the Bacteria 16S rRNA Gene and ISR

Table 2
Multiplex PCR primers established in this study

Target bacteria	Primer	Sequence (5′ to 3′)[a]	Target site[b]	Product (bp)[c]
All *Lactobacillus*	IDL03R	CCACCTTCCTCCGGTTTGTCA	1178–1198	–
All *Lactobacillus*	IDL04F	AGGGTGAAGTCGTAACAAGTAGCC	1499–1522	–
L. casei-group[d]	IDL11F	TGGTCGGCAGAGTAACTGTTGTCG	472–495	727
L. acidophilus	IDL22R	AACTATCGCTTACGCTACCACTTTGC	2079–2104	606
L. delbrueckii	IDL31F	CTGTGCTACACCTAGAGATAGGTGG	1015–1039	184
L. gasseri	IDL42R	ATTTCAAGTTGAGTCTCTCTCTC	1748–1770	272
L. reuteri	IDL52F	ACCTGATTGACGATGGATCACCAGT	94–118	1105
L. plantarum	IDL62R	CTAGTGGTAACAGTTGATTAAAACTGC	1900–1926	428
L. rhamnosus	IDL73R[e]	GCCAACAAGCTATGTGTTCGCTTGC	1922–1946	448

[a] All the primer sequences were according to *L. casei* numbering [4,17].
[b] Each target site indicates the start and end point of the complimentary sequences annealing the forward and reverse primer, respectively.
[c] Product means approximated length of each PCR product derived from primer pair composed of species-specific primer and bacterial conserved primer (IDLC3R or IDLC4F).
[d] *L. casei*-group includes all of *L. casei*-related *Lactobacillus* species such as *L. casei* and *L. rhamnosus*.
[e] Primer IDL73R were modified sequences formerly described by Ahrne et al. [24].

Kwon et al., 2004

Primer Design - rules

- Dos:

❑ 15–30 nt long
❑ T_m 55–70°C (within 5°C, for two primers)
❑ 40–60% GC (with uniform distribution)
❑ One C or G at 3′ end

- Don’ts:

❑ Secondary structure (complementarities)
❑ Direct repeats
❑ More than three G or C at 3′ end

Thermo Fischer Scientific, nd
Thermo Fischer Scientific, nd

Primer Design - example

Lactobacillus helveticus strain ATCC 15009 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer, complete sequence; and 23S ribosomal RNA gene, partial sequence

