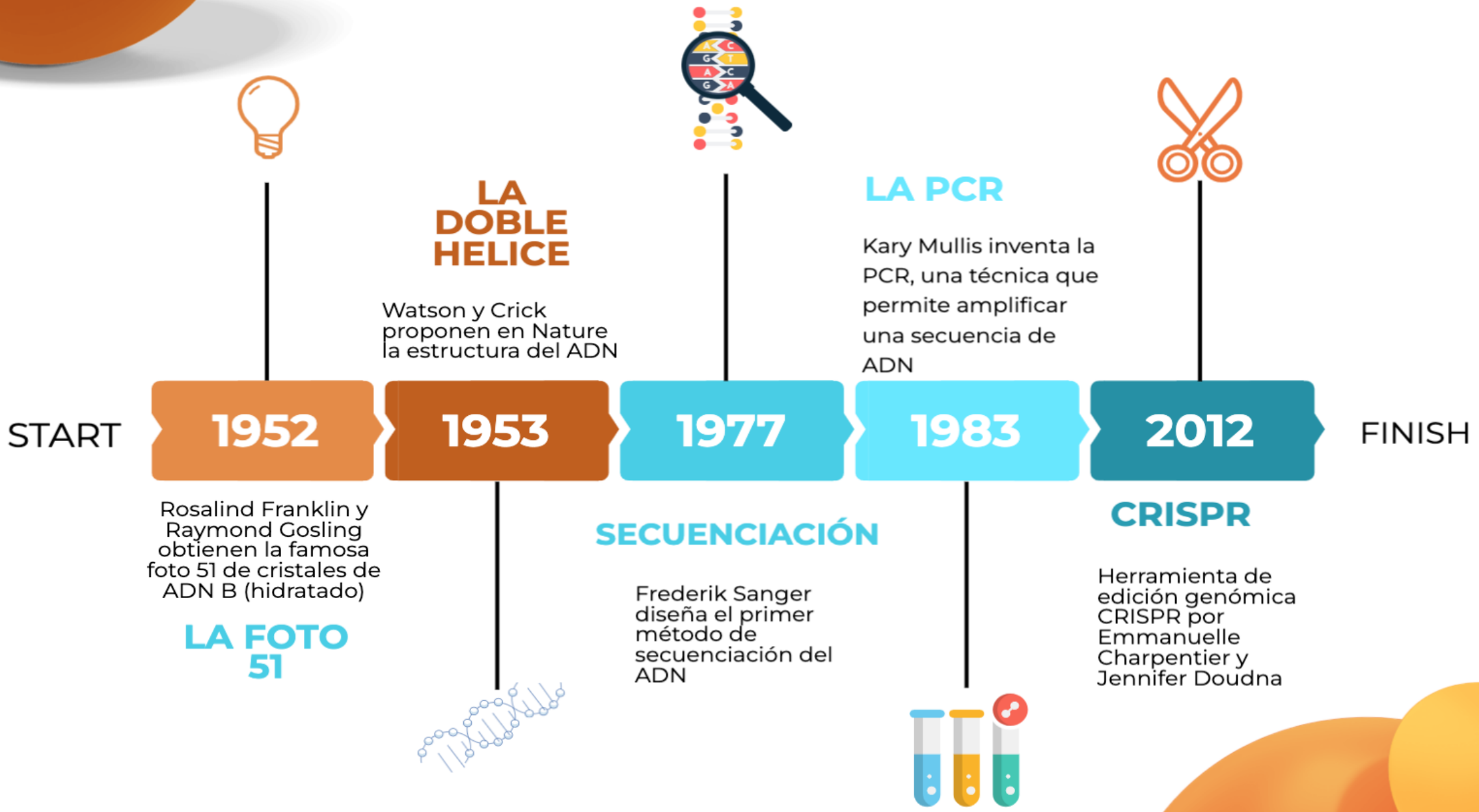
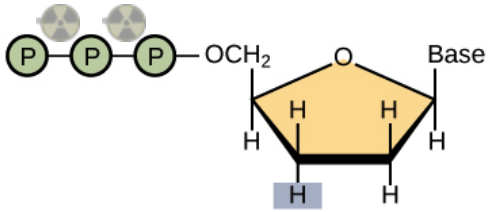
Introducción a la secuenciación



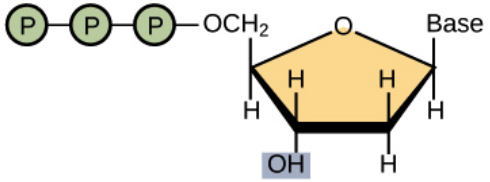
EL MÉTODO SANGER

El método Sanger “clásico”

En 1977→ F. Sanger desarrolló la metodología
En 1979→ Se logró secuenciar el genoma del bacteriófago **Phi-X174.**

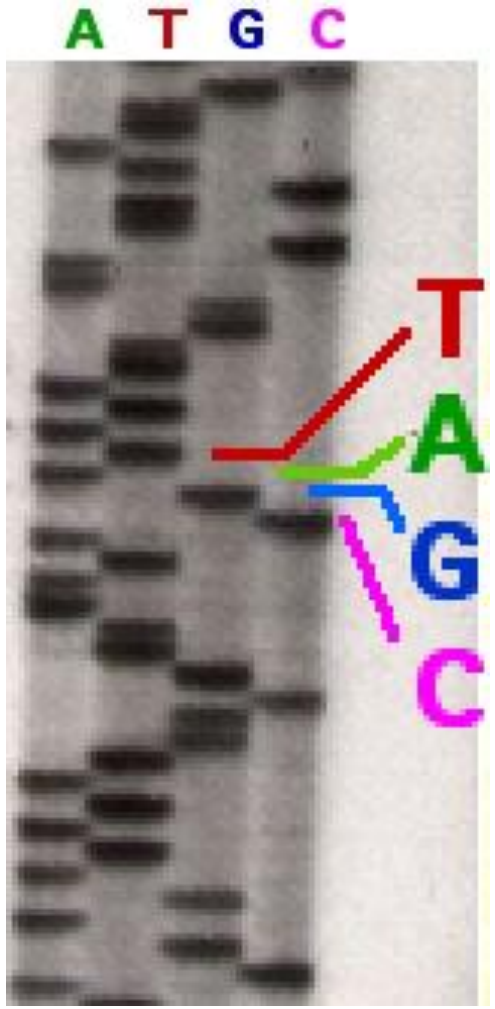


Nucleótido didesoxi **(ddNTP)**

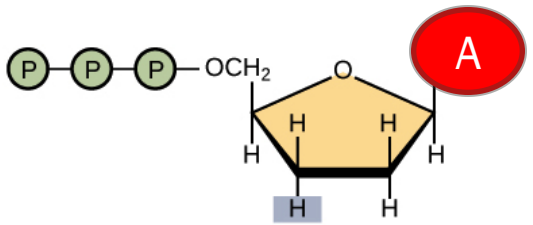


Nucleótido desoxi **(dNTP)**

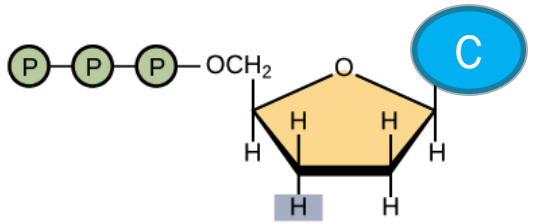
1. El proceso biológico de la replicación del ADN
2. Empleo de dideoxinucleótidos que carecen del grupo –OH del carbono 3´ (ddNTPs)
3. Los ddNTPs marcados radiactivamente (baja concentración)
4. Visualización de los fragmentos empleando geles de poliacrilamida y Rayos X



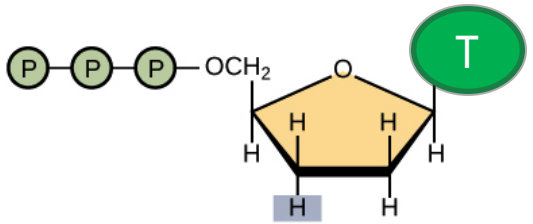
El método Sanger “Moderno”



