Final Report

Project title (Acronym)
Assessment of a generic method for the detection of begomoviruses (BegomoVal)

Project duration:

Start date:	2019-10-01
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2 Short project report

2.1 Short executive summary

The genus *Begomovirus* (Family Geminiviridae) is the largest genus of plant viruses with more than 424 species (see https://talk.ictvonline.org/taxonomy). In Europe and especially in the Mediterranean basin, several members of this genus have mainly been detected on tomato, but also on others dicotyledonous crops of economic importance.
Early diagnosis of begomoviruses based on symptoms is not reliable as biotic and abiotic factors may induce similar symptoms. Serological tests (ELISA) can also be used, but with limited success due to low antigenicity of the viral coat protein (CP). No diagnostic test is available for the generic detection of begomoviruses. A Diagnostic Protocol for tomato leaf curl virus (TYLCV) and tomato mottle virus (ToMoV) (EPPO PM7/50) is available, but it was not evaluated for other begomoviruses. This project focused on the validation of diagnostic tests in accordance with the EPPO Standard PM7/98 *Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity* and was organised in accordance with the EPPO standard PM7/122 *Guidelines for the organization of interlaboratory comparisons by plant pest diagnostic laboratories*.
Four diagnostic tests were compared: the conventional PCR from Wyatt and Brown (1996) and optimised by the Dutch National Plant Protection Organization (M. Botermans, personal communication), the conventional PCR from Saison and Gentit (2015), the conventional PCR adapted from Li *et al.* (2004) and the conventional PCR from Accotto *et al*. (2000) currently included in the EPPO standard PM7/50 tomato yellow leaf curl and tomato mottle begomoviruses. Among the four methods evaluated, the test from Wyatt and Brown (1996) modified and the test from Saison and Gentit (2015) showed the best performances. The tests adapted from Li *et al*. (2004) and the test from Accotto *et al*. (2000) did not show good analytical sensitivity and did not allow to detect all isolates included in the test performance study panel.

2.2 Project aims

This project aimed to support the diagnosis of begomoviruses by validating several diagnostic tests. A test performance study was organized to evaluate the following tests:

1. Accotto *et al*. (2000);
2. Wyatt and Brown (1996) modified in 2002 according to M Botermans (pers. comm.);
3. Li *et al*. (2004) (adapted);
4. Saison and Gentit (2015).

The validation data was used to support the development of an EPPO Diagnostic Protocol for the generic detection of begomoviruses.

2.3 Description of the main activities

2.3.1 Test performance study (TPS) setup

The project partners gathered information on the various molecular tests available for the generic detection of begomoviruses. Ten tests were identified:

- Briddon *et al*. (2002)
- Briddon et Markam (1994)
- Deng *et al*. (1994)
- Li *et al*. (2004) (adapted)
- Rojas *et al*. (1993)
- Saison and Gentit (2015)
- Umaharan *et al*. (1998)
- Wyatt and Brown (1996) modified in 2002 according to M. Botermans (pers. comm.)
- Wyatt and Brown (1996) modified in 2012 according to Lecoq and Desbiez (pers. comm.)
- Wyatt and Brown (1996)

Each primer specificity was individually validated *in-silico* using BLAST tool and Geneious® software V11.1.2. Thereafter, the primers having the best matches in silico (i.e >90%) were tested in the wet lab. The selected tests were:

- Briddon *et al*. (2002)
- Briddon et Markam (1994)
- Li *et al*. (2004) (adapted)
- Saison and Gentit (2015)
- Wyatt and Brown (1996) modified in 2002 according to M. Botermans (pers. comm.)
- Wyatt and Brown (1996)

A restricted panel of 15 begomovirus samples was selected by partner ANSES to evaluate these tests. At the end of this pre-test, three protocols having a maximum of positives results (i. e. >70%) were selected:

- Wyatt and Brown (1996) modified in 2002 according to M. Botermans (pers. comm.)
- Li *et al*. (2004) (adapted)
- Saison and Gentit (2015)

For the organization of the TPS, these three tests were selected. The conventional PCR from Accotto *et al*. (2000) currently included in the EPPO standard PM7/50 *Tomato yellow leaf curl and Tomato mottle begomoviruses* was added as a reference.
Protocols were drafted by partner ANSES and sent to all partners to agree on the tests to consider for the TPS.
A panel of 30 (25 targets and 5 non-targets) DNA samples obtained from naturally and artificially infected leaves was selected for the TPS. The detailed composition of the panel is presented in the TPS report in the Appendix.

2.3.2 Organization of the TPS

Nine laboratories registered for the TPS from AT, FR, GB, GR, GT, IT, NL, PE, SI.
The homogeneity and stability of the samples was assessed using the conventional PCR from Saison and Gentit (2015). Raw data on the homogeneity study are presented in the TPS report in the Appendix.

Due to the COVID19 pandemic and availability of partners to receive the TPS material, panels were sent at different time. Partner ANSES performed intermediate stability tests before sending the panels, and at reception of the parcel by the TPS participants. Raw data on the stability study are presented in the TPS report in the Appendix. Only two samples (one in duplicate) were not considered stable and the results from these samples were not considered for the analysis of the TPS results.

2.3.3 Evaluation of the TPS

Results were interpreted for each laboratory and for each protocol by calculating the number of positive agreements, negative agreements, positive deviations and negative deviations. The results from the different participants (anonymised) and their analysis are presented in the TPS report in the Appendix.

2.4 Main results

Among the four tests evaluated, the test from Wyatt and Brown (1996) modified was able to detect all tested isolates (up to a 10^{-3} dilution) with a high repeatability and reproducibility.
The test from Saison and Gentit (2015) allowed the detection of all begomovirus isolates selected for the TPS but with a clear lower analytical sensitivity (up to a 10^{-2} dilution).
The tests of Li *et al*. (2004) and of Accotto *et al*. (2000) did not show good analytical sensitivity and did not allow to detect all isolates included in the TPS panel. More details on the results are presented in the TPS report in the Appendix.

2.5 Conclusions and recommendations to policy makers

The test performance study allowed to identify the protocols that give the best results for the largest range detection of begomoviruses. Regulations in many countries (e.g. 2019/829EC) recommends the detection of a large number of begomoviruses, so it is essential to have the most versatile test available. The consortium recommends the use of the test from Wyatt and Brown (1996) and the test from Saison and Gentit (2015) to comply with these regulations. An EPPO Diagnostic Protocol is currently being drafted that takes into account the project results. Evaluation of tests depends very much on the choice of the begomovirus isolates selected for the test performance study, thus the use of the tests on new regulated or emerging begomovirus species would have to be evaluated before their use in routine diagnostic activities.

2.6 Benefits from trans-national cooperation

The collaboration allowed to gather a collection of begomovirus samples available at the premises of the project partners for the TPS. The project demonstrated the usefulness of extensive comparative laboratory testing for protocol validation. Although not very widespread in the field of plant pathology, they are recommended by different regional and international organizations in plant health. These studies are essential to provide guidance for reliable detection of a given pathogen. Collaboration allowed the consortium to join effort for the drafting of an EPPO Diagnostic Protocol and to make easier future collaboration between those partners that worked together for more than 2 years.

3 Publications

3.1 Article(s) for publication in the EPPO Bulletin

None.

3.2 Article for publication in the EPPO Reporting Service

None.

3.3 Article(s) for publication in other journals

In preparation.

4 Open Euphresco data

Validation data are available in the EPPO Database on diagnostic expertise.

Annex I Detailed TPS report

Plant Health Laboratory

National reference laboratory

Mandate: All viruses except viruses on bananas, tropical plants, Sharka (PPV), viruses on potatoes and citrus.

TEST PERFORMANCE STUDY (TPS)

19BEGOMOVAL

Test performance study (TPS) of molecular polyvalent detection methods for Begomoviruses

Session : 2020

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TEST CODE : 19BEGOMOVAL

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1 Introduction

The genus *Begomovirus* (Family *Geminiviridae*) is the largest genus of plant viruses (424 species) (see https://ictv.global/taxonomy/). Under electron microscopy, the begomoviruses virion form a typical twinned shape particle ("geminate"). The genome is a monopartite (DNA-A) or bipartite (DNA-A and DNA-B) circular ssDNA and might be associated with DNA satellite (α or β) or defective components. Monopartite are mainly distributed in the old world whereas bipartite are in the New world.
Begomoviruses infect a wide range of economically important dicotyledonous plants and represent an emerging problem worldwide, due to the large distribution of their vectors such as *Bemisia tabaci* in all vegetable producing areas (open field and greenhouse).
Begomoviruses represent a severe threat of agronomic and horticultural crops in many countries and historically in Americas and Asia regions. Due to the expansion of their main vector in these last decades, the Begomoviruses extend the range of their distribution in others places like Africa and Europe.
In the EPPO region, the oldest reports go back to the eighties, while these viruses continue to infect sporadically with a variable intensity different countries (see EPPO global database). Several members of this genus have mainly been detected on tomato, but also on others crops of economic importance especially in the Mediterranean basin.
Tomato yellow leaf curl virus (TYLCV) and its variants are probably the most harmful and widespread members of the genus *Begomovirus*. This virus is widespread across the globe and associated with the damaging tomato yellow leaf curl disease. Although there is some genetic variability, all isolates found in the EPPO region belong to four species: TYLCV, Tomato yellow leaf curl Sardinia virus (TYLCSV), Tomato yellow leaf curl Axarquia virus (TYLCAxV) and Tomato yellow leaf curl Malaga virus (TYLCMaV).
Recently, another Begomovirus Tomato leaf curl New Delhi virus (ToLCNDV) has been officially reported in several Mediterranean countries, i.e. Algeria, France, Greece, Italy, Morocco, Portugal, Spain, and Tunisia but also in Estonia.
At least two other Begomoviruses - which are currently absent in Europe - should be considered as a potential threat to tomato production: Tomato mottle virus (ToMoV) and Tomato leaf curl virus (ToLCV).

In this context and through a Euphresco project named Begomoval, the plant health laboratory organised this TPS. Its objective was to compare and identify a conventional PCR method leading to the detection of a wide range of Begomoviruses. In this purpose, four different methods were evaluated: Wyatt *et al* (2000) modified in 2002 according to M Botermans (pers comm), Accotto *et al*. (2000); Li *et al*.(2004) and Saison *et al*. (2015).

2 General information

2.1 Nature and purpose of the test performance study

The PHL organized this test performance study for the detection of Begomoviruses on host plants, in which French or foreign laboratories were invited to participate.

This test performance study aimed to evaluate among four detection methods for the detection of Begomoviruses which is the one having the most extended range of detection with the highest sensitivity and specificity.
The **accuracy** of the results was evaluated through:

- The capacity to obtain positive results from positive samples (**sensitivity**) and negative results from negative samples (**specificity**);
- The capacity to obtain the same qualitative and quantitative result from identical samples analysed under conditions of repeatability (**repeatability**).
- The capacity to obtain the same qualitative result from identical samples analysed under conditions of reproductibility (**reproductibility**).

2.2 Organiser of the interlaboratory test

The TPS is organised by the Anses Plant Health Laboratory (Anses-Laboratoire de la santé des végétaux, 7 rue Jean Dixméras, 49044 ANGERS CEDEX 01, France). The Anses Plant Health Laboratory is the French national reference laboratory for the detection of Begomoviruses in host plants. Pascaline COUSSEAU and her substitute Pascal GENTIT ensured the coordination of this study.

2.3 Participating laboratories

Following the call for applications for this TPS, 9 laboratories were registered. In this report, they are coded with the LXX format (XX being a two-digit number) to ensure the confidentiality of the results. The typology of participants is presented in **Table 1**.

Categories	Number
French national reference laboratories (NRL)	1
Other laboratories	8
TOTAL	9

Table 1: Categories of the participants

2.4 Information to participants

The participating laboratory agreed to perform analyses in its laboratory according to the instructions of the organiser (participants' contract), and in its usual conditions of work. A technical sheet describing the implementation of the proficiency test was provided to the participants before their registration.

The participants received the parcel containing the samples together with:

- an instruction sheet describing the instructions for the sample storage, the analysis, and the result submission;
- an acknowledgement of receipt form for reporting to the organiser any problem concerning the sample integrity;
- a result form to standardise presentation of results.

2.5 Framework of the interlaboratory study

This TPS was conducted according to the schedule summarised in the **Table 2**.

Stages	Deadlines
Call for applicants	18th of February 2020
End of registration	6th of March 2020
Sample dispatch*	4th of June 2020 (6 parcels) 6th of July 2020 (2 parcels) 28th of September 2020 (1 parcel)
Deadline for performing analyses*	1st of July 2020 (6 parcels) 4th of September 2020 (2 parcels) 16th of November 2020 (1 parcel)
Deadline for submitting results*	8th of July 2020 (5 parcels) 4th of September 2020 (3 parcels) 20th of November 2020 (1 parcel)
Final report and individual summary sheets transmitted to the participants	End of 2020

Table 2: Schedule of the Test performance study n°19Begomoval.

*Due to the COVID19 pandemic situation all over the world, the organisers accepted some delay during the TPS. The subsequent panels were treated with regard to the changes dates.

3 Samples

3.1 Composition of the panel

A panel of 30 coded (25 target and 5 non-target) samples of DNA extract obtained from natural or artificially infected leaves were prepared in 1.5 mL Eppendorf tubes and sent to each participant. The detailed composition of each panel is presented in the **Table 3**.

Sample Id	Type	Name of virus	Isolate	Concentration	Assigned value
A	target	Abutilon mosaic virus (bipartite)	17/87 AbMV	1	Positive
B	target	African cassava mosaic virus (bipartite)	17/22.2 ACMV	1	Positive
C	target	Bean golden mosaic virus (bipartite)	17/23.2 BGMV	1	Positive
D	target	Chili leaf curl virus (monopartite)	17/104.1 ChiLCV	1	Positive
E	target	Pepper golden mosaic virus (bipartite)	17/104.3 PepGMV	1	Positive
F	target	Potato yellow mosaic virus (bipartite)	16/257.4 PYMV	1	Positive
G	target	Tomato leaf curl Mali virus (bipartite)	EL 20 30031 ToLCMLV	1	Positive
H	target	Tomato leaf curl New Delhi virus (bipartite)	14/433 (index EL35) ToLCNDV	10^{-2}	Positive
H	target	Tomato leaf curl New Delhi Virus (bipartite)	14/433 (index EL35) ToLCNDV	10^{-2}	Positive
I	target	Tomato leaf curl New Delhi virus (bipartite)	14/433 (index EL35) ToLCNDV	10^{-3}	Positive
I	target	Tomato leaf curl New Delhi virus (bipartite)	14/433 (index EL35) ToLCNDV	10^{-3}	Positive
J	target	Tomato mottle virus (bipartite)	14/14 (EL28) ToMoV	1	Positive
K	target	Tomato severe rugose virus (bipartite)	EL6 00/749A B ToSRV	1	Positive
L	target	Tomato yellow leaf curl virus (monopartite)	16/34.2 et .3 TYLCV	10^{-2}	Positive
L	target	Tomato yellow leaf curl virus (monopartite)	16/34.2 et .3 TYLCV	10^{-2}	Positive
M	target	Tomato yellow leaf curl virus (monopartite)	16/34.2 et .3 TYLCV	10^{-3}	Positive
M	target	Tomato yellow leaf curl virus (monopartite)	16/34.2 et .3 TYLCV	10^{-3}	Positive
N	target	Tomato yellow leaf curl virus (monopartite)	11/480 TYLCV	1	Positive
O	target	Tomato leaf curl virus Comores (monopartite)	12/49 (EL25) ToLCYTV	1	Positive
P	target	Tomato yellow leaf curl virus Sardinia (monopartite)	EL18 09/12/98 TYLCSV	1	Positive
Q	target	Tomato yellow leaf curl virus (monopartite)	16/116.3 TYLCV	1	Positive
R	target	Sri Lankan cassava mosaic virus (bipartite)	19/182 SLCMV	1	Positive
S	target	Sri Lankan cassava mosaic virus (bipartite)	19/183 TYLCTHV	1	Positive
T	target	African cassava mosaic virus (monopartite)	19/184 ACMV	1	Positive
U	target	Watermelon chlorotic stunt virus (bipartite)	19/185 WmCSV	1	Positive
V	Non target	Banana bunchy top virus (Babuvirus)	17/74 BBTV	1	Negative
W	Non target	Maize streak virus (Mastrevirus)	17/75 MSV	1	Negative
X	Non target	Pea necrotic yellow dwarf virus (Nanovirus)	17/86 PNYDV	1	Negative
Y	Non target	Pepino mosaic virus (Potexvirus)	CH2 serre PepMV	1	Negative
Z	Non target	Tomato chlorosis virus (Crinivirus)	14/003 ToCV	1	Negative

Table 3: Target and non target DNA extracts prepared and included in each panels (*see section 3.3)

The composition of the panel was chosen to allow the evaluation, from the qualitative results, of the following performance criteria (see section 5 and **Table 4**):

- Sensitivity: presence of 25 target samples;
- Specificity: presence of 5 non target samples;
- Repeatability: each diluted sample was sent in duplicate and all samples were analysed in replicate;
- Accuracy: this summarizes the three above criteria;
- Reproducibility: identical samples for different participants.