Sequence ID: AF429596.1

```
  1 agagtttgta acacccaaag ccggtggggt aacctttag gagctagccg tctaaggtgg
 61 gacagatgat tagggtgaag tcgtaacaag gtagccgtag gagaacctgc ggctggatca
121 cctcctttct aaggaataaa cggaaaccca cactatgtca aagttttgtt tagttttgag
181 aggtttactc tcaattttgt tctttgaaac ctgattaata tctataattc tctaattcta
241 attggttaat cattattaac ccgagagaaa ccgcgtaatt taaagagttt taaaccagat
301 tagttcaaaa gattatcgct aactcaatta atccacttta ccgtaggttg aagattaggt
361 taagttagga agggcgcatg gtgaatgcct tggtactagg agccgatgaa ggacgggact
421 aacaccgata tgcctcgggg agctgtaagt aagctttgat ccggggattt ccgaatgggg
481 caacccaata gttttaacga ctattatccg catctgaata cataggatgt gtgaaggtaa
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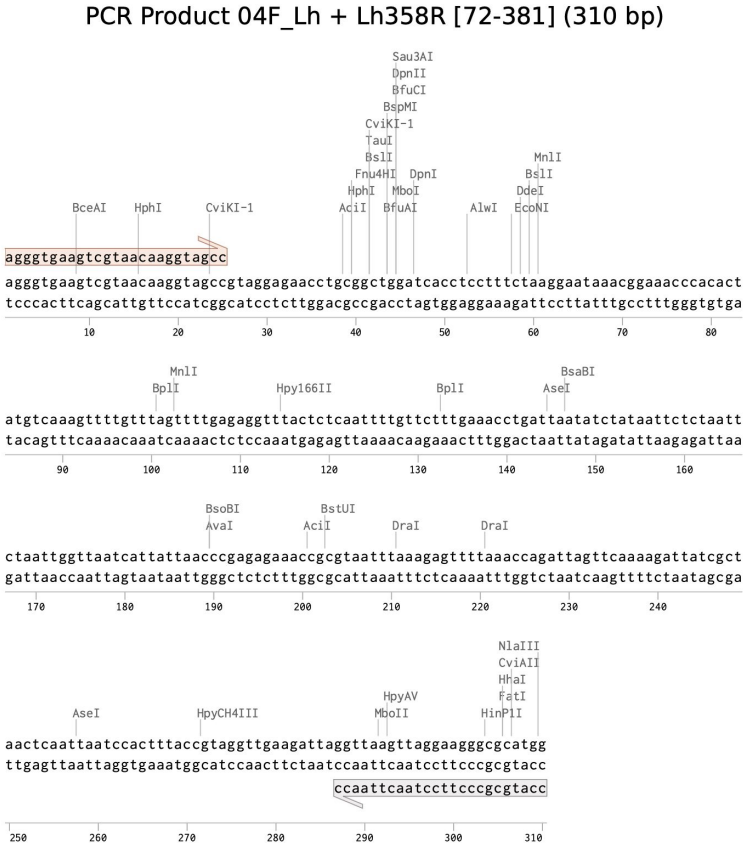
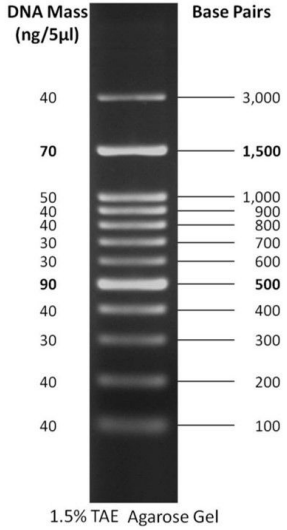
Primer Design - example



Sequence ID: [AF429596.1]

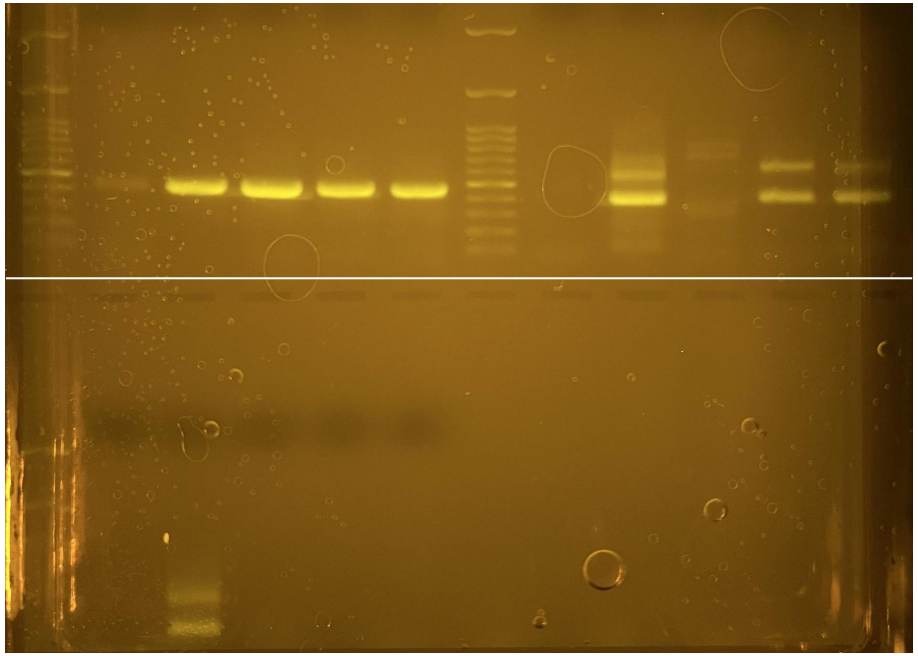
Example of data: Experiment #1: gDNA PCR

- Used sterile water, but did not use sterile 16S primers.
- Used Imilce's PCR protocol.
- Why is there a band in PCR #1– NTC?
- Why are there double bands in PCR #2 and #3?
- *Lh* primers working for *Lp*, but not for *Lh*.