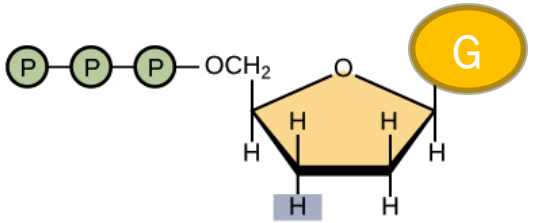
Nucleótido didesoxi **(ddNTP)**



Nucleótido didesoxi **(ddNTP)**

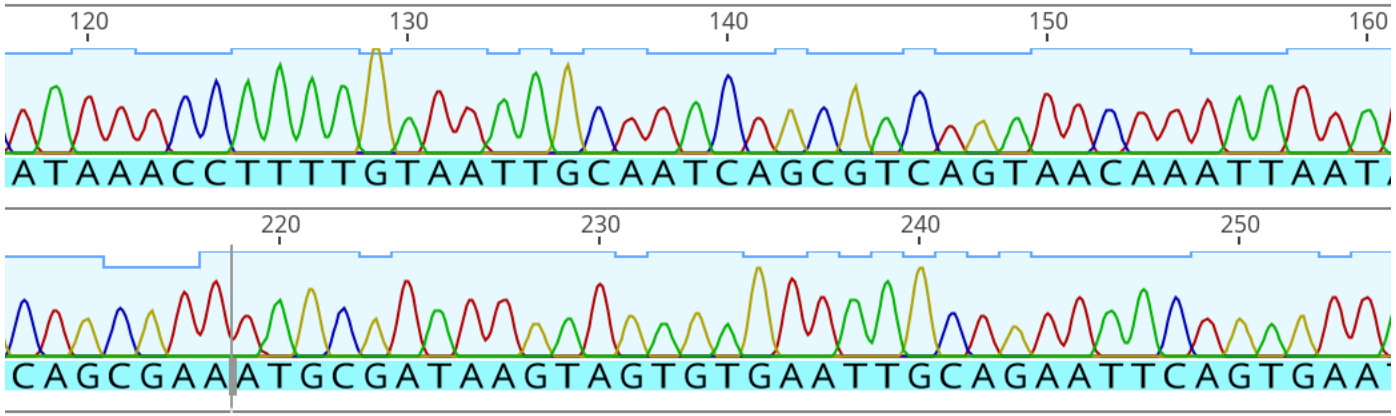


Nucleótido didesoxi **(ddNTP)**



Nucleótido didesoxi **(ddNTP)**

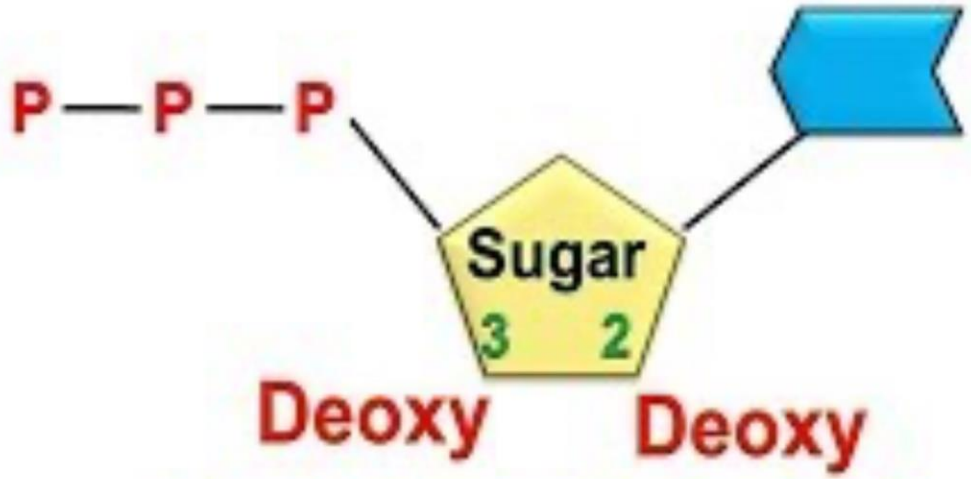
1. El proceso biológico de la replicación del ADN
2. Empleo de dideoxinucleótidos que carecen del grupo –OH del carbono 3´ (ddNTPs)
3. Los ddNTPs marcados con fluorosforos que permiten su diferenciación
4. Visualización de los fragmentos empleando geles de poliacrilamida y Rayos X



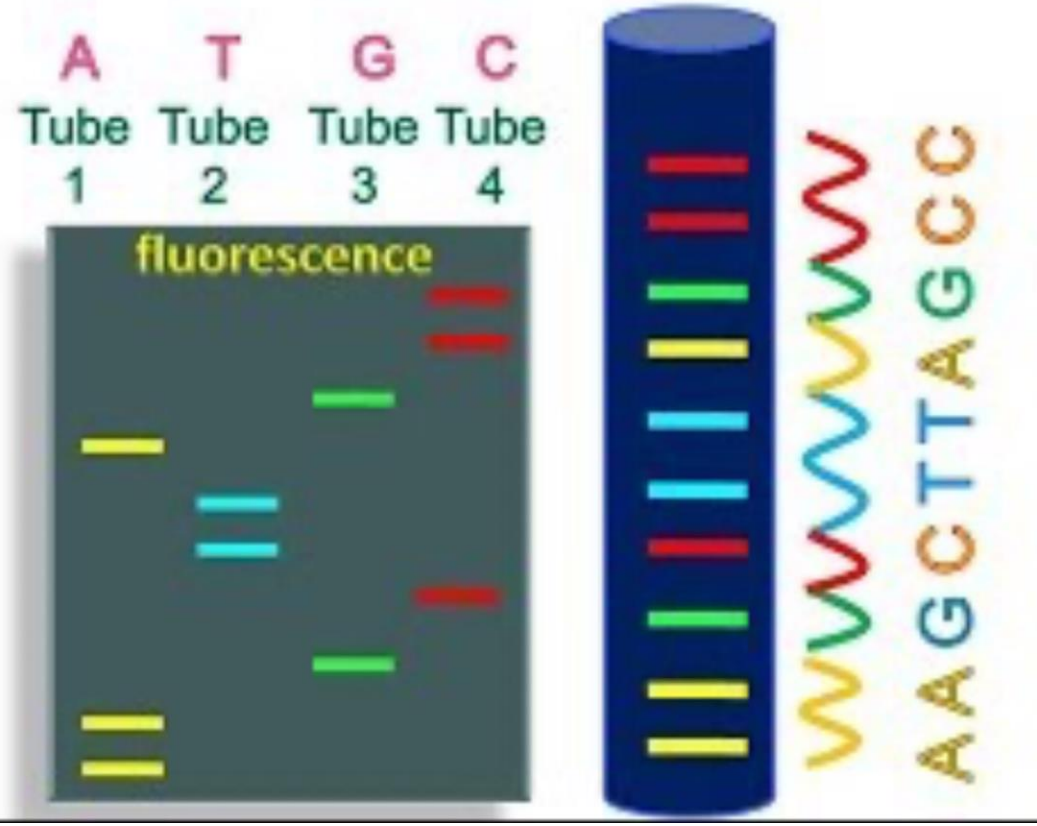
Cromatograma

Quickly understand

Sanger's sequencing



Di deoxy nucleotide



Pasos de la secuenciación

- Pasos previos a la secuenciación (Preparación del ADN)
  1. *Obtención del ADN diana*
  2. *Purificación de ácidos nucleicos*