Samples of the panel	Evaluated criteria				
	Sensitivity	Specificity	Repeatability	Accuracy	Reproducibility
Sample A	X		X	X	X
Sample B	X		X	X	X
Sample C	X		X	X	X
Sample D	X		X	X	X
Sample E	X		X	X	X
Sample F	X		X	X	X
Sample G	X		X	X	X
Sample H	X		X	X	X
Sample H	X		X	X	X
Sample I	X		X	X	X
Sample I	X		X	X	X
Sample J	X		X	X	X
Sample K	X		X	X	X
Sample L	X		X	X	X
Sample L	X		X	X	X
Sample M	X		X	X	X
Sample M	X		X	X	X
Sample N	X		X	X	X
Sample O	X		X	X	X
Sample P	X		X	X	X
Sample Q	X		X	X	X
Sample R	X		X	X	X
Sample S	X		X	X	X
Sample T	X		X	X	X
Sample U	X		X	X	X
Sample V		X	X	X	X
Sample W		X	X	X	X
Sample X		X	X	X	X
Sample Y		X	X	X	X
Sample Z		X	X	X	X

Table 4: Identification of the samples used for the evaluation of the different performance criteria.

3.2 Codification of the samples

Each sample was coded from 01 to 30 on the following basis: TPS code -panel code-sample code. *e.g. 19-BEGO-L01-05: sample N°5 of panel 1.*
The coding was assigned randomly to each participant (and also for extra panels). These precautions were intended to allow participants to perform blind analyses and to ensure their confidentiality.

3.3 Validation of the samples

To ensure that the TPS was reliable, samples were validated in terms of status (assigned value), homogeneity and stability.

3.3.1 Assigned value

The assigned value is the value attributed to a particular property of a panel sample. This TPS concerns a qualitative method. So, the assigned value is qualitative and corresponds to the status attributed to the sample: “positive” or “negative” (the target samples were chosen above the required detection level, consequently, the uninterpretable status was judged inappropriate).

The samples used during these assays are reference material issue from the PHL-UBVO (ANSES) and DSMZ and previously tested with the end-point PCR of Saison *et al.* (2015, internal method), Wyatt *et al.* (1996) modified, Accotto *et al.* (2000) and Li *et al.* (2004). The assigned value of samples (status assigned to the samples) resulted from the experimental work of the ANSES. It was independently defined from the participants’ results.

The experimental works of the ANSES allow establishing:

- an *a priori* assigned value based on the analytical characterisation of the reference materials used to produce the test matrices and the manufacturing by formulation of the test matrices.
- confirmed during the homogeneity study by repeated analyses on the samples packaged in their final form.

3.3.2 Homogeneity

The assessment of homogeneity was performed with the end-point PCR of Saison *et al* (2015) and after the samples had been packaged in their final form and before their distribution to participants.

The homogeneity was assessed for:

- 4 tubes (amplification repeated twice) for each batch of non-diluted target sample production and for each batch of non-target samples of the test;
- 10 tubes (amplification repeated twice) by batch for batches of diluted target samples.

The analytical method used for homogeneity study being qualitative. Qualitative results (homogeneity status) were used for the analysis of homogeneity results. Samples were tested the 6th of March 2020. Raw data of the homogeneity study are available in **appendix** 1.

All samples used during this study to evaluate the analytical specificity can be considered as homogeneous.

3.3.3 Stability

The stability was assessed for all samples with the end-point PCR of Saison *et al* (2015) and after their packaging in their final form. The stability was performed on 2 tubes of each batch of non-diluted target and non-target sample production.
The analytical method used for stability study being qualitative, qualitative results (stability status) were used for the results.

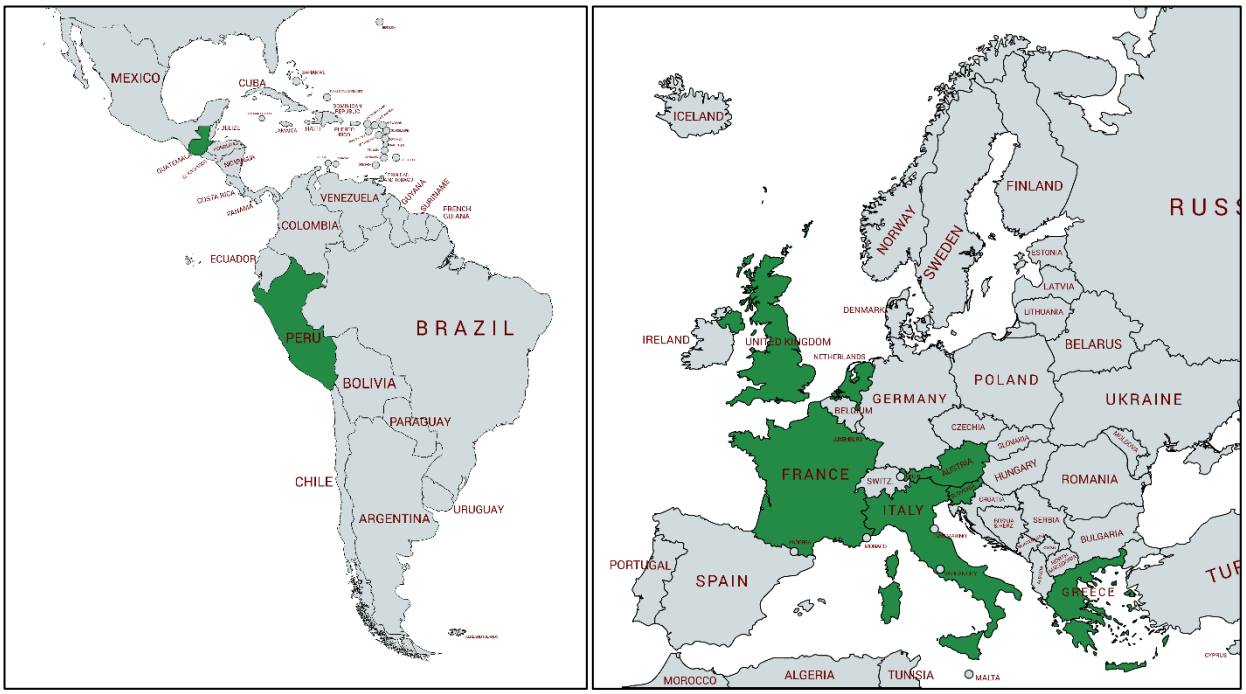
Due to the COVID19 pandemic and availability of partners to receive their panel, panels were send at different time (see 4.2). So, to insure sample stability at the time of sending, we did an intermediate stability. Then we did the last stability after all partners have received their parcel.

Samples were tested the 29th of May 2020 for the intermediate stability (Appendix 2). All samples used during this study were considered stable. The final stability was performed the 5th of November 2020 (Appendix 2bis). Apart from two samples, all the samples used during this study can be considered as stable. The sample I (duplicated) corresponding to the most important level of dilution (1×10^{-3}) of the ToLCNDV target and sample S with a low concentration were not considered as stable. The results of those 3 samples were excluded from the following analysis of data.

4 Practical implementation of the interlaboratory test

4.1 Registration

The call to applicants for participating in the begomoviruses TPS was launched in February 2020 and closed the 06th of March 2020. Nine laboratories registered within the deadline.
9 participants registered to participate to the TPS. There were mostly from European Union (6) and 3 from outside the EU.



4.2 Shipment and receipt of the parcels

The TPS panels were dispatched from Angers (France) by express shipping service in different times due to the covid19 pandemic:

- The 04th of June 2020: France Express for laboratory in France and DHL for 5 foreign laboratories. For these 6 participants, the packages were received in less than 10 days.
- The 06th of July 2020: DHL for 2 foreign laboratories. One participant received its package in less than 10 days. The other participant received its package in 14 days.
- The 28th of September 2020: DHL for 1 foreign laboratories. This package was arrived the 7th of October 2020.

4.3 Sample conditions

The samples were shipped at ambient temperature. All laboratories indicated to have received the samples in good condition.
The L02 participant indicated that the samples number 1, 2, 23, 28 and 29 contained not enough extract to perform the method of Li *et al*. (2004). Complementary DNA extracts were sent to this participant the 3rd of August 2020.
The L08 participant indicated that samples did not contain enough DNA extracts to replicate each sample for each method. This laboratory did not make replicate.

4.4 Delay for analysing the samples and submitting the results

For the first sending, the deadline for performing analyses was set on the 1st of July 2020 and the deadline for submitting the results was set on the 8th of July 2020.
For the second sending, the deadline for performing and submitting analyses was on the 4th of September 2020.
For the last sending, the deadline for performing and submitting analyses was on the 16th of October 2020.

The L08 participant asked for a delay to send its results. They have performed the analyses the 13rd of November 2020. Their results were received the 26th of November 2020. All the other laboratories respected the deadlines.

4.5 Studied methods

The objective of this TPS was to compare and identify a conventional PCR methods leading to the detection of a wide range of Begomoviruses. In this purpose, four different methods were evaluated: Wyatt *et al* (1996) modified in 2002 according to M Botermans (pers comm), Accotto *et al*. (2000), Li *et al*. (2004) and Saison *et al*. (2015) (See protocols in Appendix 3).

All participants implemented these four methods according to the organizer's instructions with the following modification (**Table 5**):

- L06, L07, L08 and L10 did not analyse samples in duplicate.
- L10 did not perform the ramping cycles for the PCR Wyatt et al (1996) modified and Li et al (2004). This participant did not obtained any amplification with the Accotto et al (2000) method.
- L08, L09 and L10 did not used the recommended amplification kit.
- L09 did not used the recommended final volume of amplification (10 µL vs 25µL) and DNA extract volume (1µL vs 2µL).

Laboratories	L01	L02	L03	L05	L06	L07	L08	L09	L10
Amplification Kit	Invitrogen Platinum Taq DNA polymerase (lot 2129415)	Invitrogen Platinum Taq DNA polymerase (lot 2129416)	Invitrogen- Platinum Taq DNA polymerase (lot 202419)	invitrogen Platinum Taq DNA Polymerase (lot 2129416)	Invitrogen, Platinum Taq DNA Polymerase (Lot 208644)	Invitrogen Platinum Taq DNA Polymerase, (lot 2177288)	GoTaq Flexi DNA Polymerase, PROMEGA (lot 396121)	Solis Biodyne, 5x FirePol Mastermix	Jumpstart Redtaq Readymix - Sigma (P0982)
Comments			Duplicate were not perform in the same day		No duplicate	No duplicate	No duplicate	1µL DNA extract	1-No ramping for Wyatt and Li methods. 2-No duplicate
Final volume per well	25µL	25µL	25µL	25µL	25µL	25µL	25µL	10µL	25µL

Table 5: Summary of the implementation of the test by the different laboratories. The divergent modalities of the initial protocol are indicated in red.

5 Analysis of results

Each method was evaluated according to the qualitative results submitted by the participants. These results are shown in the **appendices** 4 to 7 and the photos of agarose gels are shown in the **appendices** 8 to 11.

5.1 Statistical criteria used to interpret the results

The results were interpreted for each laboratory and for each method by calculating the number of positive agreements (PA), negative agreements (NA), positive deviations (PD) and negative deviations (ND), according to the **Table 6**.

Reference / Laboratory	Positive assigned value	Negative assigned value
Positive laboratory result	PA = positive agreement	PD = positive deviation
Negative laboratory result	ND = negative deviation	NA = negative agreement
undetermined laboratory result	ND = negative deviation	PD = positive deviation

Table 6: Definition of parameters.

The same interpretation was used when participant attributed the result "undetermined" to a sample. The interpretation was always in disfavour of the test. If the expected result was positive (target), an undetermined result was interpreted as negative deviation whereas if the expected result was negative (non-target), an undetermined result was interpreted as positive deviation. In summary, inconclusive results were estimated as non-concordant.
These parameters were used to calculate the performance criteria defined in the **Table 7**.

Performance criteria	Definition	Calculation
Sensitivity (SE)	Closeness of agreement between the test result and the assigned value for samples for which the assigned value is positive (definition which has been adapted from **EN ISO 16140-1**).	SE= sum PA /N+ x 100% Comments: the result of the calculation (1-SE) gives the number of false negatives obtained by the laboratory.
Specificity (SP)	Closeness of agreement between the test result and the assigned value for samples for which the accepted assigned value is negative (definition of which has been adapted from **EN ISO 16140-1**). *Comments: as far as possible, the evaluation of specificity must include:* *-healthy samples, i.e. symptomless samples not contaminated by any particular non-target organism. These healthy samples aim to check the absence of interference with the plant matrix.* *-samples contaminated by non-target organisms that can be found in routine samples and that can interfere (non-target plant pathogens attacking the same host plant or saprophytic organisms naturally present in the plant).*	SP= sum NA/N- x 100% Comments: the result of the calculation (1-SP) gives the number of false positives obtained by the laboratory.
Repeatability	Closeness of agreement between independent test results obtained under conditions of repeatability *i.e.* conditions under which independent test results are obtained by the same method, on identical test. Samples in the same laboratory, by the same operator, using the same equipment, within a short period of time (**ISO 5725-1**).	= Percentage chance of obtaining the same result (positive, negative) from two identical samples analysed in the same laboratory. Reference of the calculation: adapted from Van der Voet et *al.* (2004).
Accuracy	Closeness of agreement between the test result and the assigned value (definition of which has been adapted from **EN ISO 16140-1**). It is worth noting that the accuracy is a global criterion which can be subdivided, to refine the analysis, into three other criteria: sensitivity, specificity and repeatability.	AC= (sum PA + sum NA)/N
Reproducibility	Closeness of agreement between independent test results obtained under conditions of Reproducibility *i.e.* conditions under which independent test results are obtained by the same method, on identical test in different laboratories, with different operators and using different equipment **(ISO 5725-1**). We are in conditions of maximum variation.	= Percentage chance of obtaining the same result (positive, negative) from two identical samples analysed in two different laboratories. Reference of the calculation: adapted from Van der Voet et *al.* (2004).

Table 7: Definition and calculation of performance criteria.
N=total number of samples. N+ = number of samples for which the accepted reference value is positive. N- = number of samples for which the accepted reference value is negative.

5.2 Requirements for analysis of data

The performance criteria of each method that must be reached were not predefined. This study should lead to verify and specify the performance criteria obtained during the different validation trials conducted at the PHL.

The ISO 16140 standard (ISO, 2016) stipulates that collaborative studies should be based on data from laboratories with high competence on the techniques that are being compared. Consequently, results of a participant can be excluded for a given method (considered as outliers *i.e.* far removed from the rest of the laboratories), as example, (i) when the expected result for at least one control is not obtained or (ii) when the number of ND or PD results obtained by this laboratory represent more than 40% of ND or PD results obtained for the method and when ≥ 50% of ND or PD results is recorded from the panel of samples.

6 Assessment of performance criteria of each method

Results from the different participants are available in **appendices** 4 to 7. According to the previous statements listed in the section 5.1.2 and the fact that this participant being too far from the recommended protocol (different volume of amplification different volume of extract and different Taq polymerase), the results of the L09 participant have been excluded from the results taken in consideration for the final calculation of methods performance criteria.
Due to the results of the homogeneity, three samples (sample I in duplicate and sample S) were not included in the exploitation of the results from the different participants. In conclusion, the full data set consisted of 8 participants (L01, L02, L03, L05, L06, L07, L08 and L10) and 27 samples (5 non-targets and 22 targets).

6.1 Results by targets

The performance of each evaluated tests was analysed through their capability to detect each target and non-target samples. The full set of results are displayed in **appendices** 4 to 7. Number of concordant results for each of the 27 samples (targets and non-targets) and for each method is represented on the stacked bar chart (Figure 1) and on the **Table 8**.

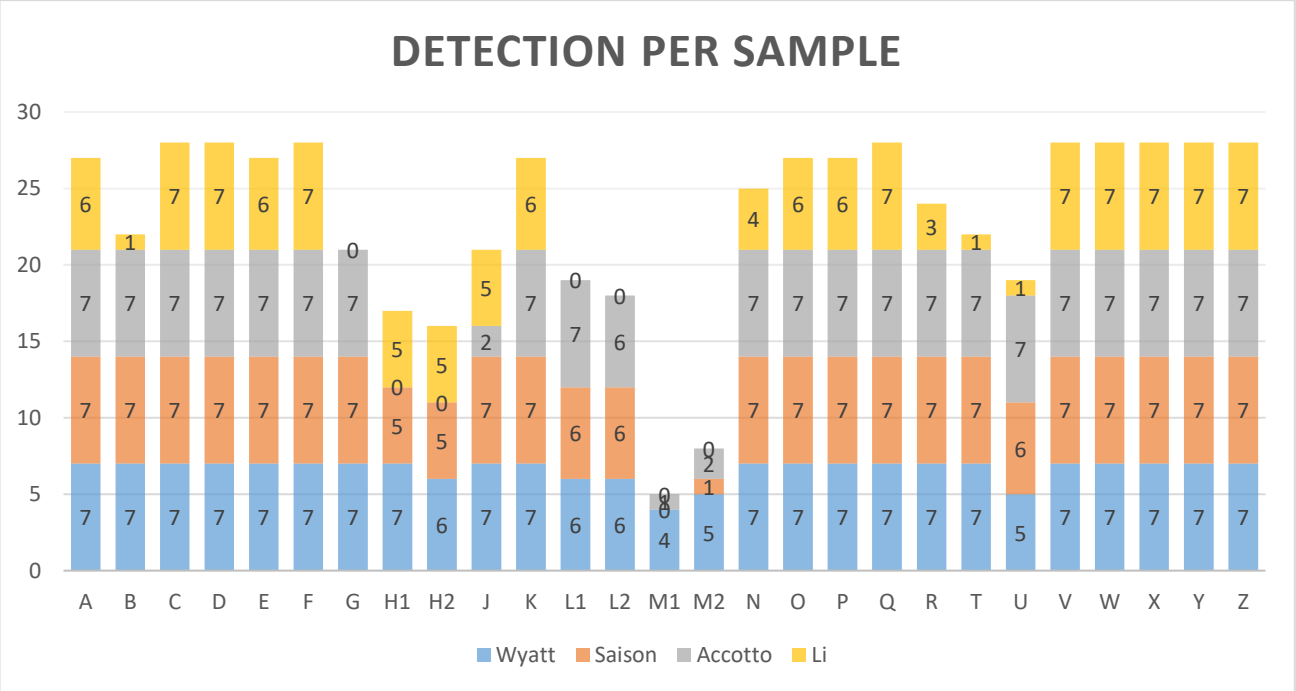


Figure 1: Stacked bar chart representing the number of concordant for each sample and for each method.