PCR #1: 341F + 805R (16S)

PCR #2: 04F + 62R (*Lp*)



PCR #3: 04F_Lh + Lh358R (*Lh*)

Gold Biotechnology, n.d.

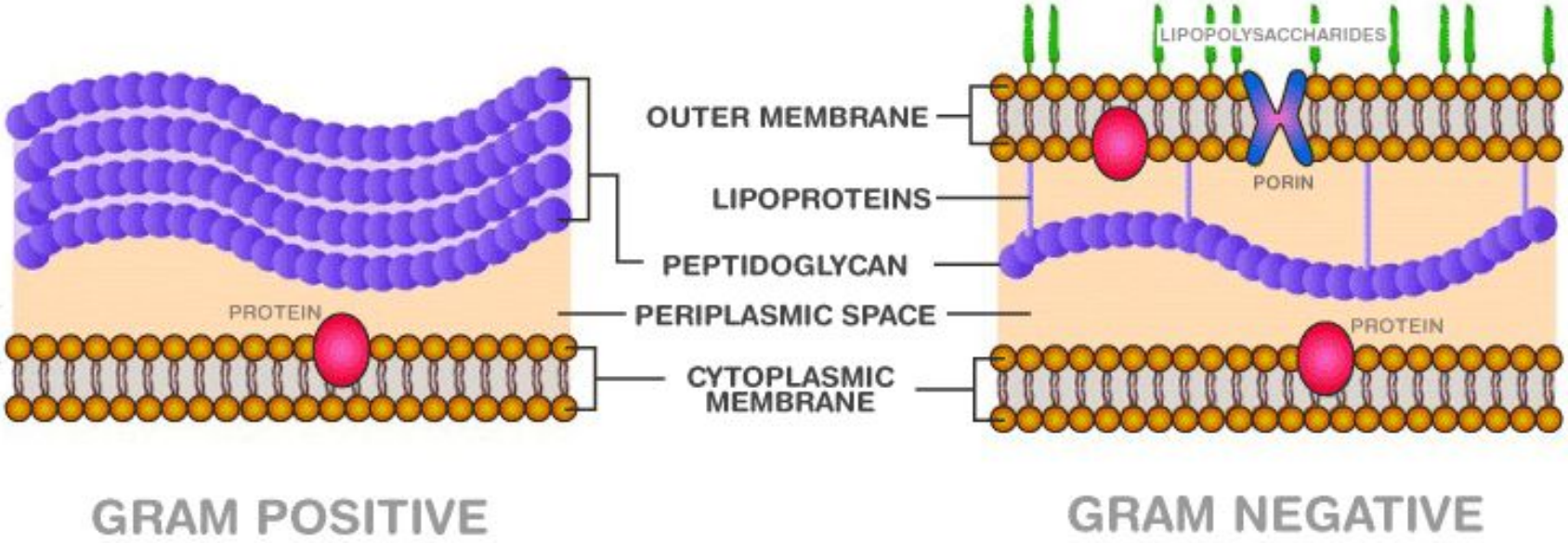
Today’s PCR experiment

Goal: Learn to do Colony PCR.

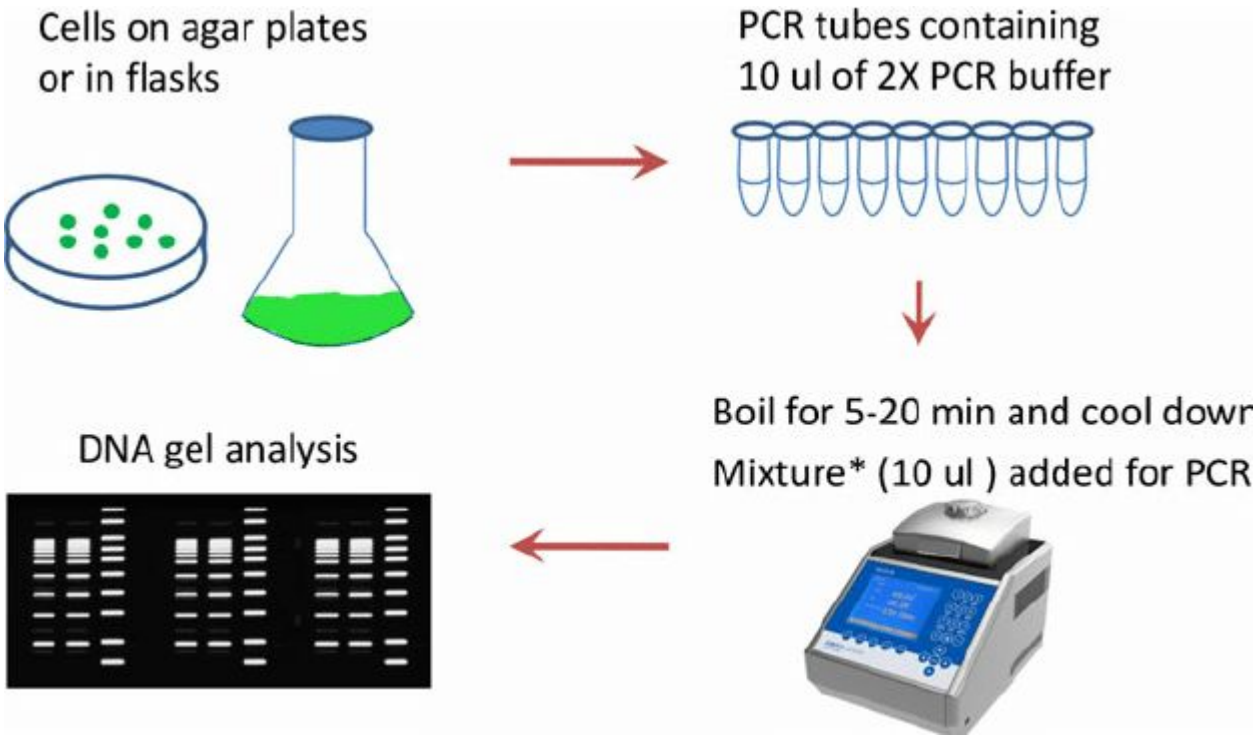
What is Colony PCR?

Is a PCR in which you add lysed bacteria instead of extracted genomic DNA (gDNA)

GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA



Colony PCR

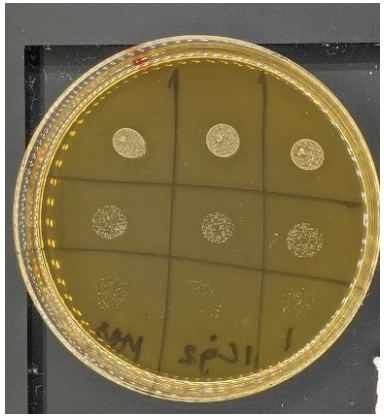


Link to image HERE

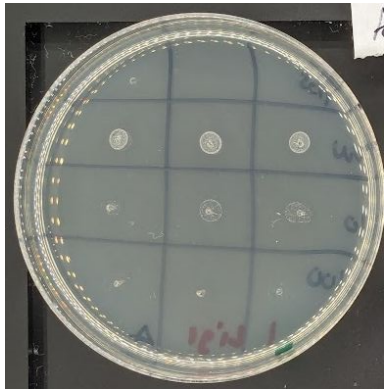
Today's PCR experiment

Experiment overview:

1. We will have 2 selective plates with bacteria colonies.
   - MRS for *Lactobacillus*, gram positive bacteria
   - Acetobacter for *Acetobacteria*, gram negative bacteria
2. Will take the colony, resuspend in YPER buffer to lyze and obtain gDNA
3. Make a 1:20 dilution, use this as PCR template.
4. Run PCR

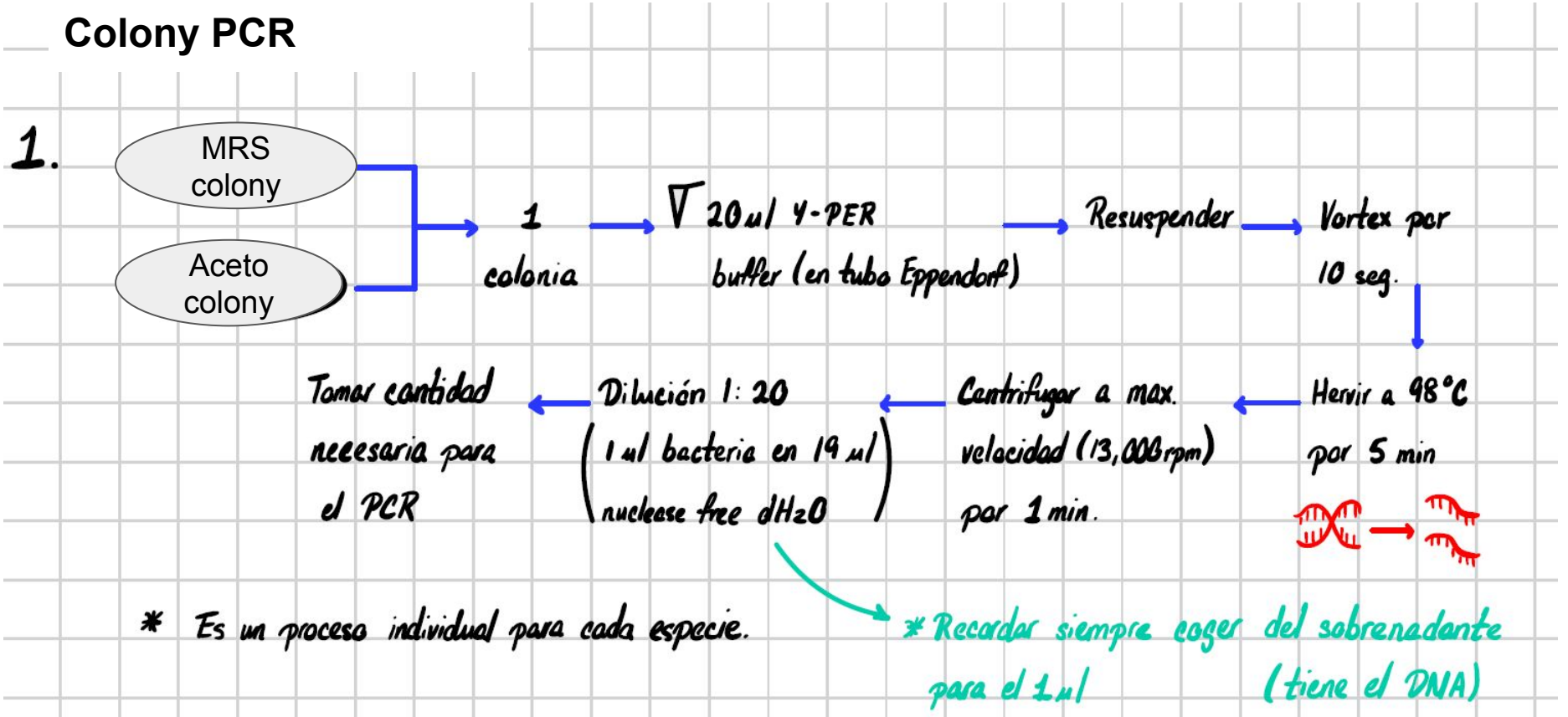


MRS



Acetobacter

Colony PCR



Protocolo por Xavier Maldonado

Primer Specifications - the ones we will use

Primer	Sequence (5' to 3')	nt	T_m (°C)	GC %
341F	CCTACGGGAGGCAGCAG	17	57.8	70.6
805R	GACTACCAGGGTATCTAATCC	21	50.9	50.9
04F	AGGGTGAAGTCGTAACAAGTAGCC	25	60	52