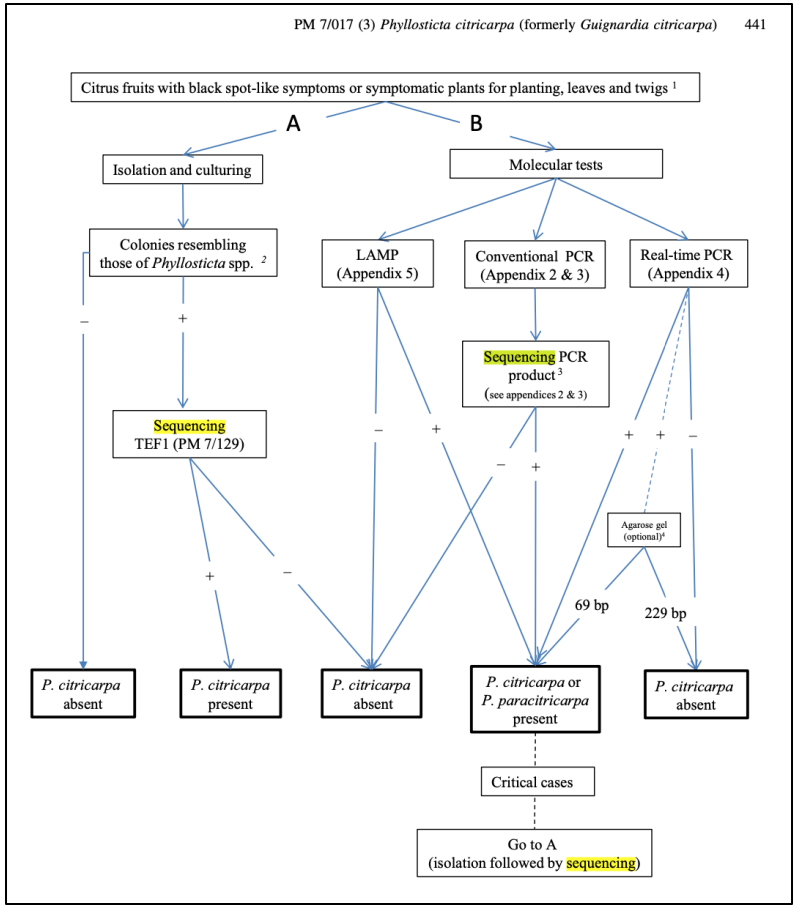
- **Pasos de la secuenciación (Secuenciación de nucleótidos)**
  1. *Amplificación*
  2. *Purificación*
  3. *Electroforesis capilar*

Principales aplicaciones de la secuenciación en patología vegetal

1. **Identificación de Patógenos**
2. Resistencia Genética de las plantas
3. Detección de Resistencias de patógenos
4. **Estudios de diversidad genética**
5. Estudio de Interacciones Planta-Patógeno
6. **Control de Enfermedades Emergentes**
7. Optimización de Estrategias de Control

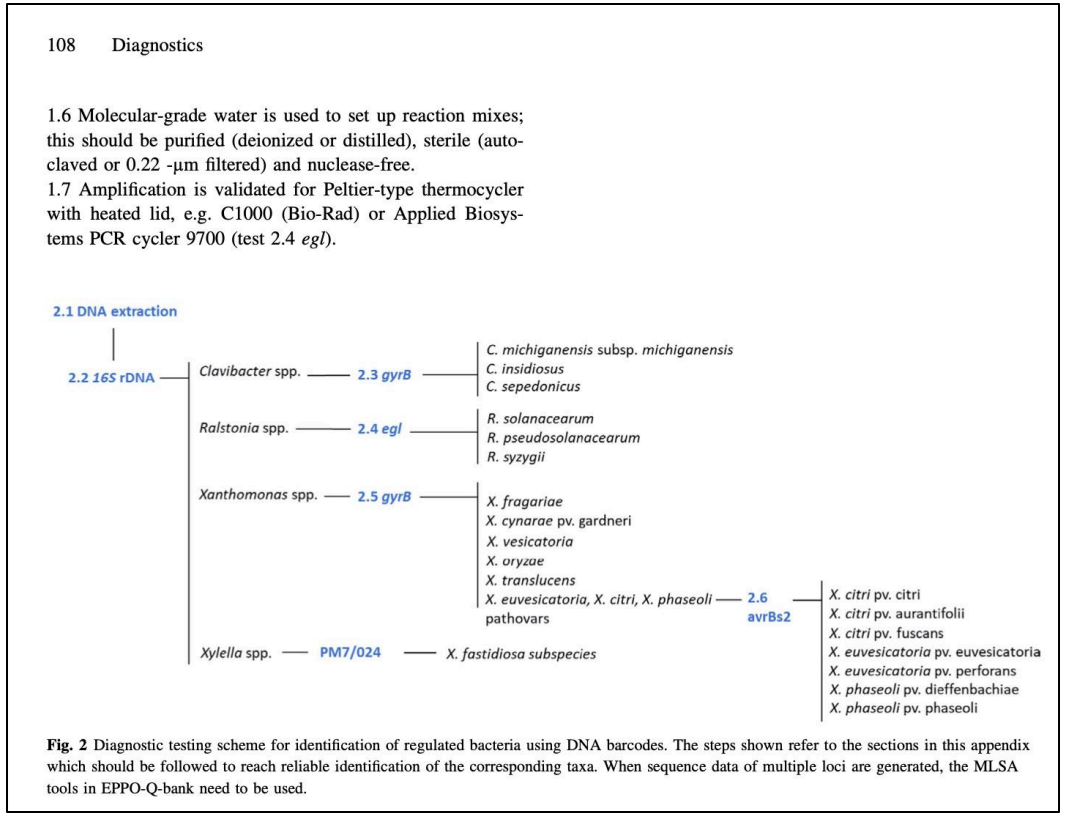
Identificación de Patógenos

Hongos



PM 7/017 (3) *Phyllosticta citricarpa*

Bacterias



PM7/129(2) DNA barcoding as an identification tool for a number of regulated pests

Identificación de Patógenos

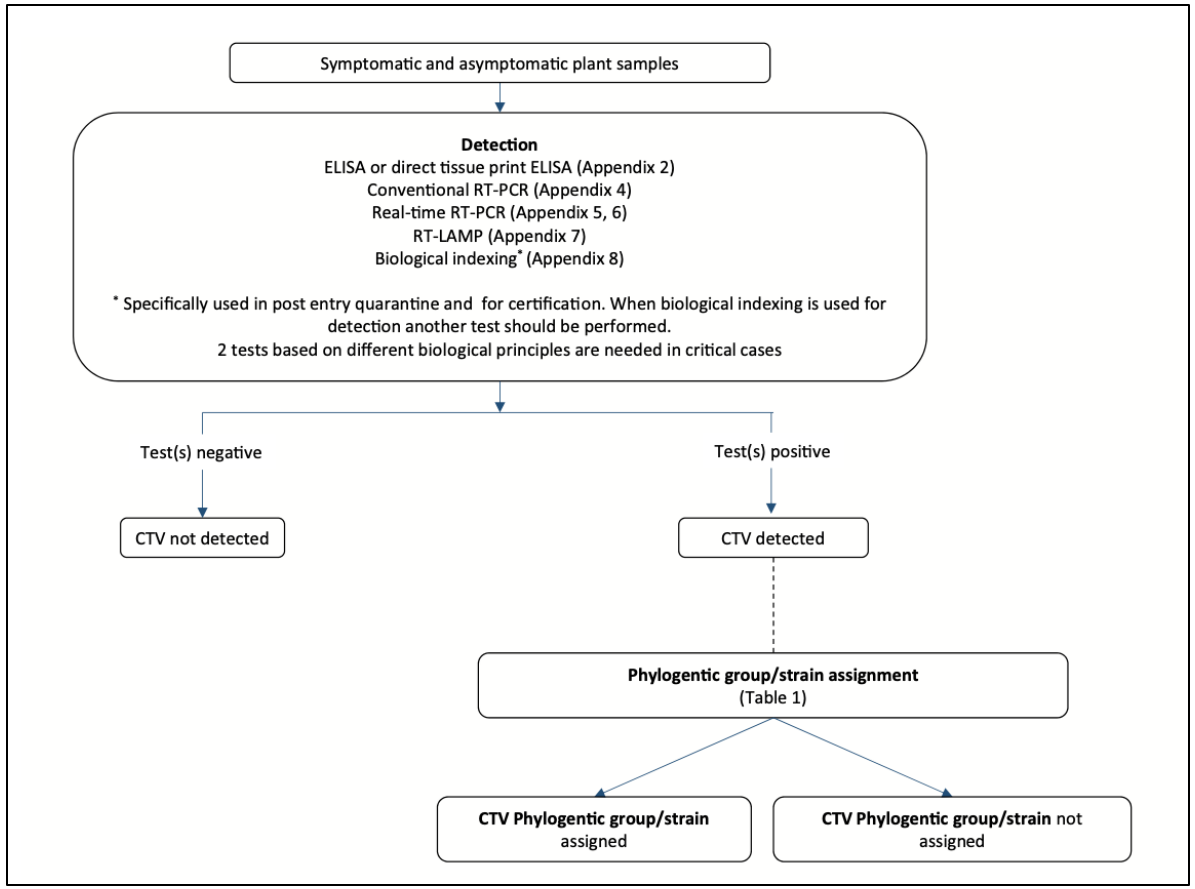


TABLE 2 Available tests to characterize CTV isolates

Phylogenetic group	T36[a]	T68[a]	RB[a,1]	T3[b]	VT[b]	T30[b]
Method/Test						
Biological indexing (Appendix 8) (assignment to strains)	SY, QD, SP	SP	Potentially all types of symptoms	SY, SP	All types of symptoms	Leaf yellowing, vein clearing, leaf cupping
HTS	✓	✓	✓	✓	✓	✓
Multiplex conventional RT-PCR (Roy et al., 2010)	✓[2]	✓	✓[2]	✓	✓	✓
Multiplex real-time RT-PCR (Yokomi et al., 2010)	✓	✓[3]	✓	✓[3]	✓[3]	✓[4]
Conventional RT-PCR (Roy et al., 2013)	–	–	✓	–	–	–
Conventional RT-PCR (Cook et al., 2016)[5]	✓	–	✓	–	–	–
Two-step conventional RT-PCR (Saponari et al., 2019)	–	–	✓	–	–	–
One-step real-time RT-PCR (Saponari et al., 2019)	–	–	✓	–	–	–