For the non-target samples (V to Z), the method from Accotto et al (2000) gave a full concordance with all non-target samples. The method from Li et al (2004) gave one false positive (undetermined results according to the participant L06). The detailed analysis of the gel from the L06 participant (in **appendix** 10) revealed a pattern of several bands without bands at the expected size (sample X or L06-02). The method from Saison et al (2015) gave two non-concordant results for the L06 participant. On the gel (in **appendix** 11), one sample (W or L06-13) correspond to a clear band at the expected size, not observed with any of the rest of the participants or methods. Therefore, we considered this result as a doubtful non-concordant result. The other sample (V or L06-04) gave a faint band on the gel and was considered as undetermined. The method from Wyatt et al (1996) modified gave two false positive. One from the participant L06 for the sample X (or L06-02). The detailed analysis of the gel from the L06 participant (in **appendix** 8) revealed a pattern of several bands without bands at the expected size. Another one

corresponds to the sample W (or L05-18) for the participant L05. The detailed analysis of its gel revealed a faint band above the expected size and could not be considered as concordant results.
In conclusion, for the five non-target samples, 3 methods gave non concordant results. The detail analysis of the results per sample revealed that among the 5 non-concordant results, 4 could be considered as misinterpretation of the gel or mistake during the manipulation and 1 presenting faint band might be considered as undetermined. According to the previous statement (§5.2), the results of the laboratory L06 was excluded for 3 out of 4 methods. The alone dataset obtained by the participant L06 for the method Accotto et al (2000) was considered.

For the target samples, none of the method allowed the detection of all samples (**Table 8**). If we considered the sensitivity of the methods, the Wyatt et al (1996) modified and Saison et al (2015) methods detected all undiluted DNA extract species. The Accotto et al (2000) did not detect the undiluted species Tomato mottle virus (ToMoV) and the diluted species Tomato leaf curl New Dehli virus (ToLCNDV) species. The Li et al (2004) method did not detect the Tomato yellow leaf curl virus (ToLCMLV) and the diluted species of Tomato yellow leaf curl virus isolate (TYLCV). This method detected with a low sensitivity the following species isolates: African cassava mosaic virus, Sri Lankan cassava mosaic virus, African cassava mosaic virus and Watermelon chlorotic stunt virus.
If we considered the diluted samples (H, L, M), the Wyatt et al (1996) modified method detected 34 replicates out of 42 (81%) with a detection of all sample at least once (min 4 out of 7). The method of Saison et al (2015) detected 23/42 replicates (54.8%) with no detection for one sample (M1) and one replicate for one sample (M2). The method of Accotto et al (2000) detected 16/42 replicates (38.1%) with no detection of two samples (H1, H2) and 3 replicates for sample M (1 and 2 combined). The method of Li et al (2004) detected 10/42 replicates (23.8%) with no detection for two samples (L, M).
Even if this performance criteria was not fully evaluated, the relative analytical sensitivity (i.e ability to detect a low concentration of a given substance in a biological sample) of the method develop by Wyatt et al (1996) modified showed the best results to this criteria far from the other tested methods.

Laboratory				Conformity			
Samples	**Isolate - dilution**	**Assigned value**	**Ech**	Wyatt	Saison	Accotto	Li
Abutilon mosaic virus	17/87 AbMV	**Positive**	A	7	7	7	6
African cassava mosaic virus	17/22.2 ACMV	**Positive**	B	7	7	7	1
Bean Golden Mosaic Virus	17/23.2 BGMV	**Positive**	C	7	7	7	7
Chili leaf curl virus	17/104.1 ChiLCV	**Positive**	D	7	7	7	7
Pepper golden mosaic virus	17/104.3 PepGMV	**Positive**	E	7	7	7	6
Potato Yellow mosaic virus	16/257.4 PYMV	**Positive**	F	7	7	7	7
Tomato Leaf Curl Mali Virus	EL 20 30031 ToLCMLV	**Positive**	G	7	7	7	0
Tomato leaf Curl New Delhi Virus	14/433 (index EL35) ToLCNDV diluted 10^{-2}	**Positive**	H1	7	5	0	5
Tomato leaf Curl New Delhi Virus	14/433 (index EL35) ToLCNDV diluted 10^{-2}	**Positive**	H2	6	5	0	5
Tomato Mottle Virus	14/14 (EL28) ToMoV	**Positive**	J	7	7	2	5
Tomato Severe Rugose Virus	EL6 00/749A B ToSRV	**Positive**	K	7	7	7	6
Tomato yellow leaf curl virus	16/34.2 et .3 TYLCV diluted 10^{-2}	**Positive**	L1	6	6	7	0
Tomato yellow leaf curl virus	16/34.2 et .3 TYLCV diluted 10^{-2}	**Positive**	L2	6	6	6	0
Tomato yellow leaf curl virus	16/34.2 et .3 TYLCV diluted 10^{-3}	**Positive**	M1	4	0	1	0
Tomato yellow leaf curl virus	16/34.2 et .3 TYLCV diluted 10^{-3}	**Positive**	M2	5	1	2	0
Tomato yellow leaf curl virus	11/480 TYLCV	**Positive**	N	7	7	7	4
Tomato leaf curl virus Comores	12/49 (EL25) ToLCYTV	**Positive**	O	7	7	7	6
Tomato yellow leaf curl virus Sardinia	EL18 09/12/98 TYLCSV	**Positive**	P	7	7	7	6
Tomato yellow leaf curl virus	16/116.3 TYLCV	**Positive**	Q	7	7	7	7
Sri Lankan cassava mosaic virus	19/182 SLCMV	**Positive**	R	7	7	7	3
African cassava mosaic virus	19/184 ACMV	**Positive**	T	7	7	7	1
Watermelon chlorotic stunt virus	19/185 WmCSV	**Positive**	U	5	6	7	1
Banana bunchy top virus	17/74 BBTV	**Negative**	V	7	7	7	7
Maize streak virus	17/75 MSV	**Negative**	W	7	7	7	7
Pea necrotic yellow dwarf virus	17/86 PNYDV	**Negative**	X	7	7	7	7
Pepino Mosaic Virus	CH2 serre PepMV	**Negative**	Y	7	7	7	7
Tomato Chlorosis Virus	14/003 ToCV	**Negative**	Z	7	7	7	7

Table 8 : Number of concordant replicates per sample and per method for all datasets.

6.2 Analysis of the qualitative results

The analysis of the performance criteria corresponding to the full dataset sent by the laboratories is presented in **Table 9.**

	Wyatt et al. (1996) modified	Saison et al. (2015)	Accotto et al. (2000)	Li et al. (2004)
Number of results	**189**	**189**	**189**	**189**
Number of PA	144	134	123	83
Number of NA	35	35	35	35
Number of PD	0	0	0	0
Number of ND	10	20	31	71
Sensitivity	**93,5%**	**87,0%**	**79,9%**	**53,9%**
Specificity	**100,0%**	**100,0%**	**100,0%**	**100,0%**
Repeatability	**97.1%**	**97.4%**	**97.3%**	**100%**
Accuracy	**94,7%**	**89,4%**	**83,6%**	**62,4%**
Reproducibility	**85,8%**	**87,6%**	**92,2%**	**82,2%**

Table 9: Results of the performance criteria of the evaluated methods for the detection of Begomoviruses.

The sensitivity of the evaluated methods ranges from 53.9% to 93.5%, whereas the specificity was 100% for all methods and the repeatability ranges from 97.1% to 100% reinforcing results obtained for both criteria. The resulting accuracy of the evaluated methods ranges from 62.4% to 94.7%. For these three parameters and the resulting parameter the accuracy, the methods are by order of the end-point PCR developed par Wyatt *et al*. (1996) modified, Saison et al (2015), Accotto *et al*. (2000). The method of Li *et al*. (2004) had the worse results for sensitivity and accuracy.

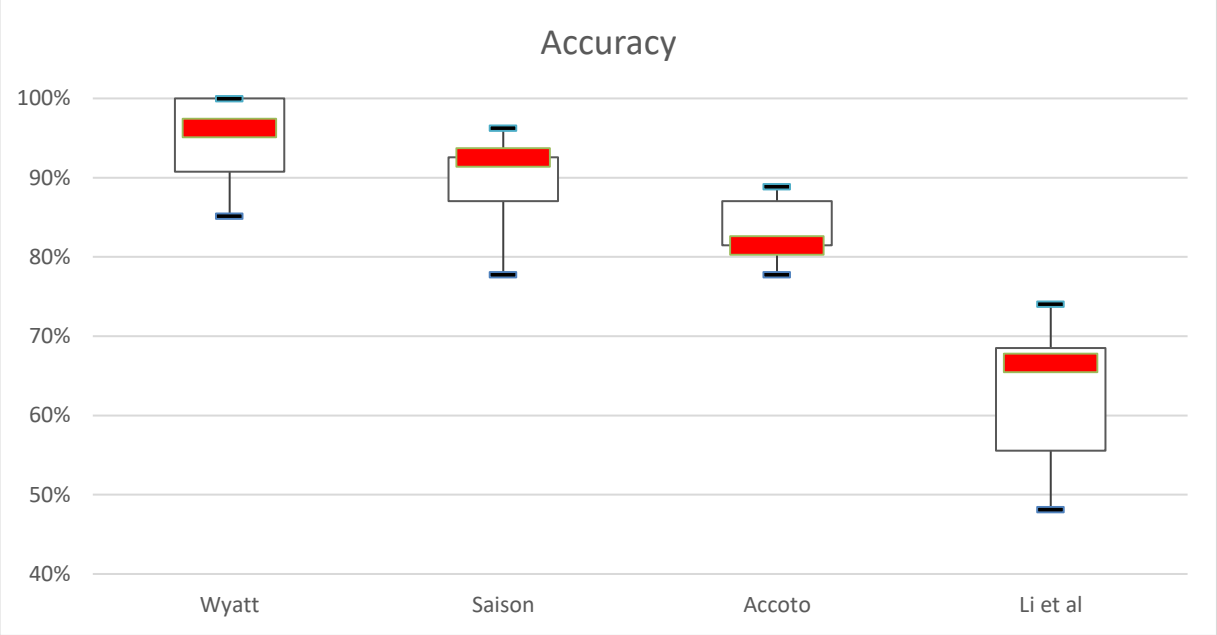


Figure 2: Comparative box plots of accuracy criteria distribution for each evaluated methods

If your look in detail for the accuracy criteria (Figure 2), the box plot shows a better homogeneity in the results for three methods (Wyatt, Saison, Accotto) compare to the Li method. The median for both methods Wyatt and Saison are close (96% vs 93%) but the distribution of the Wyatt method is more symmetric compare to the others methods even if for the Saison and Accotto methods results are more concentrated in a restricted range of values. The Accotto method had also a better homogenous response for the virus species detected compare to the Li method showing the widest distribution. The high variability observed between the extremities of the box plot for the Saison method is clearly associated to the diluted samples used for this TPS whereas the distribution observed for the Li method is due to the absence of detection for several virus species and the diluted samples.

The repeatability and reproducibility results for the four methods is presented in a scatter plot (Figure 3). This type of graph is helping to display values for these two variables and for the set of data.

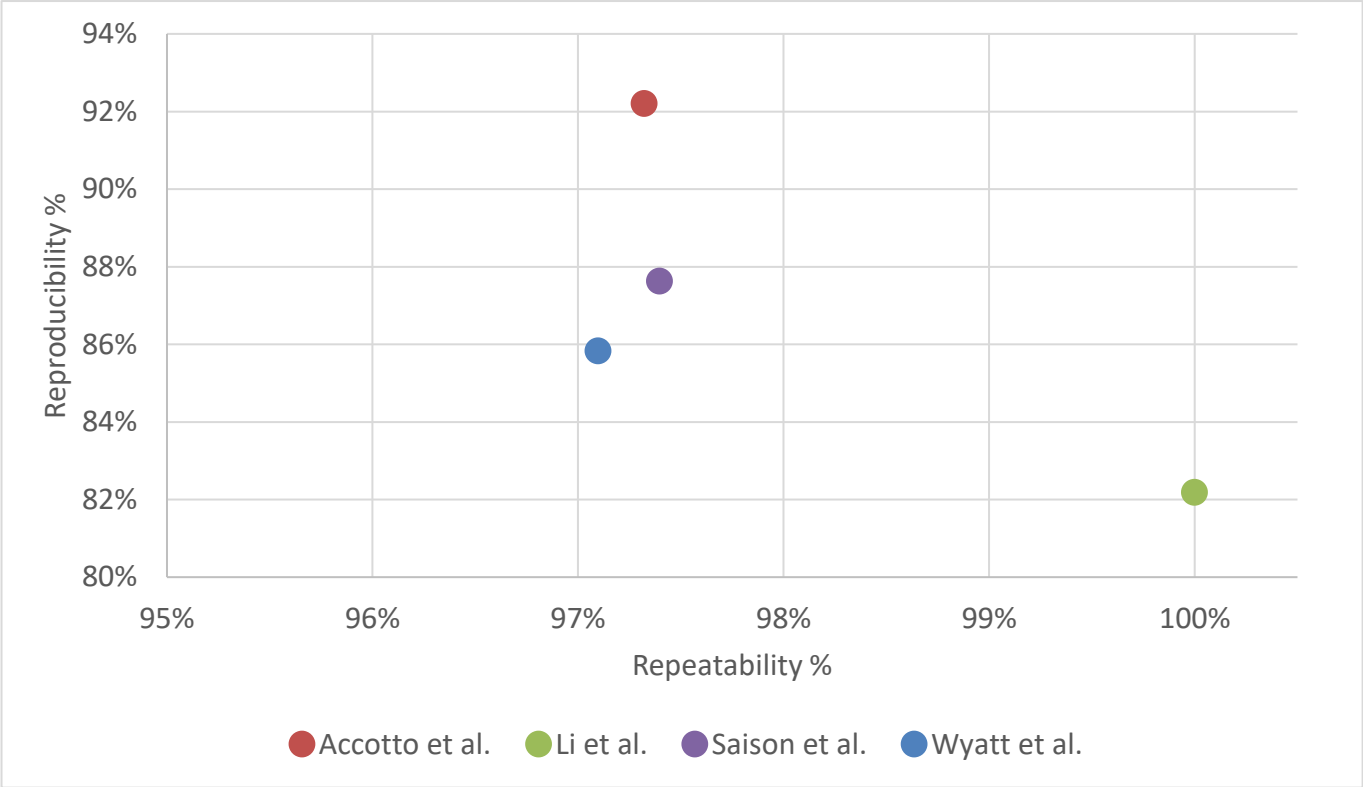


Figure 3 : Scatter plot of repeatability (X axis) and reproducibility value (Y axis) for the four methods evaluated.

The Accotto method presents the best reproducibility (92.2%) whereas the Li method had the worst result with 82.2%. At the contrary, this last method had the best repeatability (100%). For both criteria, the Saison method (Repeatability 97.4% / Reproducibility 87.6%) had a slightly better result compare to the Wyatt method ((Repeatability 97.1% / Reproducibility 85.8%).

7 Conclusion

The main target of this TPS was to compare and identify among several conventional PCR methods the best one for the detection of virus species belonging to the Begomovirus genus. To fit to this purpose, this test has to detect ideally the widest range of isolates (sensitivity) with lowest false positives (specificity) and the most reliable results (repeatability, reproducibility). The analytical sensitivity was also an interesting criteria to take in consideration.
Thus, four different methods were compare: a method initially develop by Wyatt *et al.* (1996) and optimised by NPPO (NL) (M Botermans, personal communication), a method develop by Saison *et al*. (2015), a method from Li *et al.* (2004) and the method listed in the EPPO standard PM7/50 and develop by Accotto *et al*. (2000).

Among the four methods evaluated, the method from Wyatt *et al*. (1996) modified was able to detect all tested isolates even when they were diluted (up to 10^{-3}) with a high repeatability and reproducibility. This method seems to be the most fit for purpose especially when you look in detail the conditions of the TPS. The L10 participant tested this method without the ramping amplification protocol due to a lack of PCR machine adapted. Thus, he was not able to detect the samples L and M corresponding to diluted samples. His result lead to a decrease of the sensitivity performance criteria of this method. In other way his results shows that the ramping amplification increases the sensitivity of the Wyatt method.