62R	CTAGTGGTAACAGTTGATTAAAACTGC	27	54.3	37
04F_Lh	AGGGTGAAGTCGTAACAAGGTAGCC	25	59.3	52
Lh358R	CCATGCGCCCTTCCTAACTTAACC	24	59.1	54

PCR Primer Combinations and Samples

PCR #	Primer Set	PCR Product	Target Sequence
1	04F + 62R	428 bp	*L. plantarum* 16S

Sample #	Sample
1	NTC (no template control)
2	*MRS* colony PCR
3	*Acetobacter* colony PCR

Manual

EconoTaq PLUS 2X Master Mix

5.2. **Reaction setup.** Set up PCR amplifications of the desired size, according to the following:

				Final concentration
EconoTaq PLUS 2X Master Mix	12.5 µL	25.0 µL	50.0 µL	1 X
Forward primer (100 µM)	0.25 µL	0.5 µL	1.0 µL	1 µM
Reverse primer (100 µM)	0.25 µL	0.5 µL	1.0 µL	1 µM
DNA template (10 ng/µL)	1.0 µL	1.0 µL	1.0 µL	
Water, nuclease-free	11.0 µL	23.0 µL	47.0 µL	
Total reaction volume	25.0 µL	50.0 µL	100.0 µL	

5.2. Gently mix the PCR components in a thin-walled reaction tube and spin briefly in a microcentrifuge. Add a drop of mineral oil if the thermal cycler does not have a heated lid.

6. PCR cycling conditions

6.1. Pre-heat the thermal cycler to 94 °C.

6.2. For initial denaturation of target template DNA, incubate the reactions at 94 °C for two minutes.