Abbreviations: QD, quick decline; SP, stem pitting; SY, seedling yellows;
✓, test (or combination of tests) considered suitable to assign isolates to phylogenetic groups;
–, test does not detect isolates from the phylogenetic group.
[1]Able to overcome *Citrus trifoliata* resistance.
[2]The test will detect T36 and RB isolates, but these cannot be distinguished.
[3]The test will detect T68, T3 and VT isolates, but these cannot be distinguished.
[4]Results obtained from the four different probes should be combined to assign an isolate to the T30 phylogenetic group.
[5]Two specific RT PCR tests are described in this publication.
Note: a and b are additional information that relate to the presence of the phylogenetic groups in EU countries or not.
[a]Not present in EU countries.
[b]Present in EU and also in non-EU countries.

- Conventional simplex and multiplex RT-PCR tests followed by sequencing and sequence analysis of the amplicon.
- Simplex and multiplex real-time RT-PCR tests using specific primers (and probes) for particular phylogenetic groups.
- High-Throughput Sequencing (HTS) and analysis of the sequences obtained.

PM7/031(2) *Citrus tristeza virus*

Estudios de diversidad genética

GPGV RT-qPCR

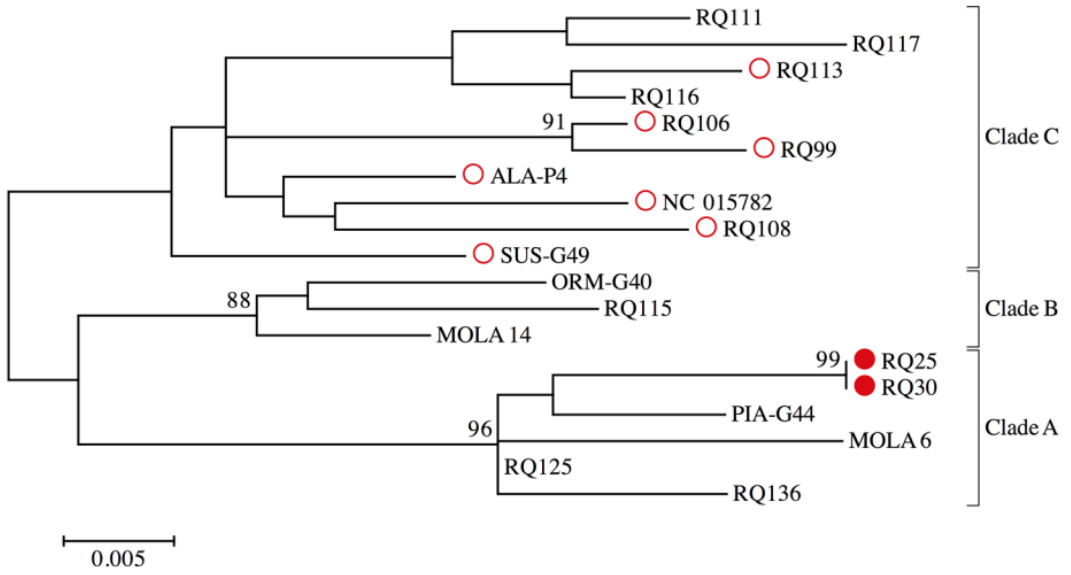


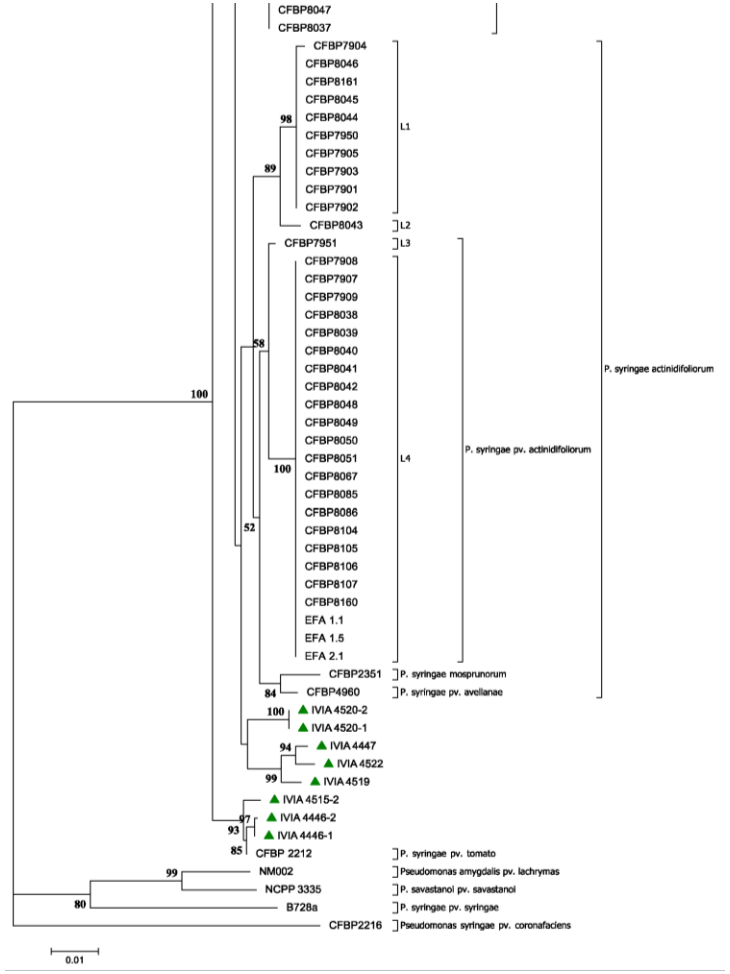
Fig 4. Phylogenetic analysis of Spanish GPGV isolates by Maximum likelihood method. Trees were generated under the Tamura 3 model of nucleotide substitution. Branches supported by a minimum of 75% of 2,000 bootstrap replicates.

https://doi.org/10.1371/journal.pone.0197237.g004

Análisis filogenético de **12 aislados de GPGV españoles y 8 secuencias de referencia representativas de los tres clados (A, B y C)** identificados por Saldarelli *et al.,* (2015) y Bertazzon *et al., (2017)* basado en 588 nt de la región MP/CP

La caracterización de los aislados de GPGV ha demostrado la presencia de un nuevo polimorfismo, con baja prevalencia, en su genoma que implica una señal de parada traduccional que acorta la proteína de movimiento (MP) en cinco aminoácidos