The method developed by Saison *et al*. (2015) allowed the detection of all isolates but with a clear lower analytical sensitivity (max 10^{-2}). It could be clearly interesting to extend the range of tested species to identify clearly which method is the most suitable technic for the detection of a wide range of Begomovirus species with the highest analytical sensitivity. Moreover, the results of this TPS clearly demonstrate that the two others methods do not correspond to the define criteria and do not lead to the detection of all isolates with a good analytical sensitivity.

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Acknowledgments

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Appendix 1: Results of the homogeneity study

Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
A1	Target	+	+	Positive
A2		+	+	Positive
A3		+	+	Positive
A4		+	+	Positive
B1	Target	+	+	Positive
B2		+	+	Positive
B3		+	+	Positive
B4		+	+	Positive
C1	Target	+	+	Positive
C2		+	+	Positive
C3		+	+	Positive
C4		+	+	Positive
D1	Target	+	+	Positive
D2		+	+	Positive
D3		+	+	Positive
D4		+	+	Positive
E1	Target	+	+	Positive
E2		+	+	Positive
E3		+	+	Positive
E4		+	+	Positive
F1	Target	+	+	Positive
F2		+	+	Positive
F3		+	+	Positive
F4		+	+	Positive
G1	Target	+	+	Positive
G2		+	+	Positive
G3		+	+	Positive
G4		+	+	Positive
H1	Target	+	+	Positive
H2		+	+	Positive
H3		+	+	Positive
H4		+	+	Positive
H5		+	+	Positive
H6		+	+	Positive
H7		+	+	Positive
H8		+	+	Positive
H9		+	+	Positive
H10		+	+	Positive
I1	Target	+	+	Positive
I2		+	+	Positive
I3		+	+	Positive
I4		+	+	Positive
I5		+	+	Positive
I6		+	+	Positive
I7		+	+	Positive
I8		+	+	Positive
I9		+	+	Positive
I10		+	+	Positive
J1	Target	+	+	Positive
J2		+	+	Positive
J3		+	+	Positive
J4		+	+	Positive
K1	Target	+	+	Positive
K2		+	+	Positive
K3		+	+	Positive
K4		+	+	Positive
L1	Target	+	+	Positive
L2		+	+	Positive
L3		+	+	Positive

Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
L4	Target	+	+	Positive
L5		+	+	Positive
L6		+	+	Positive
L7		+	+	Positive
L8		+	+	Positive
L9		+	+	Positive
L10		+	+	Positive
M1	Target	+	+	Positive
M2		+	+	Positive
M3		+	+	Positive
M4		+	+	Positive
M5		+	+	Positive
M6		+	+	Positive
M7		+	+	Positive
M8		+	+	Positive
M9		+	+	Positive
M10		+	+	Positive
N1	Target	+	+	Positive
N2		+	+	Positive
N3		+	+	Positive
N4		+	+	Positive
O1	Target	+	+	Positive
O2		+	+	Positive
O3		+	+	Positive
O4		+	+	Positive
P1	Target	+	+	Positive
P2		+	+	Positive
P3		+	+	Positive
P4		+	+	Positive
Q1	Target	+	+	Positive
Q2		+	+	Positive
Q3		+	+	Positive
Q4		+	+	Positive
R1	Target	+	+	Positive
R2		+	+	Positive
R3		+	+	Positive
R4		+	+	Positive
S1	Target	+	+	Positive
S2		+	+	Positive
S3		+	+	Positive
S4		+	+	Positive
T1	Target	+	+	Positive
T2		+	+	Positive
T3		+	+	Positive
T4		+	+	Positive
U1	Target	+	+	Positive
U2		+	+	Positive
U3		+	+	Positive
U4		+	+	Positive
V1	Non target	-	-	Negative
V2		-	-	Negative
V3		-	-	Negative
V4		-	-	Negative
W1	Non target	-	-	Negative
W2		-	-	Negative
W3		-	-	Negative
W4		-	-	Negative
X1	Non target	-	-	Negative
X2		-	-	Negative

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Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
X3		-	-	Negative
X4		-	-	Negative
Y1	Non target	-	-	Negative
Y2		-	-	Negative
Y3		-	-	Negative
Y4		-	-	Negative
Z1	Non target	-	-	Negative
Z2		-	-	Negative
Z3		-	-	Negative
Z4		-	-	Negative

Appendix 2: Results of the intermediate stability study

Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
A1	Target	+	+	Positive
A2		+	+	Positive
B1	Target	+	+	Positive
B2		+	+	Positive
C1	Target	+	+	Positive
C2		+	+	Positive
D1	Target	+	+	Positive
D2		+	+	Positive
E1	Target	+	+	Positive
E2		+	+	Positive
F1	Target	+	+	Positive
F2		+	+	Positive
G1	Target	+	+	Positive
G2		+	+	Positive
H1	Target	+	+	Positive
H2		+	+	Positive
I1	Target	+	+	Positive
I2		+	+	Positive
J1	Target	+	+	Positive
J2		+	+	Positive
K1	Target	+	+	Positive
K2		+	+	Positive
L1	Target	+	+	Positive
L2		+	+	Positive
M1	Target	+	+	Positive
M2		+	+	Positive

Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
N1	Target	+	+	Positive
N2		+	+	Positive
O1	Target	+	+	Positive
O2		+	+	Positive
P1	Target	+	+	Positive
P2		+	+	Positive
Q1	Target	+	+	Positive
Q2		+	+	Positive
R1	Target	+	+	Positive
R2		+	+	Positive
S1	Target	+	+	Positive
S2		+	+	Positive
T1	Target	+	+	Positive
T2		+	+	Positive
U1	Target	+	+	Positive
U2		+	+	Positive
V1	Non target	-	-	Negative
V2		-	-	Negative
W1	Non target	-	-	Negative
W2		-	-	Negative
X1	Non target	-	-	Negative
X2		-	-	Negative
Y1	Non target	-	-	Negative
Y2		-	-	Negative
Z1	Non target	-	-	Negative
Z2		-	-	Negative

Appendix 2bis: Results of the final stability study

Samples of the panel	Type	PCR results		Final results
		Repetition 1	Repetition 2	
A1	Target	+	+	Positive
A2		+	+	Positive
B1	Target	+	+	Positive
B2		+	+	Positive
C1	Target	+	+	Positive
C2		+	+	Positive
D1	Target	+	+	Positive
D2		+	+	Positive
E1	Target	+	+	Positive
E2		+	+	Positive
F1	Target	+	+	Positive
F2		+	+	Positive
G1	Target	+	+	Positive
G2		+	+	Positive
H1	Target	+	+	Positive
H2		+	+	Positive
I1	Target	-	-	Negative
I2		-	-	Negative
J1	Target	+	+	Positive
J2		+	+	Positive
K1	Target	+	+	Positive
K2		+	+	Positive
L1	Target	+	+	Positive
L2		+	+	Positive
M1	Target	+	+	Positive
M2		+	+	Positive
N1	Target	+	+	Positive
N2		+	+	Positive
O1	Target	+	+	Positive
O2		+	+	Positive
P1	Target	+	+	Positive
P2		+	+	Positive
Q1	Target	+	+	Positive
Q2		+	+	Positive
R1	Target	+	+	Positive
R2		+	+	Positive
S1	Target	-	-	Negative
S2		-	-	Negative
T1	Target	+	+	Positive
T2		+	+	Positive
U1	Target	+	+	Positive
U2		+	+	Positive
V1	Non target	-	-	Negative
V2		-	-	Negative
W1	Non target	-	-	Negative
W2		-	-	Negative
X1	Non target	-	-	Negative
X2		-	-	Negative
Y1	Non target	-	-	Negative
Y2		-	-	Negative
Z1	Non target	-	-	Negative
Z2		-	-	Negative

Appendix 3: End-point PCR for the detection of begomoviruses

General Information

Controls

For a reliable test result, the following (external) controls should be included for each series of nucleic acid amplification of the target organism and target nucleic acid, respectively

- Negative amplification control (NAC): amplification of molecular grade water that was used to prepare the reaction mix to rule out false positives due to contamination during the preparation of the reaction mix
- Positive amplification control (PAC): amplification of nucleic acid of the target organism to monitor the efficiency of the amplification.

Interpretation of results:

Verification of the controls

- NAC should produce no amplicons
- The PAC should produce amplicons of the expected size.

When these conditions are met:

- A test will be considered positive if PCR product is at the expected size,
- A test will be considered negative, if PCR produces no band or a band of a different size,
- Tests should be repeated if any contradictory or unclear results are obtained.

Method 1 derived from Wyatt et al. (1996)

General information

- The following PCR protocol is an End-point PCR for the detection of Begomoviruses.
- The primers were designed by Wyatt *et al.* (1996).
- The amplification size of PCR is around **580 bp.**
- Platinum Taq DNA Polymerase kit (Invitrogen) is recommended; it contains Taq DNA Polymerase, 10X Buffer, Magnesium Chloride solution 50mM but other Taq polymerase can be used. The reaction final volume is 25µL.
- dNTP mix (Invitrogen) (Conc.: 5mM each) is recommended and concentrations of oligonucleotides are given here beneath.

Protocol

Primers	Sequence
AV494	5'- GCCYATRTAYAGRAAGCCMAG -3'
AC1048	5'- GGRTTDGARGCATGHGTACATG -3'

Reagent	Working concentration	Final concentration	Volume per reaction (µL)
Molecular grade water*	N.A.	N.A.	14.80
10X Taq polymerase® buffer	10X	1X	2.50
$MgCl_2$	50 mM	2 mM	1.00
dNTP	5 mM each	0.1 mM each	0.50
Forward Primer AV494	10 µM	0.8 µM	2
Reverse Primer AC1048	10 µM	0.8 µM	2
Invitrogen Taq®DNA polymerase		2 U	0.20
Subtotal			23.00
DNA			2.00
Total			25.00

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

PCR parameters for PCR AV494/AC1048

	2 min at 94°C	denaturation
10 cycles	15 sec at 94°C	denaturation
	20 sec at ramping 65°C -1°C to 55°C	Annealing T°C. down 1°C/cycle
	30 sec at 72°C	extension
30 cycles	15 sec at 94°C	denaturation
	20 sec at 55°C	annealing
	30 sec at 72°C	extension
	10 min at 72°C	final extension
	∞ at 10°C	conservation

Method 2 derived from Accotto et al. (2000)

General information

- The following PCR protocol is an End-point PCR for the detection of Begomoviruses.
- The primers were designed by Accotto *et al.* (2000).
- The amplification size of PCR is around **580 bp** and **2650pb**
- Platinum Taq DNA Polymerase kit (Invitrogen) is recommended ; it contains Taq DNA Polymerase, 10X Buffer, Magnesium Chloride solution 50mM but other Taq polymerase can be used. The reaction total volume is 25µL.
- dNTP mix (Invitrogen) (Conc.: 5mM each) is recommended and concentrations of oligonucleotides are given here beneath.

Protocol

Primers	Sequence
TY1 (F)	5'- GCCCATGTAYCGRAAGCC -3'
TY2 (R)	5'- GGRTTAGARGCATGMGTAC -3'

Reagent	working concentration	Final concentration	Volume per reaction (µL)
Molecular grade water*	N.A.	N.A.	17.80
10X Taq polymerase® buffer	10X	1X	2.50
$MgCl_2$	50 mM	2 mM	1.00
dNTP	5 mM each	0.1 mM each	0.50
Forward Primer TY1	10 µM	0.4 µM	1.00
Reverse Primer TY2	10 µM	0.4 µM	1.00
Invitrogen Taq®DNA polymerase		2 U	0.20
Subtotal			24.00
DNA			1.00
Total			25.00

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

PCR parameters for PCR TY1(F)/TY2(R)

	4 min at 95 °C	denaturation
35 cycles	30 sec at 95 °C	denaturation
	30 sec at 60 °C	annealing
	30 sec at 72 °C	extension
	7 min at 72 °C	final extension
	∞ at 10 °C	conservation

Method 3 derived from Li et al. (2004)

General information

- The following PCR protocol is an End-point PCR performed for the detection of Begomoviruses.
- The primers were designed by Li *et al.* (2004).
- The expected size of PCR product is around **912 bp**.
- Platinum Taq DNA Polymerase kit (Invitrogen) is recommended; This kit contains Taq DNA Polymerase, 10X Buffer, Magnesium Chloride solution 50mM but other Taq polymerase can be used. The reaction final volume is **30µL**.
- dNTP mix (Invitrogen) (Conc.: 5mM each) is recommended and concentrations of oligonucleotides are given here beneath.

Protocol

Primers	Sequence
SPG1	5'- CCC CKG TGC GWR AAT CCA T -3'
SPG2	5'- ATC CVA AYW TYC AGG GAG CTA A -3'

Reagent	working concentration	Final concentration	Volume per reaction (µL)
Molecular grade water*	N.A.	N.A.	22.76
10X Taq polymerase® buffer	10X	1X	3.00
MgCl2	50 mM	2 mM	1.20
dNTP	5 mM each	0,1 mM each	0.60
Forward Primer SPG1	10 µM	0.2 µM	0.60
Reverse Primer SPG2	10 µM	0.2 µM	0.60
Invitrogen Taq®DNA polymerase		2 U	0.24
Subtotal			29.00
DNA			1.00
Total			30.00

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

PCR parameters for PCR SPG1/SPG2

	2 min at 94°C	denaturation
11 cycles	40 sec at 94°C	denaturation
	40 sec at ramping 61°C + 1°C to 72°C	Annealing T°C. up 1°C/cycle
	90 sec at 72°C	extension
24 cycles	40 sec at 94°C	denaturation
	40 sec at 60°C	annealing
	90 sec at 72°C	extension
	10 min at 72°C	final extension
	∞ at 10°C	conservation

Method 4 derived from Saison et al. (2015)

General information

- The following PCR protocol is an End-Point PCR for the detection of Begomoviruses.
- The primers were designed by Saison et *al.* (2015).
- The amplification size of PCR is around **580 bp** and **950pb**.
- Primers Beg-CP-F and Beg-580-R
- Platinum Taq DNA Polymerase kit (Invitrogen) is recommended ; it contains Taq DNA Polymerase, 10X Buffer, Magnesium Chloride solution 50mM but other Taq polymerase can be used. The reaction total volume is 25µL.
- dNTP mix (Invitrogen) (Conc.: 5mM each) is recommended and concentrations of oligonucleotides are given here beneath.

Protocol

Reagent	working concentration	Final concentration	Volume per reaction (µL)
Molecular grade water*	N.A.	N.A.	17.50
10X Taq polymerase® buffer	10X	1X	2.50
$MgCl_2$	50 mM	1.5 mM	0.80
dNTP	5 mM each	0.1 mM each	0.5
Forward Primer Beg-CP	10 µM	0.3 µM	0.75
Reverse Primer Beg-580	10 µM	0.3 µM	0.75
Invitrogen Taq®DNA polymerase		2 U	0.20
Subtotal			23.00
DNA			2.00
Total			25.00

*Molecular grade water should be used preferably or prepared purified (deionised or distilled), sterile (autoclaved or 0.45 µm filtered) and nuclease-free.

PCR parameters for PCR Beg-CP-F/Beg-580-R

	3 min at 94 °C	denaturation
35 cycles	30 sec at 94 °C	denaturation
	35 sec at 58 °C	annealing
	30 sec at 72 °C	extension
	7 min at 72 °C	final extension
	∞ at 10 °C	conservation

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- Li, Ruhui, Sarbagh Salih, and Suzanne Hurtt. 2004. "Detection of geminiviruses in sweetpotato by polymerase chain reaction." Plant disease 88 (12):1347-1351.
- Saison, A, and P Gentit. 2015. "Development of a polyvalent detection method for Begomoviruses presenting a threat to the European tomato industry." Testa - EPPO Conference on diagnostics for plant pests, Angers, 30/11 au 04/12/2015.
- Wyatt, SD, and Judith K Brown. 1996. "Detection of subgroup III geminivirus isolates in leaf extracts by degenerate primers and polymerase chain reaction." Phytopathology 86 (12):1288-1293.

Appendix 4: Results of the participants to the PCR Wyatt *et al*. (1996) modified.

Laboratory		L01	L02	L03	L05	L06	L07	L08	L09	L10
Samples	Assigned value	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation
Ech A	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech B	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech C	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech D	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech E	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech F	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech G	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech H	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech H	**Positif**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech I	**Positif**	ND	ND	ND	PA	ND	ND	ND	ND	ND
Ech I	**Positif**	ND	PA	ND	PA	ND	ND	ND	ND	ND
Ech J	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech K	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech L	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	ND
Ech L	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	ND
Ech M	**Positif**	ND	PA	PA	PA	ND	PA	ND	ND	ND
Ech M	**Positif**	PA	PA	PA	PA	ND	PA	ND	ND	ND
Ech N	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech O	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech P	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech Q	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech R	**Positif**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech S	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech T	**Positif**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech U	**Positif**	ND	PA	ND	PA	ND	PA	PA	ND	PA
Ech V	**Négatif**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech W	**Négatif**	NA	NA	NA	PD	NA	NA	NA	NA	NA
Ech X	**Négatif**	NA	NA	NA	NA	PD	NA	NA	NA	NA
Ech Y	**Négatif**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech Z	**Négatif**	NA	NA	NA	NA	NA	NA	NA	NA	NA

Lines in grey are not take into account for the analysis as samples are not stable.
Results in red are non-expected results.