6.3. Denature, anneal and extend the DNA according to the following for subsequent cycles of amplification:

Cycling step	Temperature	Time	# of Cycles
Initial denaturation*	94 °C	2 min	1
Denaturation*	94 °C	15-30 sec	25-35
Annealing**	50-65 °C	15-30 sec	
Extension	72 °C	1 min/kb	
Final extension	72 °C	5-10 min	1
Hold	4 °C	Indefinitely	1

Master Mix we will use is called EconoTaq PLUS Green

Reagent	Volume (ul)
EconoTaq PLUS 2x Master Mix	12.5 ul
Forward primer (100 uM)	0.25 ul
Reverse primer (100 uM)	0.25 ul
DNA template	1 ul
Nuclease free H2O	11 ul
Total reaction	25 ul

Step 1. Set-up PCR reaction Master Mix

Volume for 1 sample/reaction

You multiple for the samples you have +0.5

Reagent	Volume (ul)	X 3.5
EconoTaq PLUS 2x Master Mix	12.5 ul	37.5
Forward primer (100 uM)	0.25 ul	0.875
Reverse primer (100 uM)	0.25 ul	0.875
DNA template	1 ul	-
Nuclease free H2O	11 ul	38.5
Total reaction	25 ul	

Different per sample

Step 2. Add 24 ul per each PCR tube

Step 3. Add 1 ul of 1:20 dilution

PCR Conditions

		Imilce's Protocol	
Step #	**Step**	**T (°C)**	**Time (min)**
1	Initial denaturation	95	2:00
2	Denaturation	95	0:30
3	Annealing	58	1:00