Estudios de diversidad genética

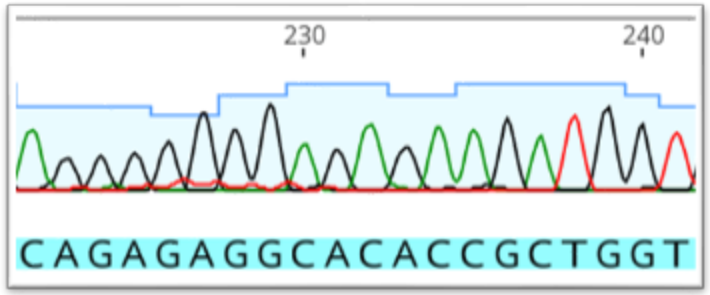


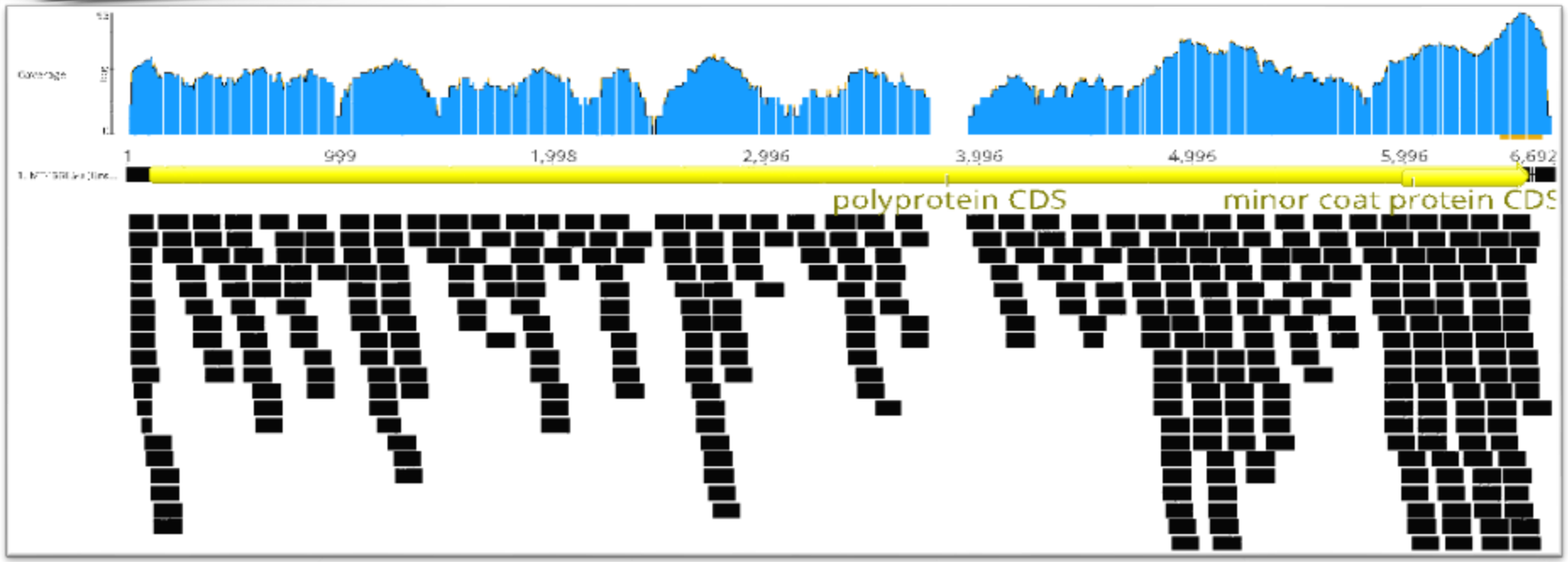
Análisis filogenético de un concatenado de cuatro genes de mantenimiento celular (*gyrB, gapA*, *gltA* and *rpoD) de cepas de Pseudomonas syringae* aisladas de kiwi

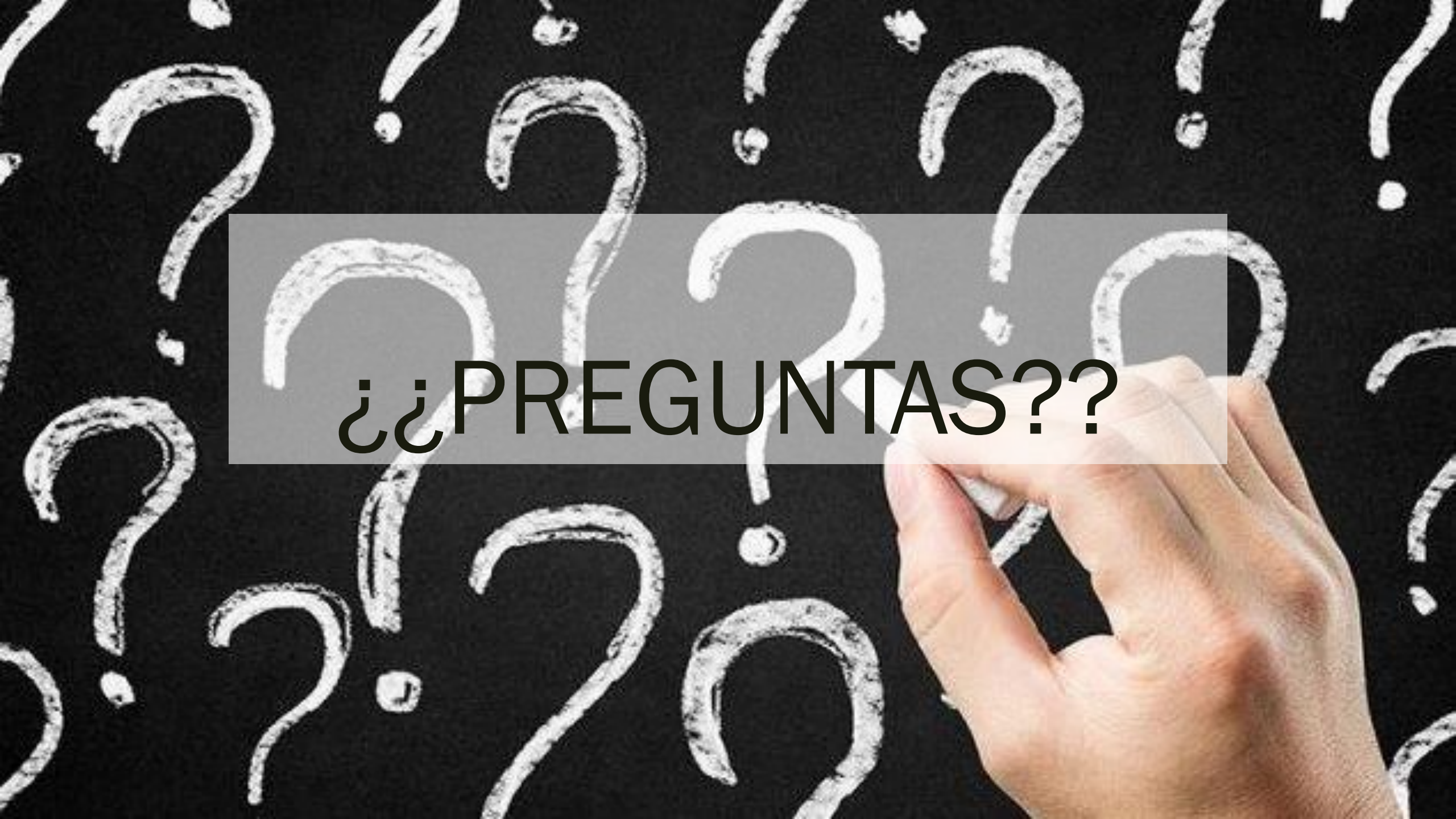
Este trabajo reveló la gran diversidad genética de *P. syringae* que afectan a kiwi y apoyó la hipótesis de que Pfm es un patógeno de baja virulencia, establecido desde hace mucho tiempo en algunas zonas de Europa y con varios linajes genéticos

Agronomy **2021**, *11*(12), 2464; https://doi.org/10.3390/agronomy11122464

Control de enfermedades emergentes

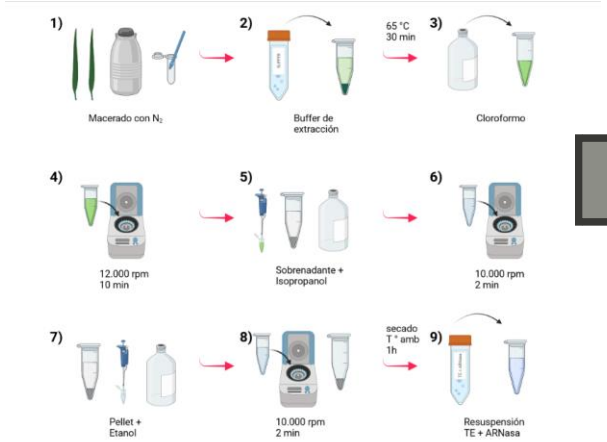


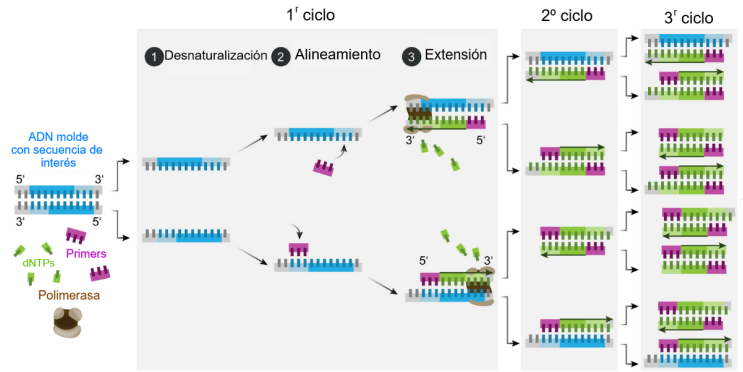


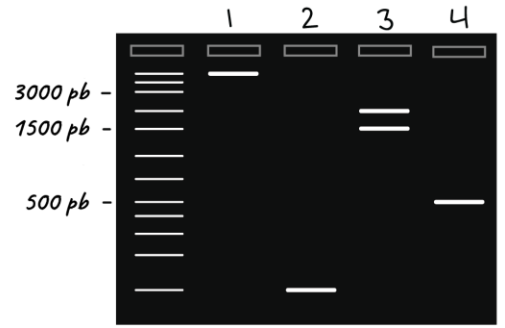

¿¿PREGUNTAS??