Appendix 5: Results of the participants to the PCR Accotto *et al*. (2000)

Laboratory		L01	L02	L03	L05	L06	L07	L08	L09	L10
Samples	Assigned value	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation
Ech A	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech B	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech C	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech D	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech E	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech F	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech G	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech H	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	
Ech H	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	
Ech I	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	
Ech I	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	
Ech J	**Positive**	ND	ND	ND	ND	ND	PA	PA	ND	
Ech K	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech L	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech L	**Positive**	PA	PA	PA	ND	PA	PA	PA	PA	
Ech M	**Positive**	ND	ND	PA	ND	ND	ND	ND	ND	
Ech M	**Positive**	ND	ND	PA	ND	ND	PA	ND	ND	
Ech N	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech O	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech P	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech Q	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech R	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	
Ech S	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	
Ech T	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech U	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	
Ech V	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	
Ech W	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	
Ech X	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	
Ech Y	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	
Ech Z	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	

Lines in grey are not take into account for the analysis as samples are not stable.
Results in red are non-expected results.

Appendix 6: Results of the participants to the PCR Li *et al.*(2004)

Laboratory		L01	L02	L03	L05	L06	L07	L08	L09	L10
Samples	Assigned value	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation	Agreement/Deviation
Ech A	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech B	**Positive**	ND	ND	ND	ND	ND	PA	ND	ND	ND
Ech C	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech D	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech E	**Positive**	PA	PA	PA	PA	PA	PA	ND	PA	PA
Ech F	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech G	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech H	**Positive**	PA	PA	PA	ND	PA	PA	ND	ND	PA
Ech H	**Positive**	PA	PA	PA	ND	PA	PA	ND	ND	PA
Ech I	**Positive**	PA	ND	PA	ND	PA	ND	ND	ND	PA
Ech I	**Positive**	ND	ND	PA	ND	PA	ND	ND	ND	PA
Ech J	**Positive**	PA	ND	PA	PA	PA	PA	ND	PA	PA
Ech K	**Positive**	PA	PA	PA	ND	PA	PA	PA	ND	PA
Ech L	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech L	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech M	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech M	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech N	**Positive**	PA	ND	PA	ND	PA	PA	ND	ND	PA
Ech O	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech P	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech Q	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech R	**Positive**	ND	ND	PA	ND	PA	PA	PA	ND	ND
Ech S	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech T	**Positive**	ND	ND	ND	ND	ND	ND	PA	ND	ND
Ech U	**Positive**	ND	ND	ND	ND	ND	ND	PA	ND	ND
Ech V	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech W	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech X	**Negative**	NA	NA	NA	NA	PD	NA	NA	NA	NA
Ech Y	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech Z	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA

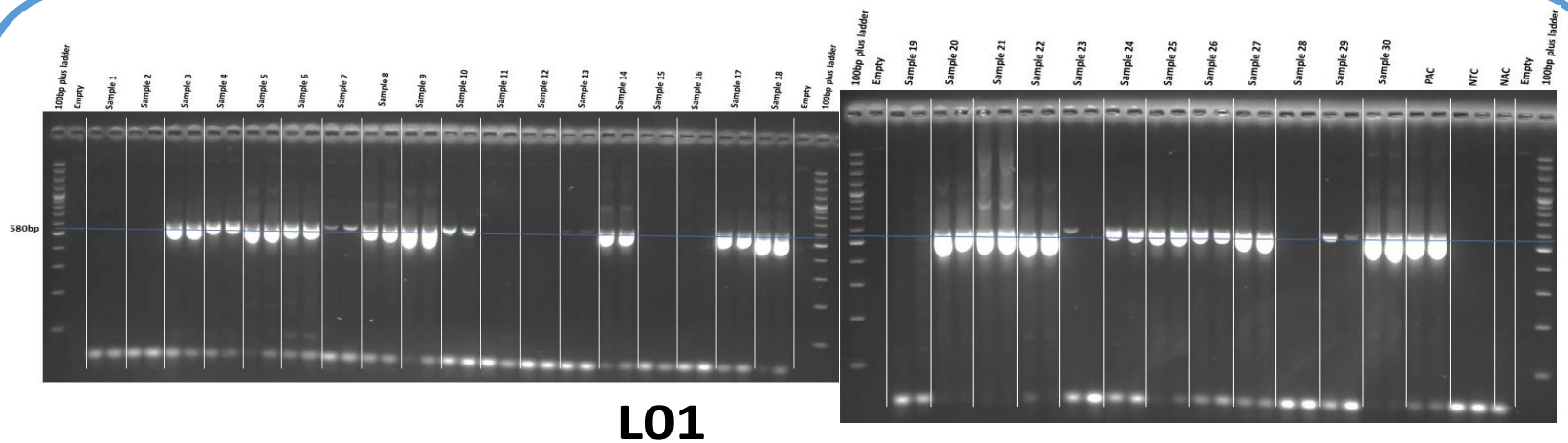
Lines in grey are not take into account for the analysis as samples are not stable.
Results in red are non-expected results.

Appendix 7: Results of the participants to the PCR Saison *et al.* (2015)

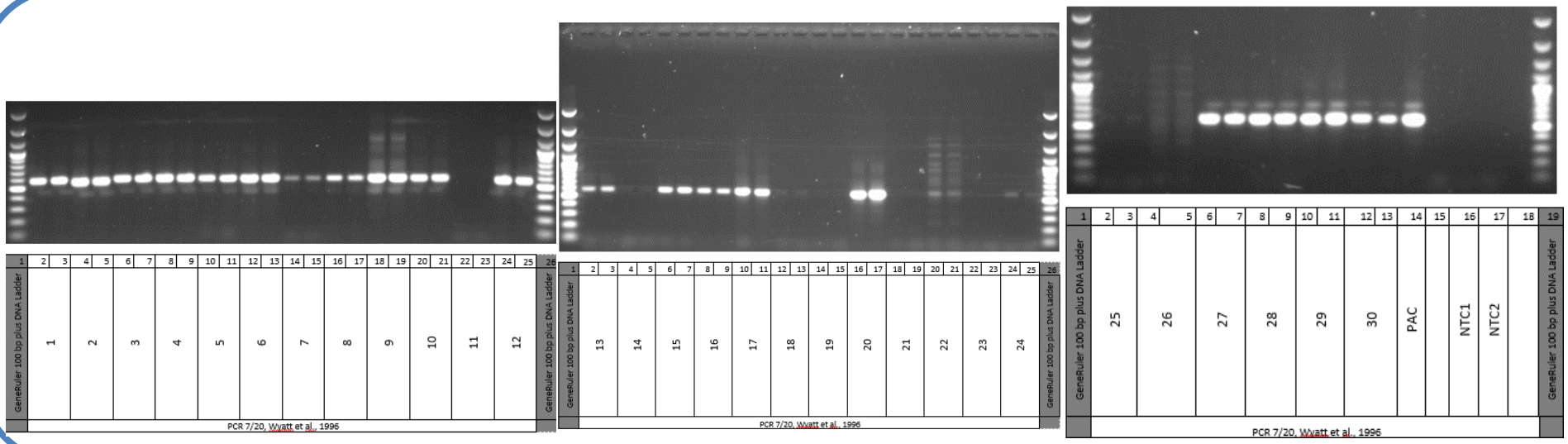
Laboratory		L01	L02	L03	L05	L06	L07	L08	L09	L10
Samples	Assigned value	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation	Agreement/ Deviation
Ech A	**Positive**	PA	PA	PA	PA	ND	PA	PA	PA	PA
Ech B	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech C	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech D	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech E	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech F	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech G	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech H	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	ND
Ech H	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	ND
Ech I	**Positive**	ND	ND	PA	ND	ND	ND	ND	ND	ND
Ech I	**Positive**	ND	ND	PA	ND	PA	ND	ND	ND	ND
Ech J	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech K	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech L	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech L	**Positive**	PA	PA	PA	PA	PA	PA	ND	ND	PA
Ech M	**Positive**	ND	ND	ND	ND	ND	ND	ND	ND	ND
Ech M	**Positive**	ND	ND	PA	ND	ND	ND	ND	ND	ND
Ech N	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech O	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech P	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	PA
Ech Q	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech R	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech S	**Positive**	ND	ND	PA	ND	PA	ND	ND	ND	ND
Ech T	**Positive**	PA	PA	PA	PA	PA	PA	PA	PA	PA
Ech U	**Positive**	PA	PA	PA	PA	PA	PA	PA	ND	ND
Ech V	**Negative**	NA	NA	NA	NA	PD	NA	NA	NA	NA
Ech W	**Negative**	NA	NA	NA	NA	PD	NA	NA	NA	NA
Ech X	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech Y	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA
Ech Z	**Negative**	NA	NA	NA	NA	NA	NA	NA	NA	NA

Lines in grey are not take into account for the analysis as samples are not stable.
Results in red are non-expected results.

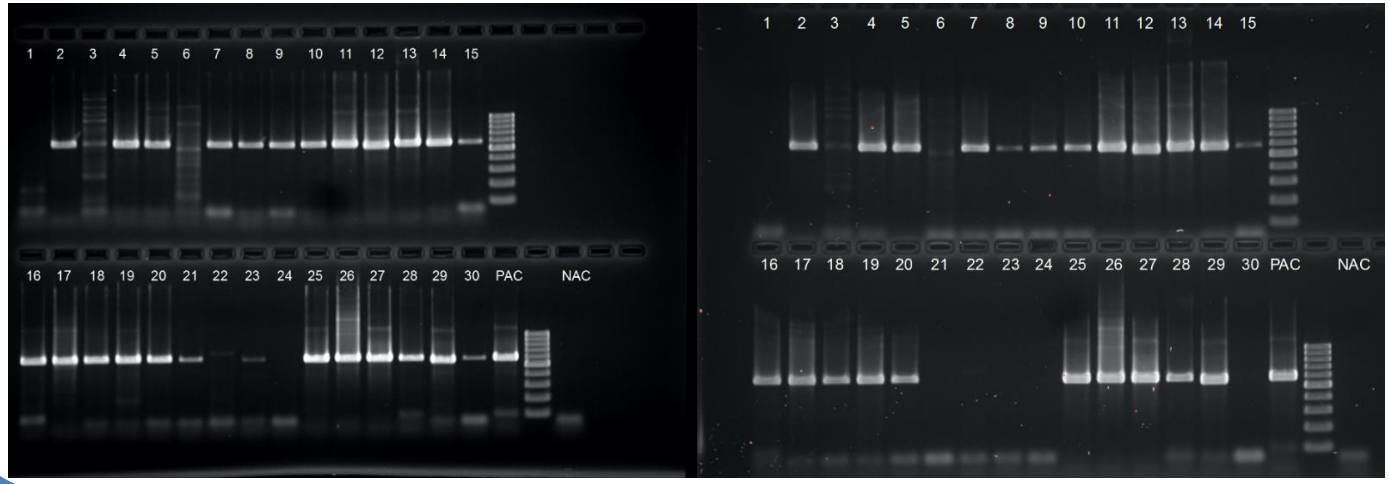
Appendix 8: Agarose gels of conventional PCR Wyatt et al. (1996) obtained by each participant.



L01



L02



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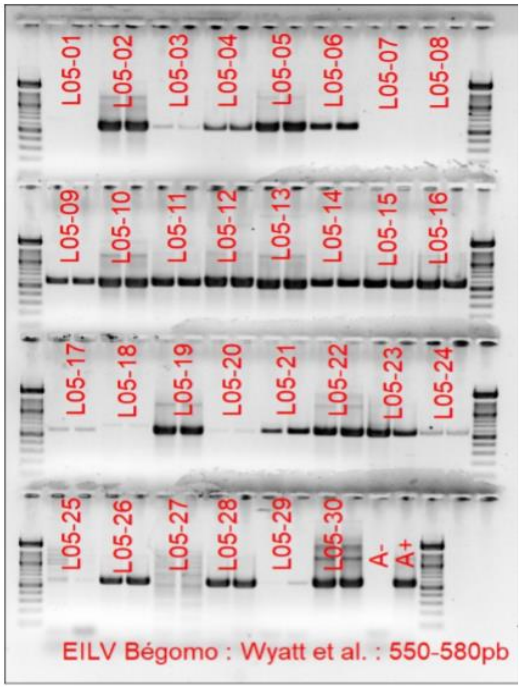
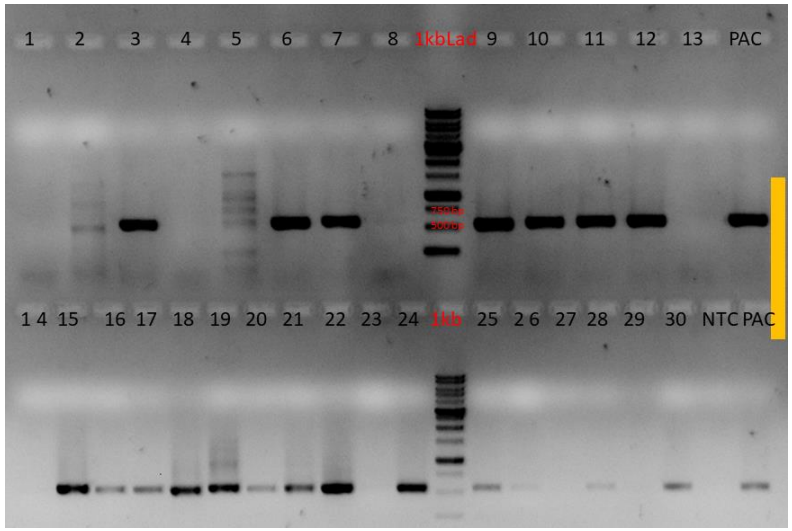
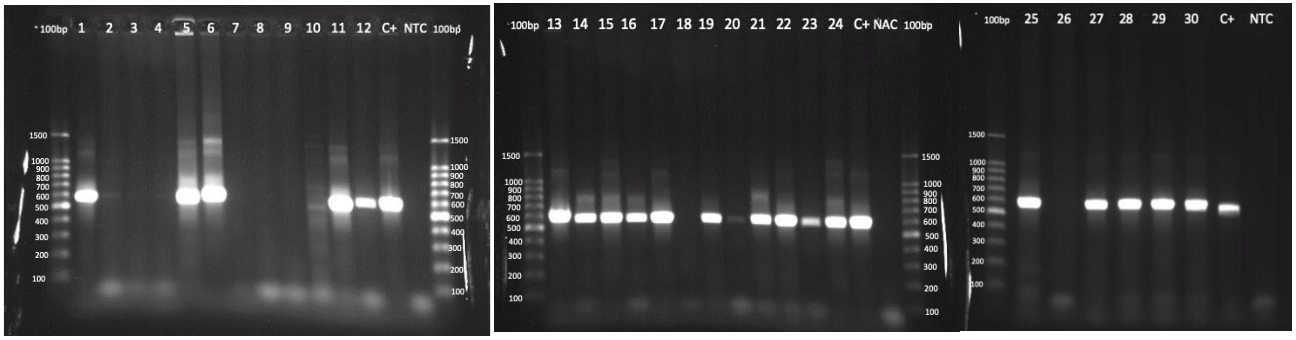