4	Extension	68	0:30 *1:00 per 1kb
5	Go to 2, 34x	-	-
6	Final extension	68	1:00
7	Cooling	10	5:00

Electroforesis

- Preparación de 1X TAE Buffer a partir de 50X TAE Buffer

$$V_1 C_1 = V_2 C_2$$

$$(1000\,mL)(1X) = V_2(50X)$$

$$V_2 = \frac{1000\,mL(1X)}{50X}$$

$$V_2 = 20\,mL$$

980 mL	+	20 mL	=	1000 mL
dH_2O		50X TAE Buffer		1X TAE Buffer

- Preparación de gel de agarosa ⅞ 1

50 mL	+ 0.5 g	+ ± 50 s	+ 5.0 µl	= Gel
1X TAE Buffer	Agarosa	Microondas	SYBR Safe	

1. Verter 50 mL de 1X TAE Buffer en un matraz Erlenmeyer 125 mL.
2. Pesar 0.5 g de agarosa y añadir al matraz.
3. Colocar matraz en el microondas y calentar por 30 s. Seguir calentando poco a poco hasta obtener una mezcla homogénea.
4. Añadir 5 µL de SYBR Safe DNA Gel Stain a la mezcla y homogenizar.
5. Colocar peines de "wells" que creas necesarios en el accesorio de vidrio que mantiene la gel en posición durante la electroforesis.
6. Verter mezcla en accesorio de vidrio. El accesorio debe estar girado y con las gomas puestas para que no se escape la mezcla. Esperar más o menos 10 min hasta que solidifique el gel.

* El máximo de gel que se puede echar en la nueva máquina de electroforesis es 45 mL.