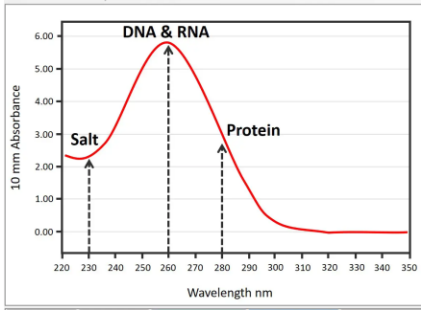
Obtención de datos de secuenciación

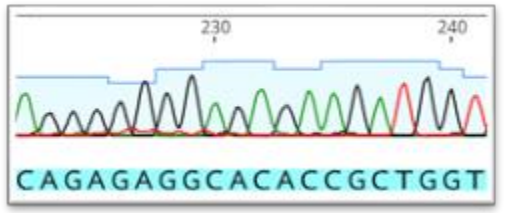
Extracción de ADN/ARN → RT-PCR PCR → Electroforesis → Purificación de Producto de PCR → Secuenciación Sanger







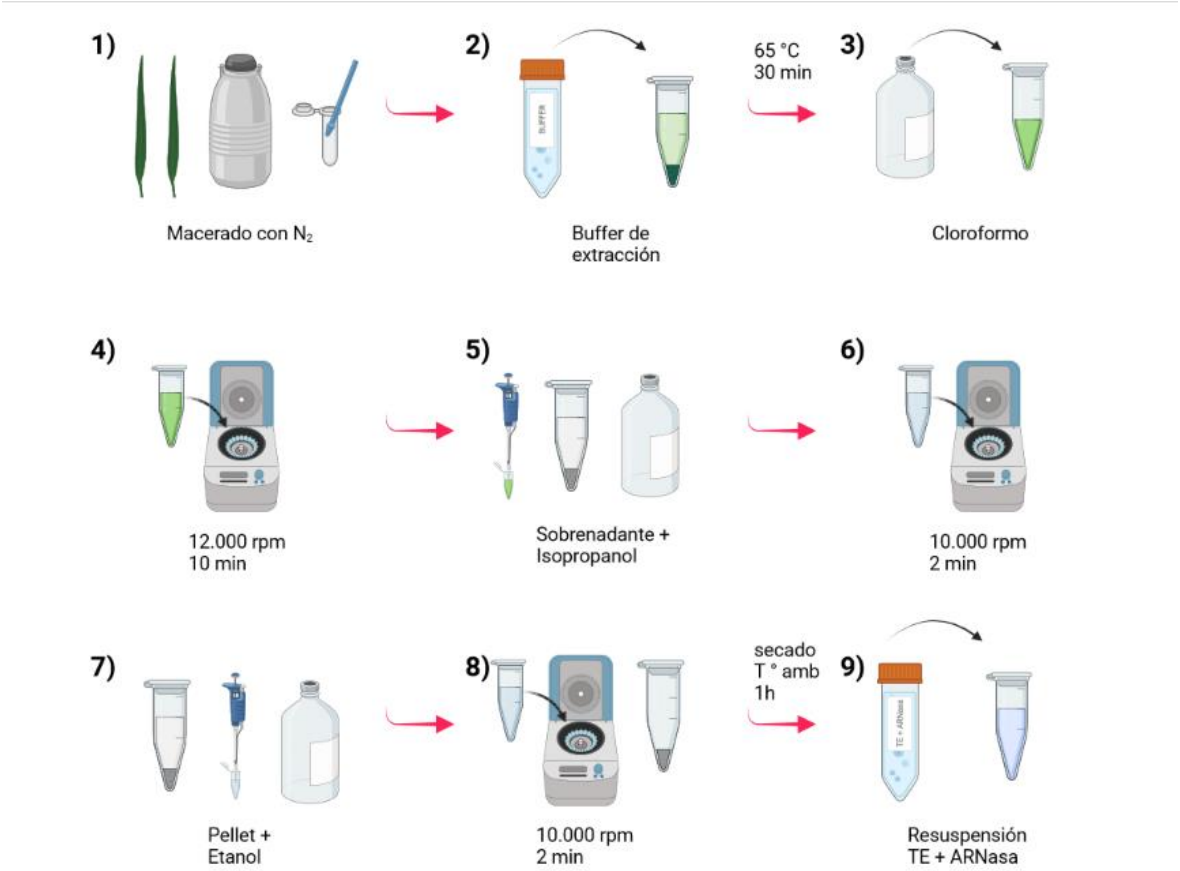




Obtención de datos de secuenciación

Extracción de ADN/ARN

- No difiere de protocolos de rutina usados en el diagnóstico
- Recomendación de protocolos validados
  - CTAB
  - *Kits* comerciales validados
- Consultar siempre PM 7 (EPPO)



Una mala purificación de los ácidos nucleico puede causar una incorrecta amplificación por PCR (falso negativo)

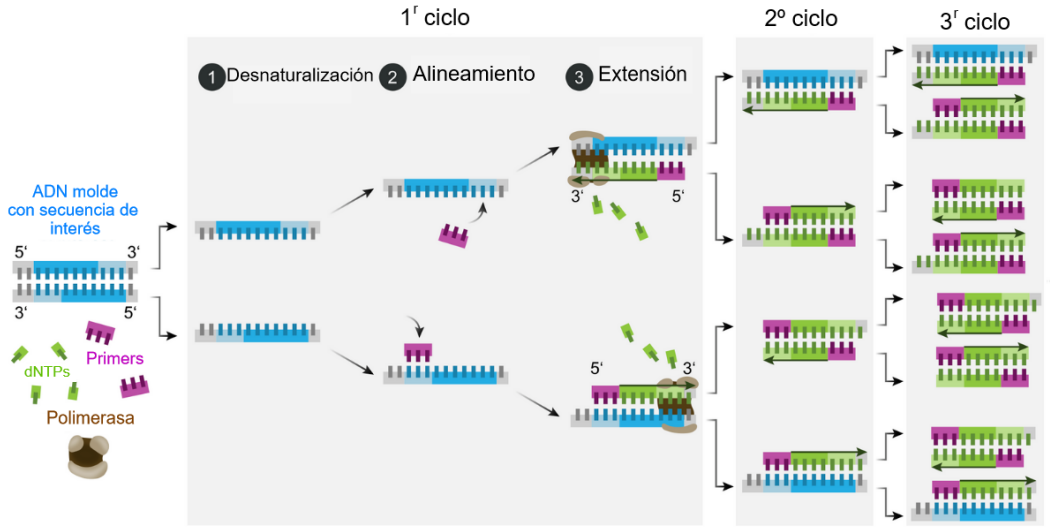
Obtención de datos de secuenciación

Extracción de ADN/ARN → RT-PCR → PCR

¿Cualquier pareja de cebadores sirve para secuenciar?