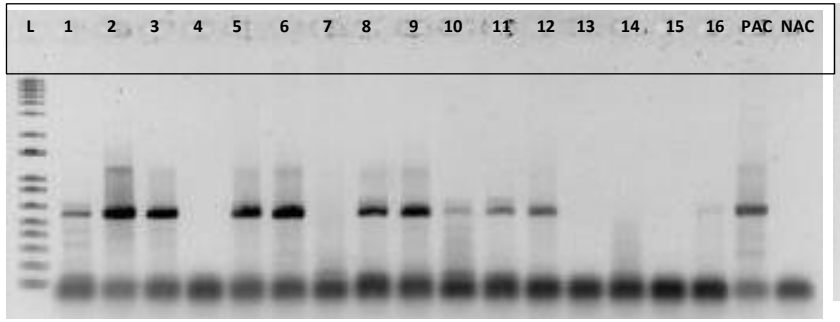
L05

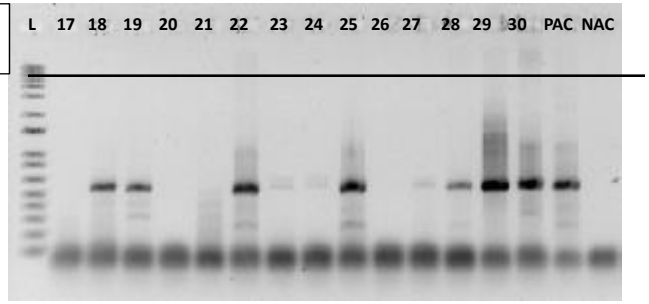


L06

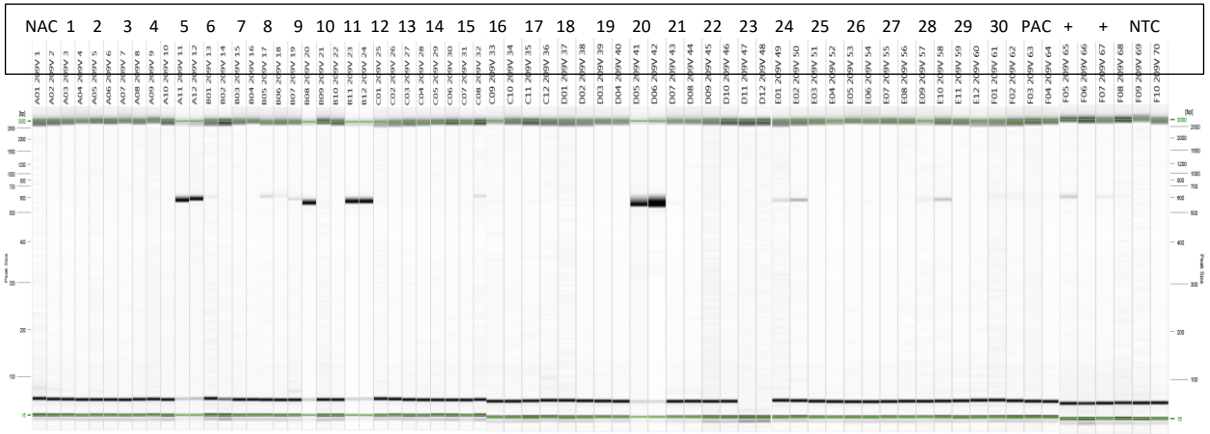


L07

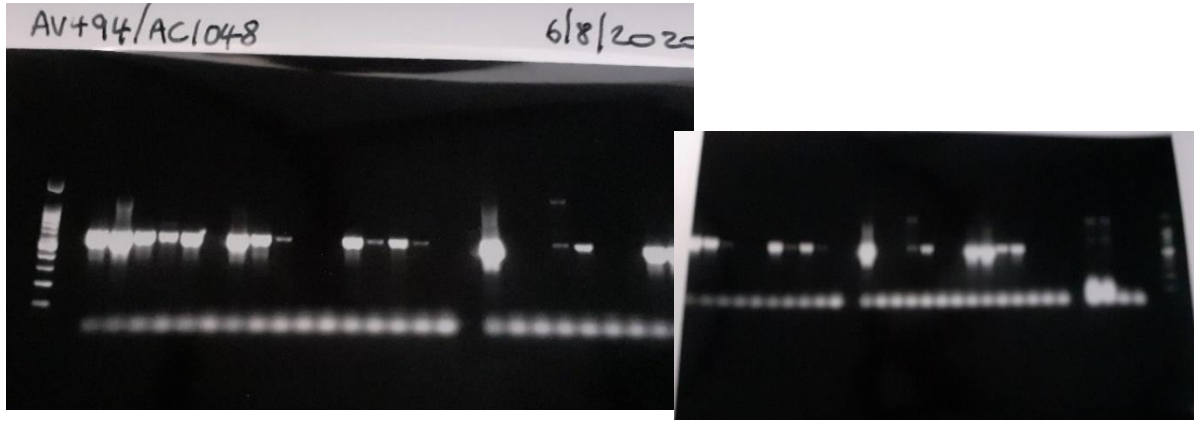

L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 PAC NAC


L 17 18 19 20 21 22 23 24 25 26 27 28 29 30 PAC NAC

L08

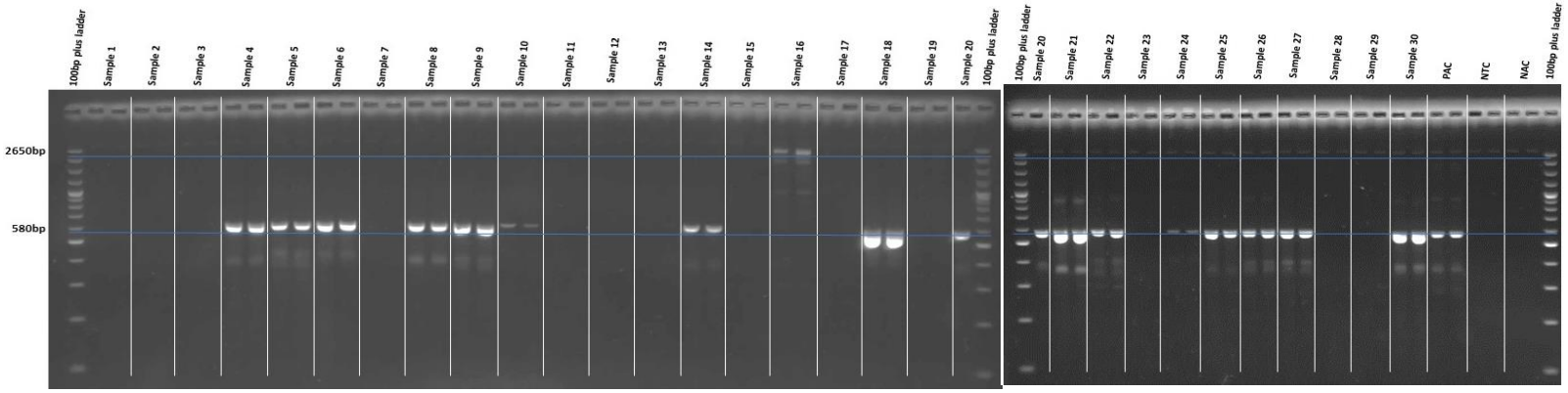

NAC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 PAC + + NTC

L09

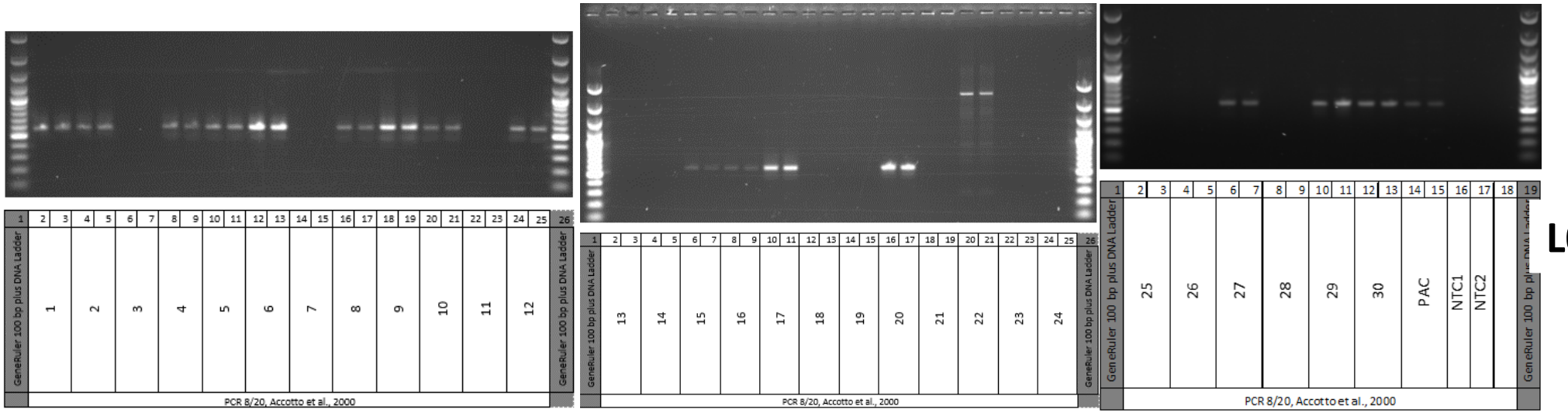

AV+94/AC1048
6/8/2025

L10

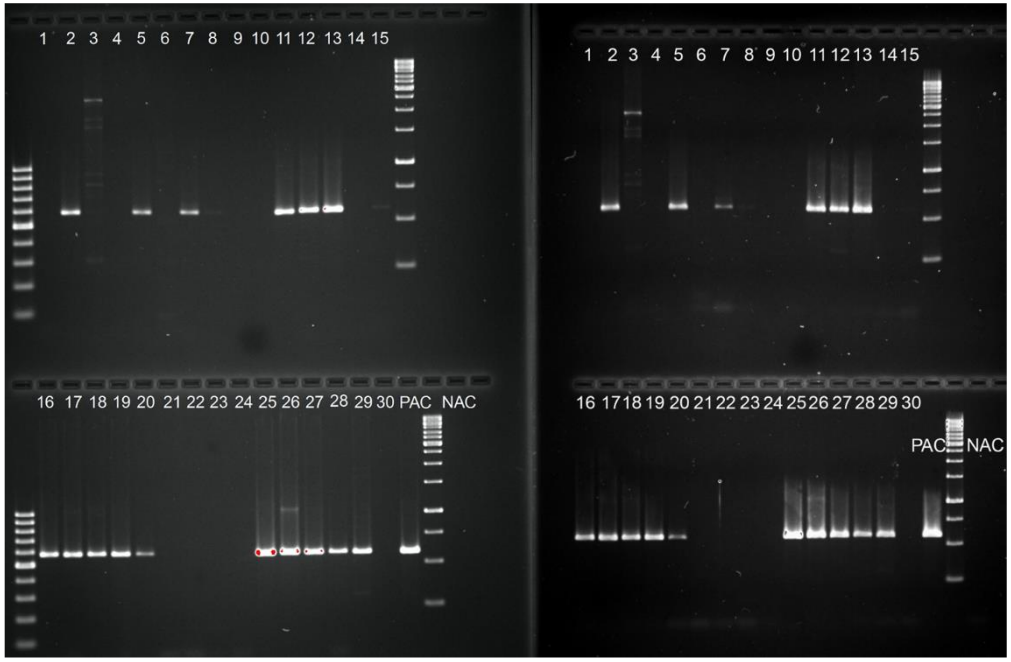
Appendix 9: Agarose gels of conventional PCR Accotto et al. (2000) obtained by each participant.



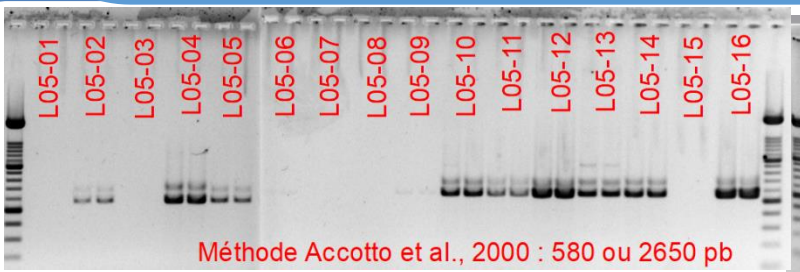
L01

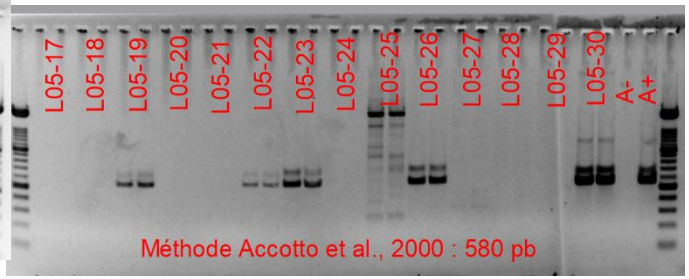


L02

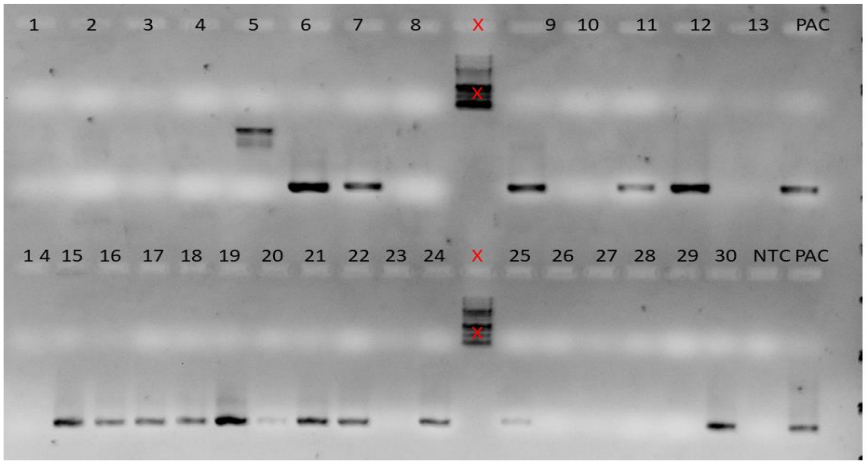


L03

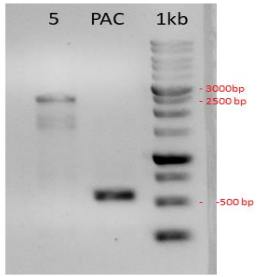




L05

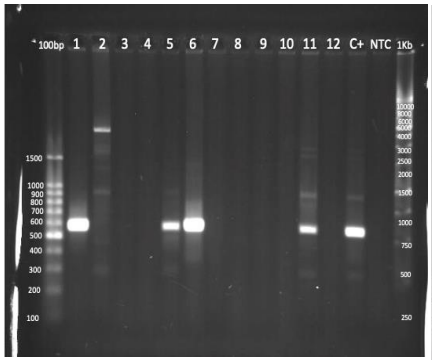


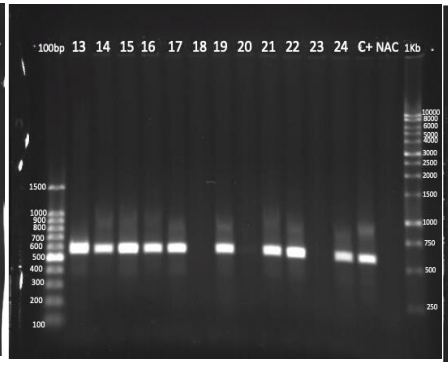
Method 2 -
Accotto et al 2000

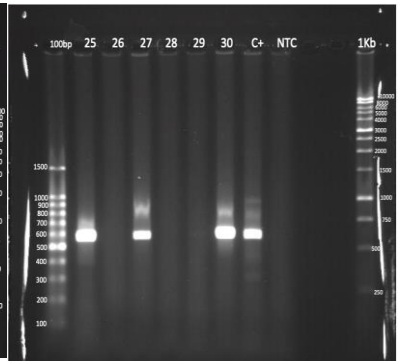


False loading of ladder,
rerun samples 5 & PAC
to confirm right size of products

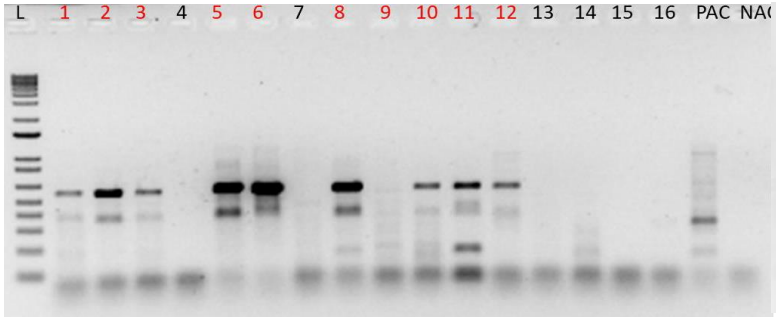
L06

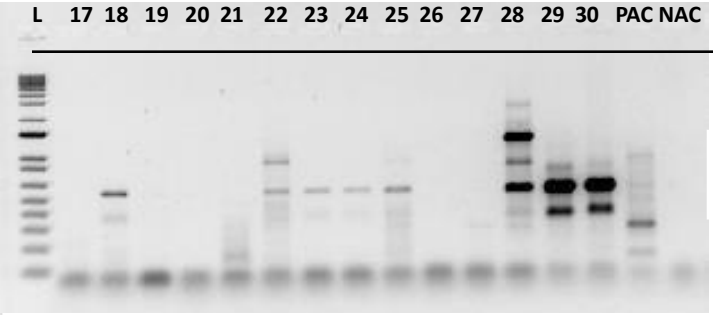




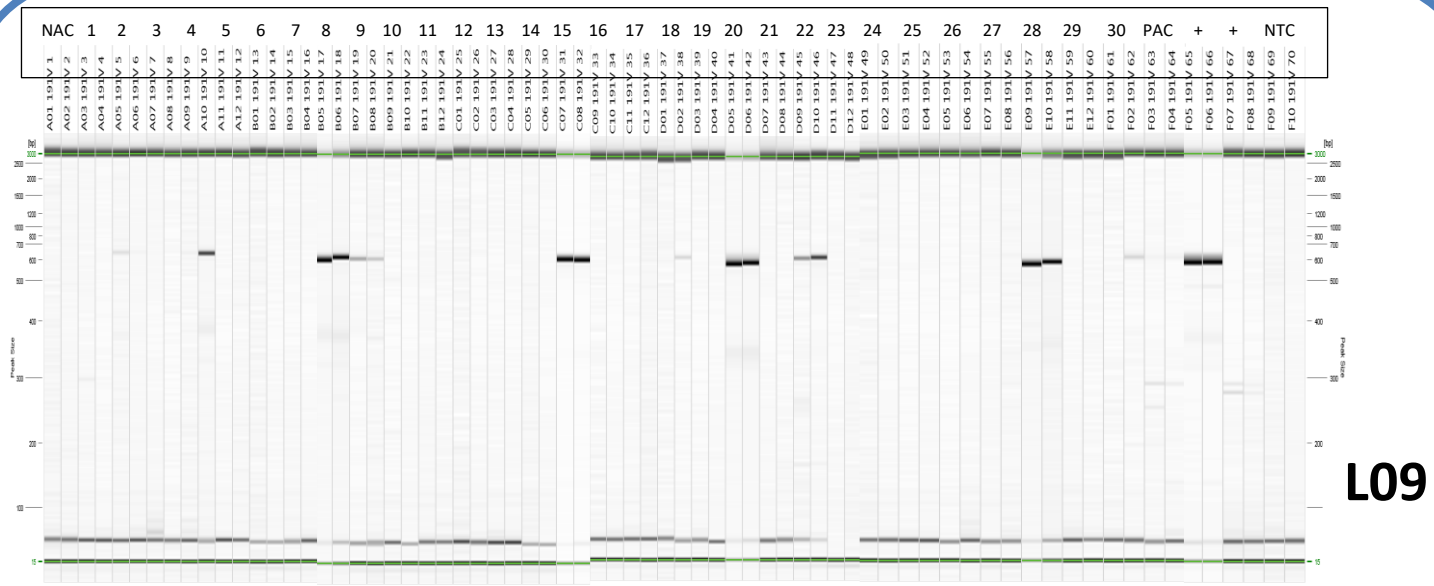


L07





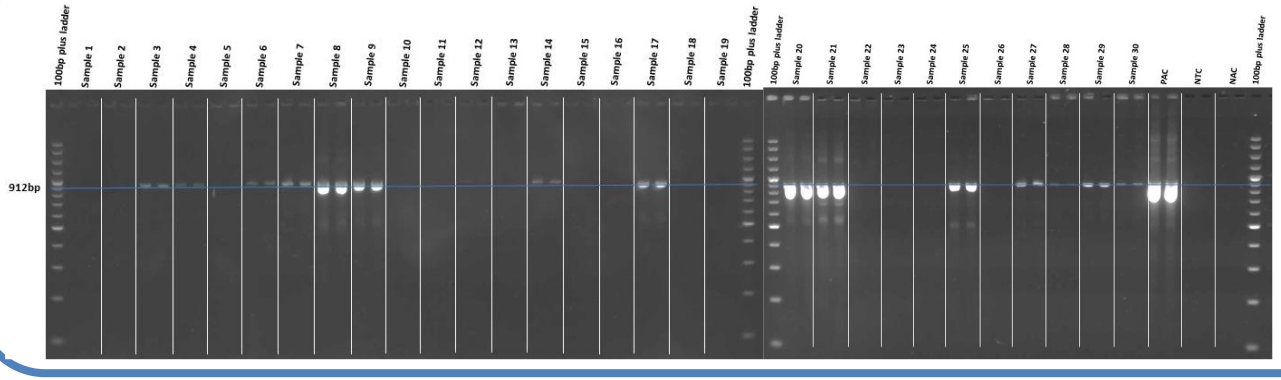
L08


NAC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 PAC + + NTC

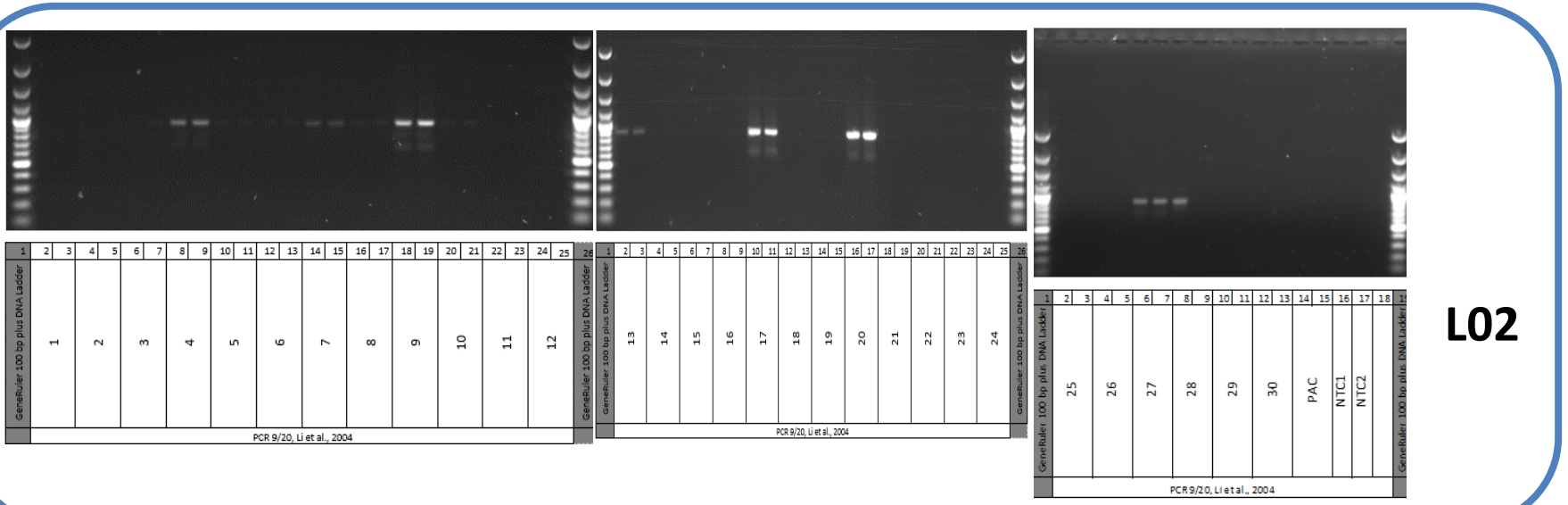
L09

L10

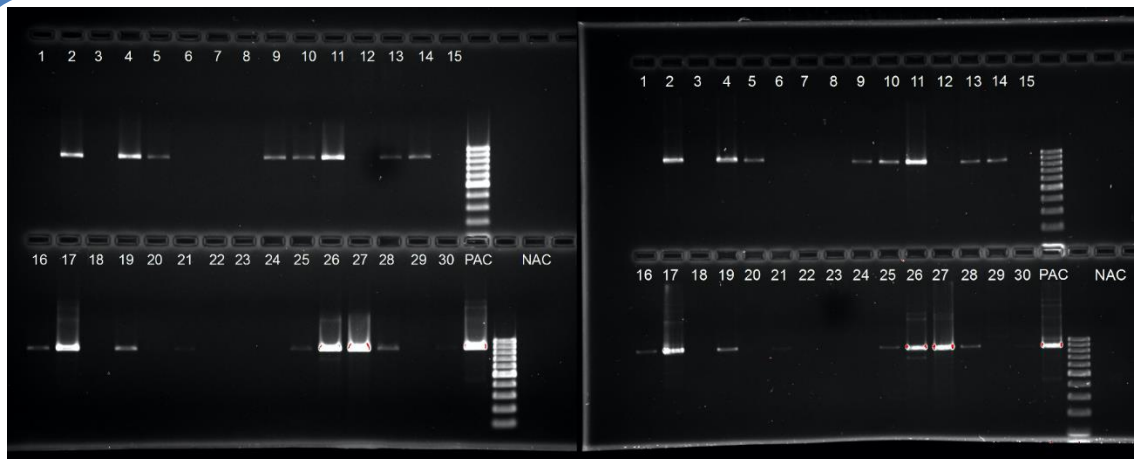
Appendix 10: Agarose gels of conventional PCR Li et al. (2004) obtained by each participant.