- Cebadores que amplifiquen regiones de interés
  - -Taxonómico
  - -Resistencias específicas
  - -Regiones codificantes o no codificantes
  - -Regiones con alta tasa de repeticiones
  - -Marcadores moleculares
  - -Plásmidos

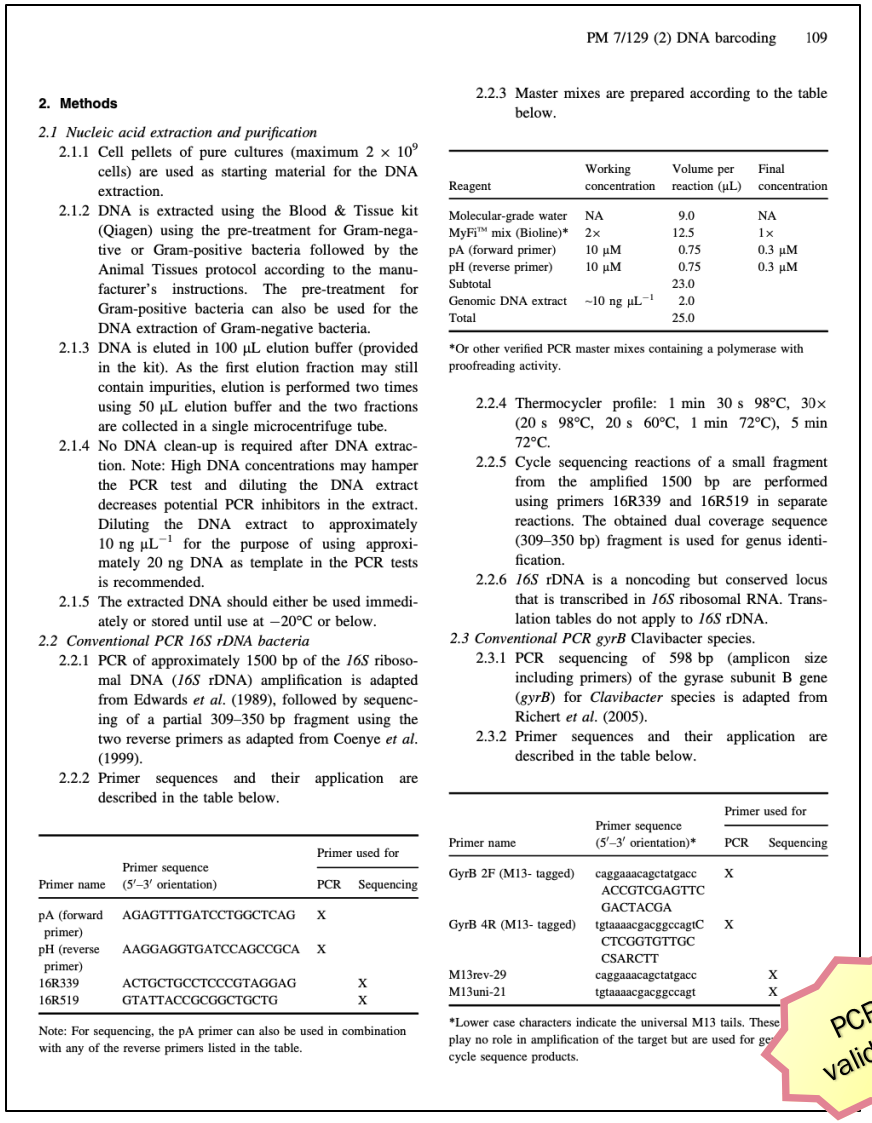
- Los protocolos de PCR empleados para la secuenciación deben estar correctamente validados



Obtención de datos de secuenciación

Extracción de ADN/ARN → RT-PCR PCR

- Los protocolos de PCR empleados para la secuenciación deben estar correctamente validados.
- Consultar los PM 7 de la EPPO.
- La amplificación y secuenciación de una región parcial que tenga escasa presencia en las bases de datos puede complicar la identificación del patógeno.



2. Methods

2.1 Nucleic acid extraction and purification

2.1.1 Cell pellets of pure cultures (maximum 2×10^9 cells) are used as starting material for the DNA extraction.

2.1.2 DNA is extracted using the Blood & Tissue kit (Qiagen) using the pre-treatment for Gram-negative or Gram-positive bacteria followed by the Animal Tissues protocol according to the manufacturer's instructions. The pre-treatment for Gram-positive bacteria can also be used for the DNA extraction of Gram-negative bacteria.

2.1.3 DNA is eluted in 100 µL elution buffer (provided in the kit). As the first elution fraction may still contain impurities, elution is performed two times using 50 µL elution buffer and the two fractions are collected in a single microcentrifuge tube.

2.1.4 No DNA clean-up is required after DNA extraction. Note: High DNA concentrations may hamper the PCR test and diluting the DNA extract decreases potential PCR inhibitors in the extract. Diluting the DNA extract to approximately 10 ng μL^{-1} for the purpose of using approximately 20 ng DNA as template in the PCR tests is recommended.

2.1.5 The extracted DNA should either be used immediately or stored until use at −20°C or below.

2.2 Conventional PCR 16S rDNA bacteria

2.2.1 PCR of approximately 1500 bp of the *16S* ribosomal DNA (*16S* rDNA) amplification is adapted from Edwards *et al.* (1989), followed by sequencing of a partial 309–350 bp fragment using the two reverse primers as adapted from Coenye *et al.* (1999).

2.2.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)	Primer used for	
		PCR	Sequencing
pA (forward primer)	AGAGTTTGATCCTGGCTCAG	X	
pH (reverse primer)	AAGGAGGTGATCCAGCCGCA	X	
16R339	ACTGCTGCCTCCCGTAGGAG		X
16R519	GTATTACCGCGGCTGCTG		X

Note: For sequencing, the pA primer can also be used in combination with any of the reverse primers listed in the table.

2.2.3 Master mixes are prepared according to the table below.

Reagent	Working concentration	Volume per reaction (µL)	Final concentration
Molecular-grade water	NA	9.0	NA
MyFi™ mix (Bioline)*	2×	12.5	1×
pA (forward primer)	10 µM	0.75	0.3 µM
pH (reverse primer)	10 µM	0.75	0.3 µM
Subtotal		23.0	
Genomic DNA extract	~10 ng μL^{-1}	2.0	
Total		25.0	

*Or other verified PCR master mixes containing a polymerase with proofreading activity.

2.2.4 Thermocycler profile: 1 min 30 s 98°C, 30× (20 s 98°C, 20 s 60°C, 1 min 72°C), 5 min 72°C.

2.2.5 Cycle sequencing reactions of a small fragment from the amplified 1500 bp are performed using primers 16R339 and 16R519 in separate reactions. The obtained dual coverage sequence (309–350 bp) fragment is used for genus identification.

2.2.6 *16S* rDNA is a noncoding but conserved locus that is transcribed in *16S* ribosomal RNA. Translation tables do not apply to *16S* rDNA.

2.3 Conventional PCR gyrB Clavibacter species.

2.3.1 PCR sequencing of 598 bp (amplicon size including primers) of the gyrase subunit B gene (*gyrB*) for *Clavibacter* species is adapted from Richert *et al.* (2005).

2.3.2 Primer sequences and their application are described in the table below.

Primer name	Primer sequence (5′–3′ orientation)*	Primer used for	
		PCR	Sequencing
GyrB 2F (M13- tagged)	caggaaacagctatgacc ACCGTCGAGTTC GACTACGA	X	
GyrB 4R (M13- tagged)	tgtaaaacgacggccagtC CTCGGTGTTGC CSARCTT	X	
M13rev-29	caggaaacagctatgacc		X
M13uni-21	tgtaaaacgacggccagt		X

*Lower case characters indicate the universal M13 tails. These play no role in amplification of the target but are used for ge cycle sequence products.

PCRs validadas

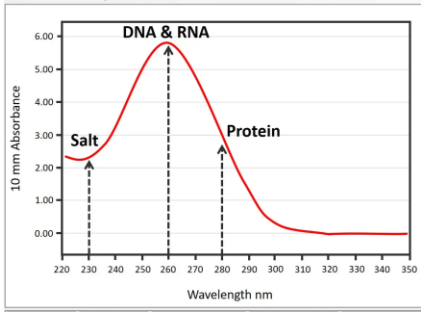
Obtención de datos de secuenciación

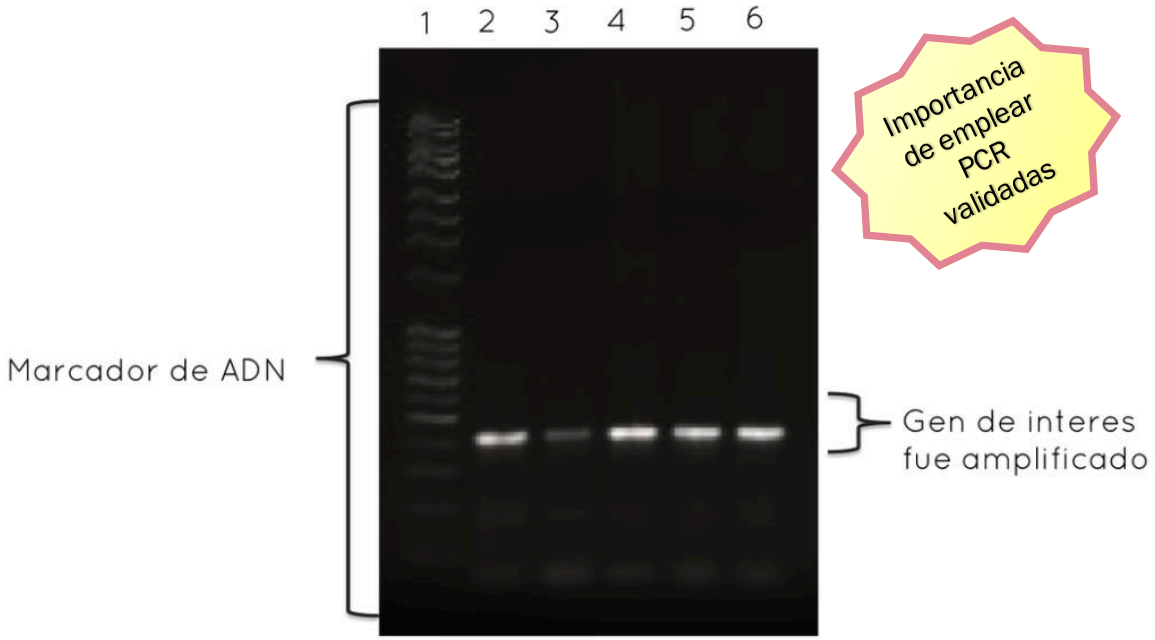
Extracción de ADN/ARN

RT-PCR

PCR

Electroforesis

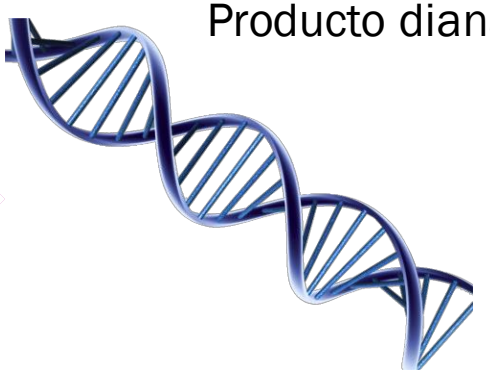




Importancia de emplear PCR validadas

Purificación de Producto de PCR

Producto diana



Esta foto de Autor desconocido

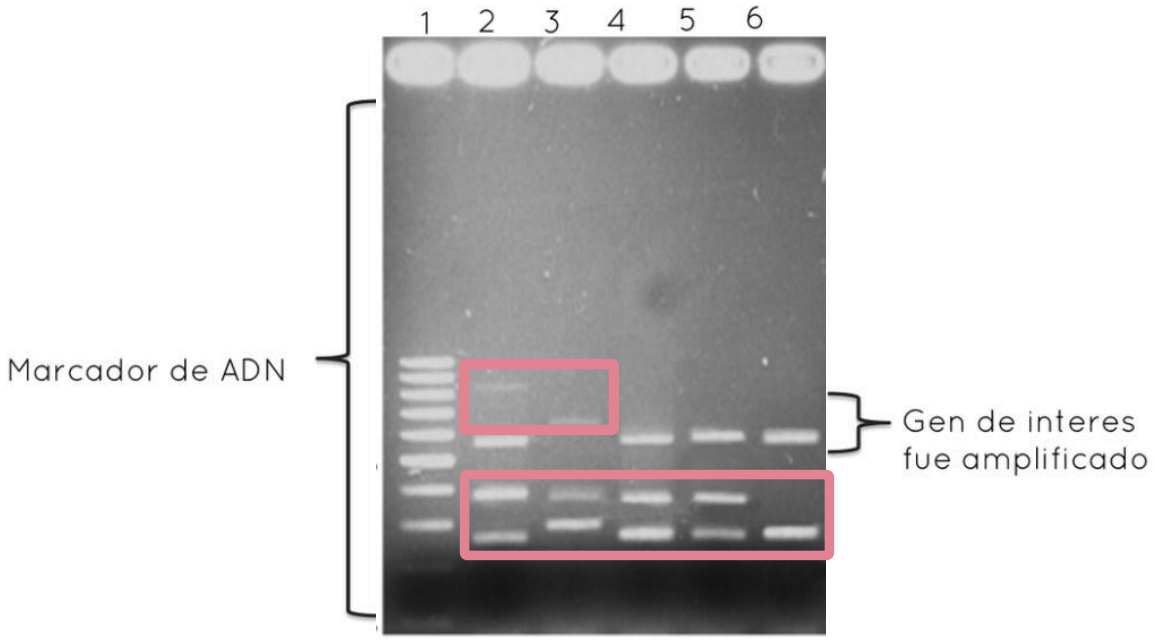
Obtención de datos de secuenciación

Extracción de ADN/ARN

RT-PCR

PCR

Electroforesis



Producto diana
+ otros productos NO diana

Purificación de Producto de PCR

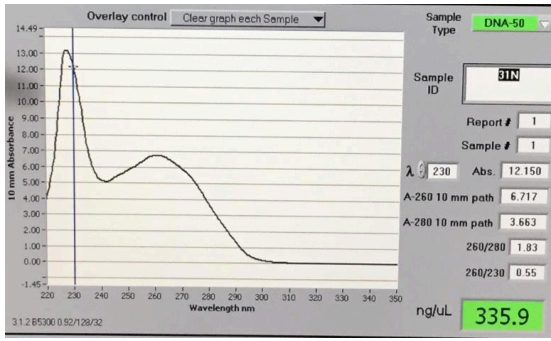
Obtención de datos de secuenciación

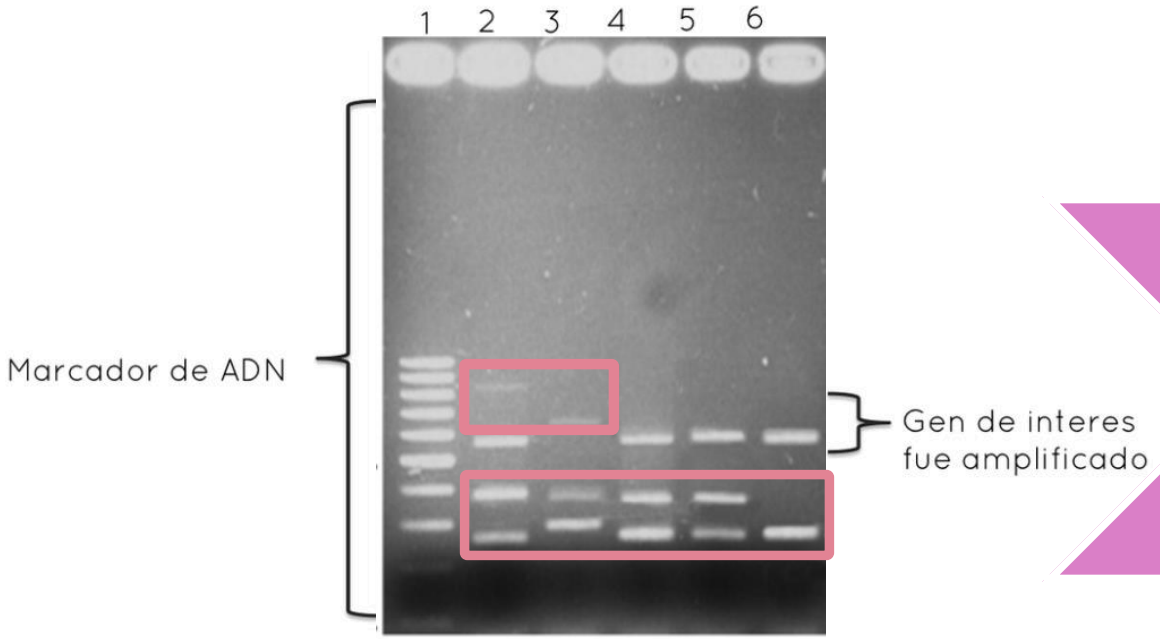
Extracción de ADN/ARN

RT-PCR

PCR

Electroforesis





Purificación de Producto de PCR Cortando Banda

Producto diana