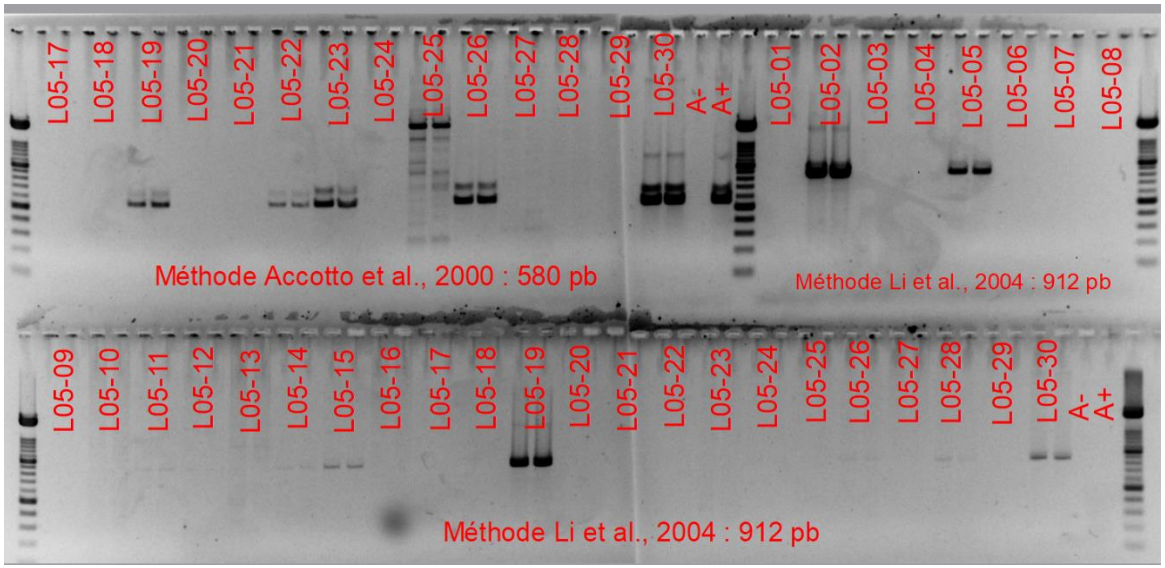
L01



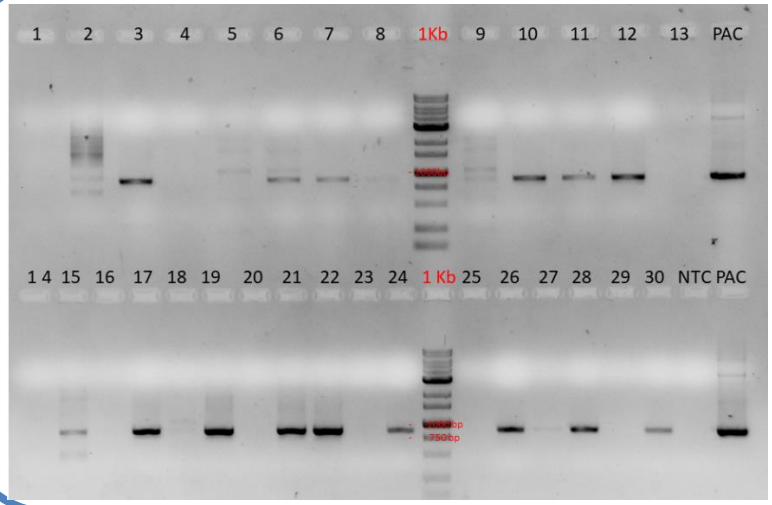
L02



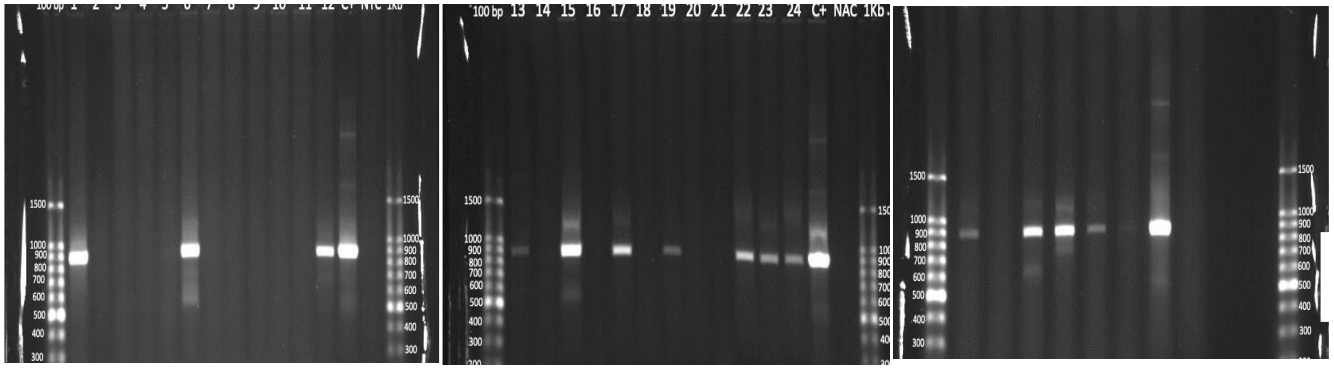
L03



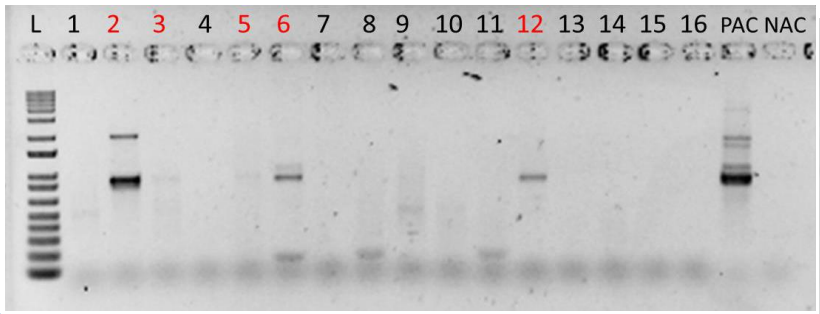
L05

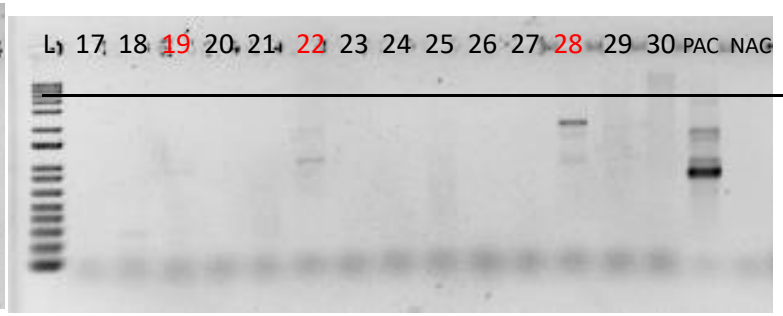


L06

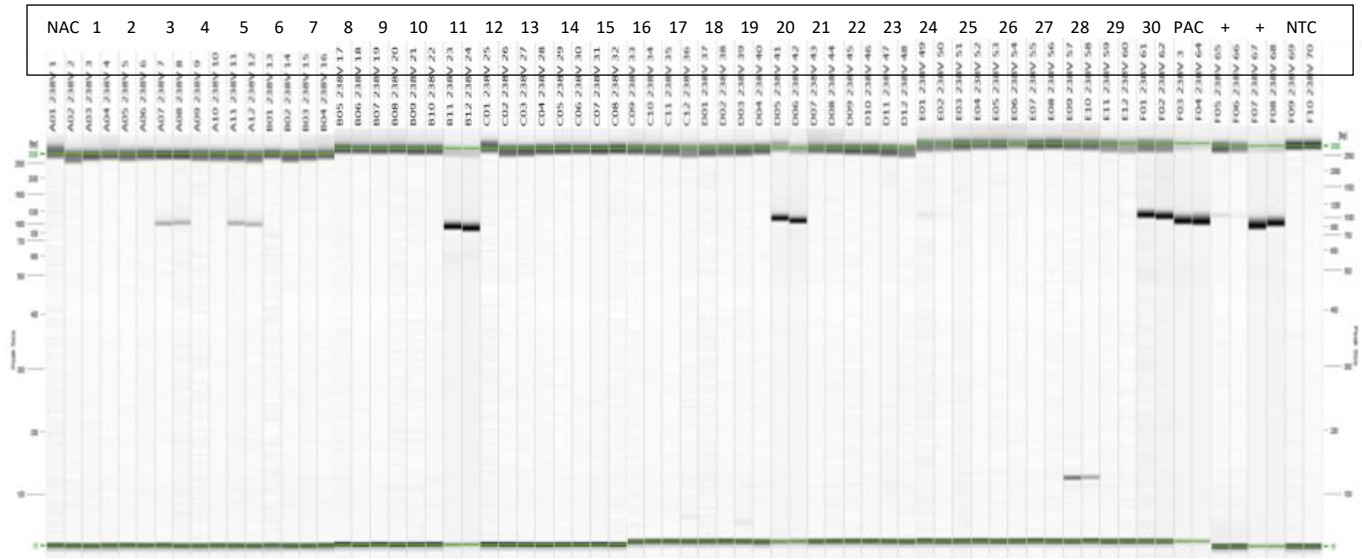


L07

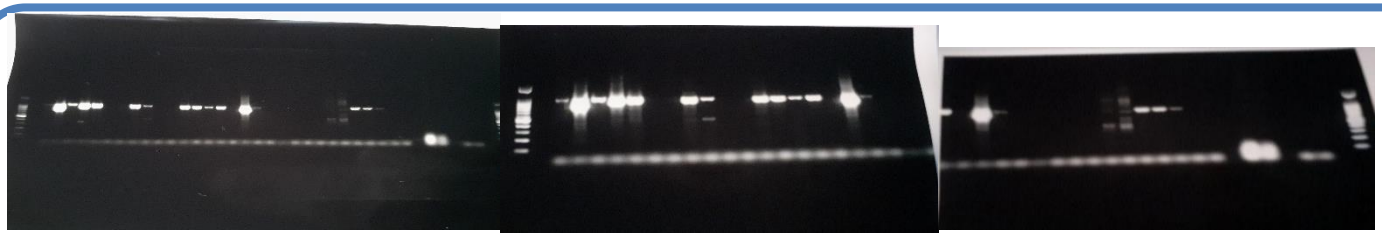




L08

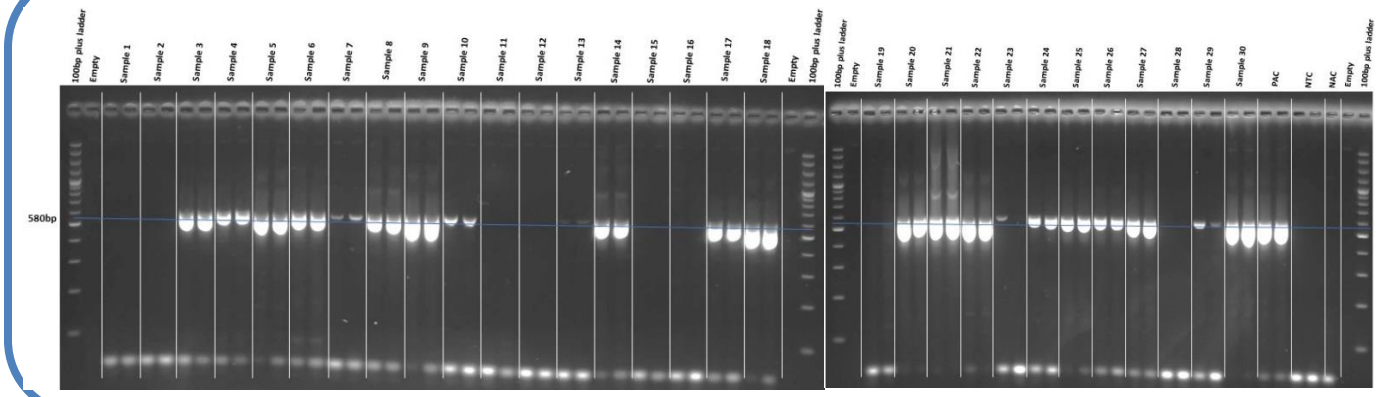


L09

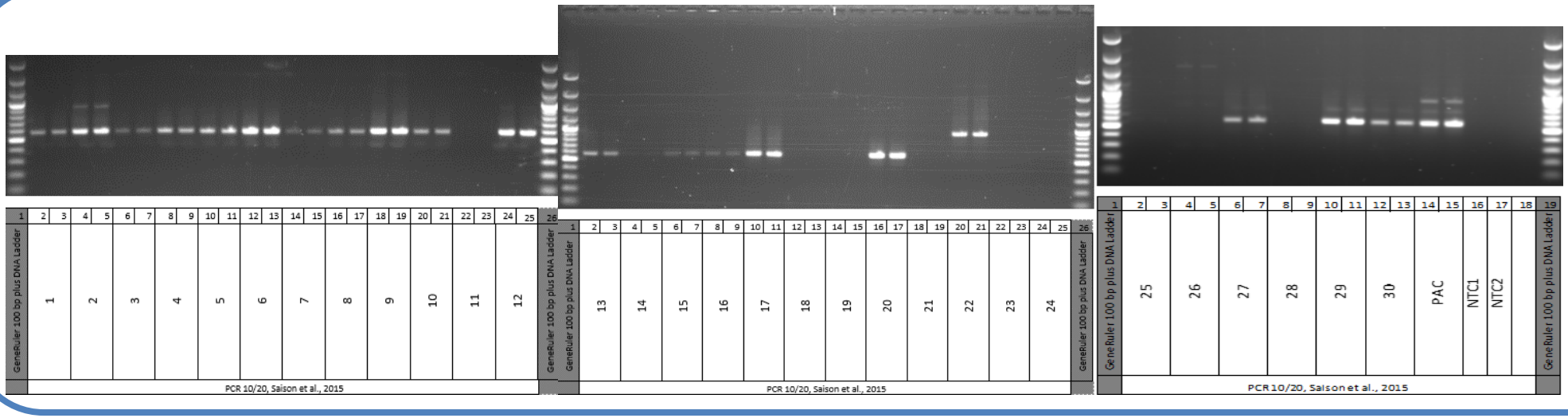


L10

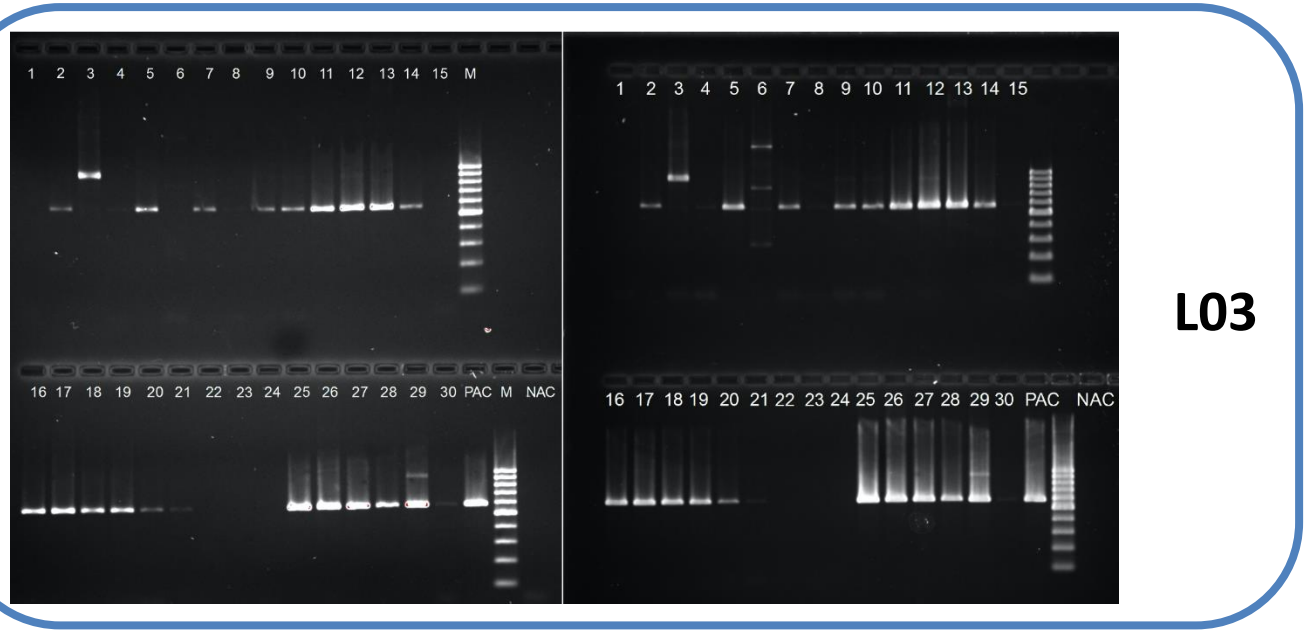
Appendix 11: Agarose gels of conventional PCR Saison et al. (2015) obtained by each participant.



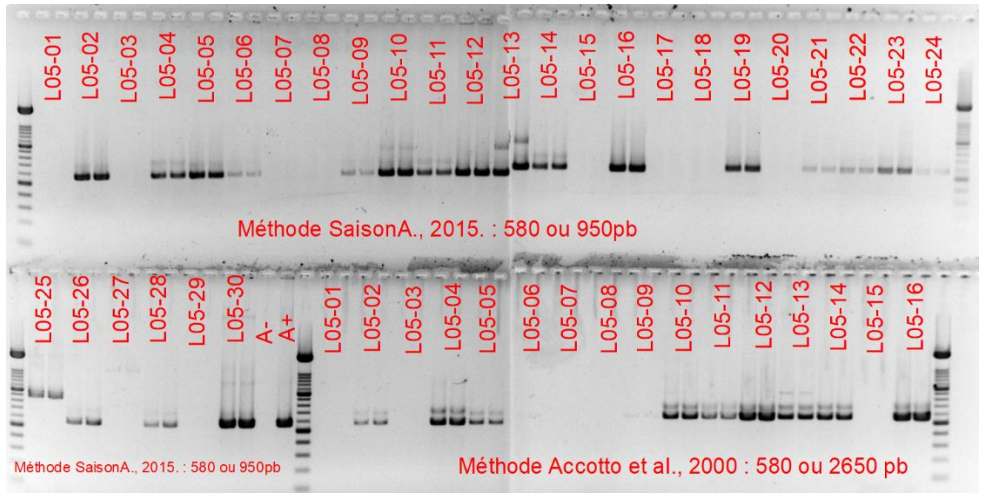
L01



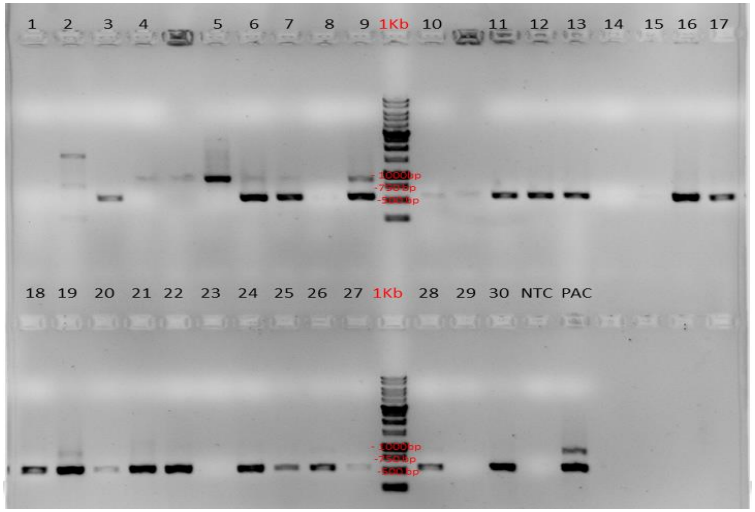
L02



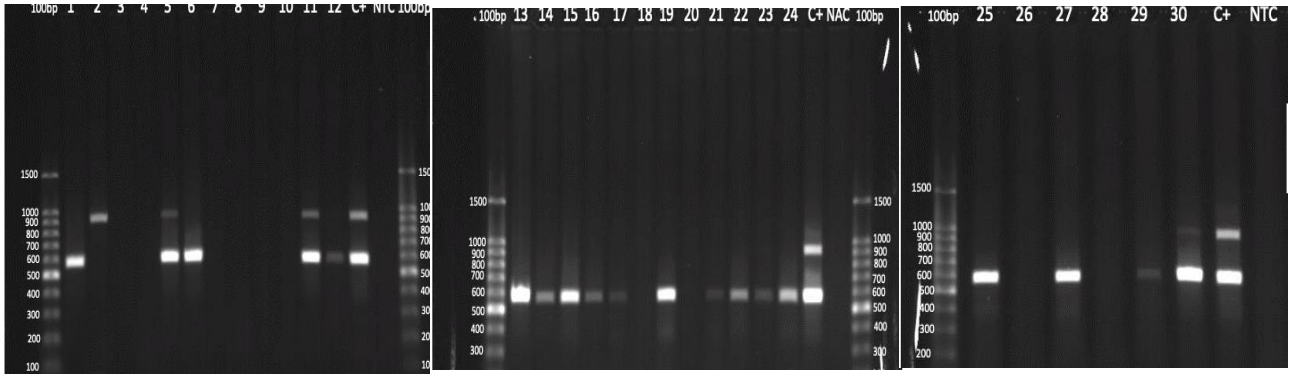
L03



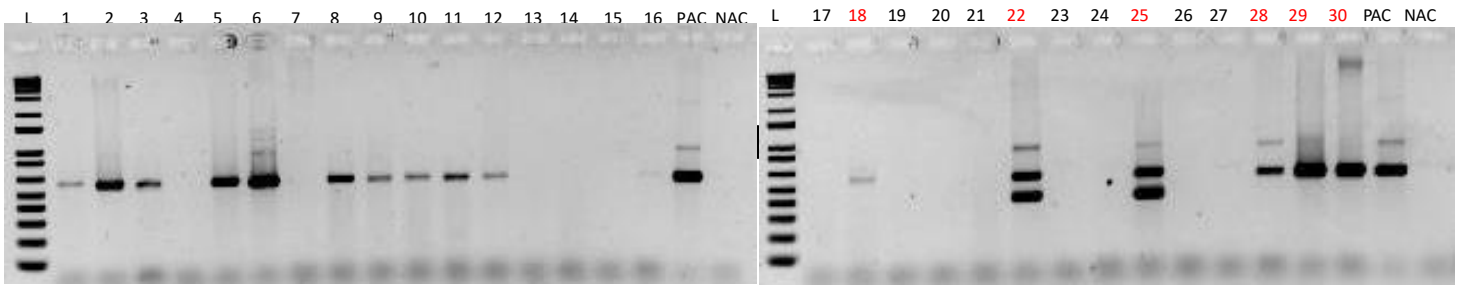
L05



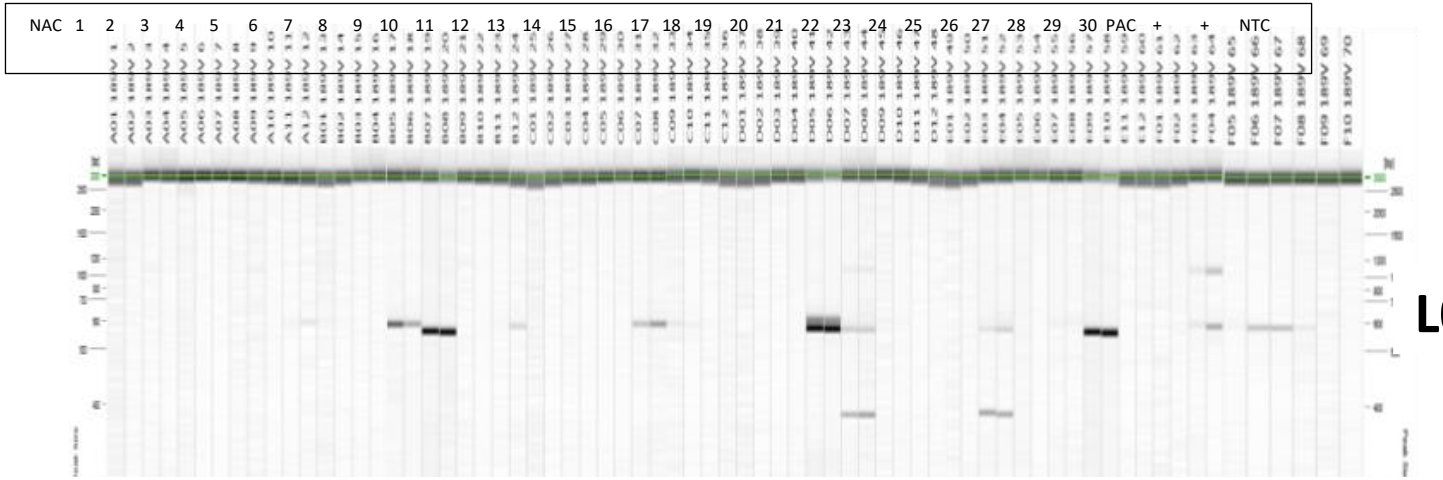
L06



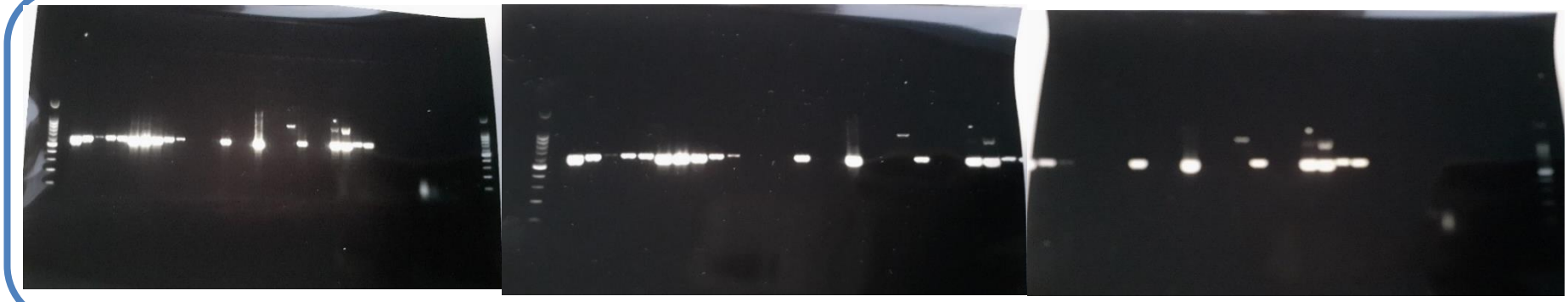
L07


L 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 PAC NAC
L 17 18 19 20 21 22 23 24 25 26 27 28 29 30 PAC NAC

L08


NAC 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 PAC + + NTC

L09



L10