Citrus tristeza virus (CTV) diagnosis and strain typing by PCR-based methods

G. Nolasco
Universidade do Algarve
Faro, Portugal

Decline of citrus plants grafted on sour orange rootstock is the commonest aspect usually associated to citrus tristeza virus. Manifestation of decline may range from being almost un-noticeable, extending over a period of years, to quick decline in which the tree dies in a matter of months. Slow decline is very frequent on the Mediterranean Basin. In some citrus producing areas outside the Mediterranean region, where sour orange is not extensively used as a rootstock, it is common to find plants exhibiting stem-pitting symptoms on the branches which are associated to a size reduction of the plants and fruits. The existence of virus strains is one cause for this wide range of symptoms. which differ heavily on their economic importance.

The current tendency to use tolerant rootstocks has allowed the virus to spread covering vast regions where a multiplicity of strains may develop. Diagnosis of CTV which since the onset of ELISA has been regarded as a matter of detection. aiming to know if the virus is present or not, is now insufficient. From a practical point of view the adoption of efficient control measures requires the knowledge about the strains that are present on a certain region or that are being introduced.

In this communication it is presented a two-step method for diagnosis of CTV. The first step aims at the detection and may also be accomplished by ELISA. The viral particles, if present in the sample to be analysed, are captured by antibodies and the coat protein (CP) gene is reverse transcribed and amplified by the polymerase chain reaction (immunocapture / RT-PCR). During the PCR reaction the DNA molecules that are produced are labelled with a rare molecule digoxigenin. These PCR products are mainly single stranded due to an imbalance of the primers concentration (asymmetric PCR). The single stranded DNA molecules are captured by hybridisation to a short capture probe immobilized on a microtitre plate and detected by digoxigenin specific antibodies. This results in a highly sensitive ability to detect the virus.

The second step aims at typing the CTV strains that may be present on the sample. The positive samples are re-amplified in a similar way and hybridised to an array of strain specific capture probes in a microtitre plate. Design of the strain specific probes was based on the alignment of a large number (160) of CP gene sequences. It was verified that these sequences clustered in 7 groups according to the biological properties (symptoms induced) of the virus. These probes have approximately 20 nucleotides and explore single nucleotide differences that exist in certain regions of the of the CP gene, resulting in cluster specific efficiencies of hybridisation. The pattern of reaction of each sample is analysed in an excel spreadsheet enabling the identification of the strains present and with their approximate proportion.

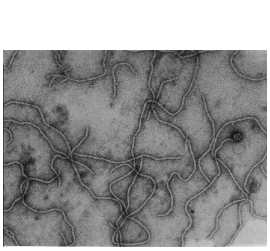
The two examples shown refer to very different epidemiological conditions. On Madeira island CTV was detected on 1995, possibly introduced from South America. Besides CTV is also present its most efficient vector *Toxoptera citricida*. Most of the samples analysed on the island contain strains from groups 3A, 4 and 5. These strains which are not widespread on the Mediterranean region produce quick decline and stem-pitting symptoms and constitute a serious threat if introduced in the Mediterranean basin. Recent results have shown that some of these strains have been in a certain moment present in Morocco, without spreading.

The second example refers to Portugal mainland where CTV has been introduced in the recent years with budwood coming from Spain. The strains present on these samples are usually of the mild type which do not produce stem-pitting or quick-decline. However it can be seen also that in some cases were detected severe strains, in varietal collections or in budwood from unknown origin present on some nurseries, for which eradication was a first priority.

CTV Diagnosis and strain typing by PCR based methods

- Praxis 2159-95, FCT, Portugal
- New approach for diagnosis and prevention of tristeza outbreaks (FIGCF 03)

1



2

Infection by CTV may originate different aspects besides tristeza in strict sense – decline of plants on sour orange:

Mediterranean basin:
no symptoms on mexican lime or other indicators

mexican lime symptoms, slow decline

mexican lime symptoms, quick decline

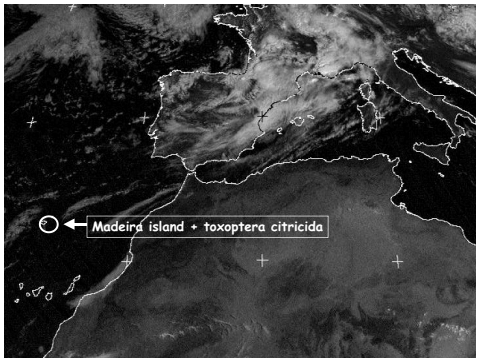
Exotic strains
Stem-pitting of sweet orange and/or grapefruit and/or decline

3

4

http://www.ecoport.org> /resources/slide shows

5



6

in some point in the future, the actual concept of CTV diagnosis based on broad spectrum antibodies will be useless due to the change to tolerant rootstocks and increase of natural transmission of CTV

7

CTV Diagnosis

Objectives

Detection → **High sensitivity, broad spectrum**
Typing → **Strain discrimination**

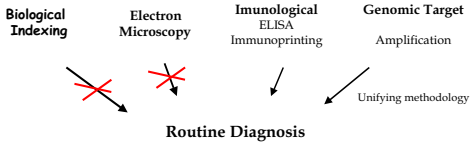
Problems

High volume of samples
Low technical skill

→ **Methods that:**
are user-friendly
are cheap
require low degree of manipulation

8

Diagnostic Methods for Virus and Virus-like agents



Routine Diagnosis

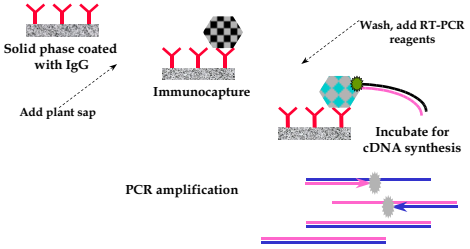
9

Bottlenecks in Amplification Based Methods

Target amplification	**Not a bottleneck: PCR, LCR, NASBA, ...**
Purifying the target	Sample preparation by: Nucleic acid extraction Probe capture Immunocapture
Getting the results	Assays for amplicon detection: Electrophoresis Heterogeneous colorimetric Homogeneous Fluorescent 5'-exonuclease Sunrise Molecular beacons

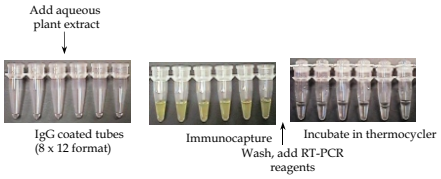
10

IC/RT-PCR



11

IC/RT-PCR – in practice



12

Bottlenecks in Amplification Based Methods

Purifying the target	Sample preparation by: Nucleic acid extraction Probe capture Immunocapture
Target amplification	Not a bottleneck: PCR, LCR, NASBA, ...
Getting the results	Assays for amplicon detection: Electrophoresis Heterogeneous colorimetric Asymmetric PCR-ELISA Homogeneous Fluorescent 5'-exonuclease (taqman) Sunrise Molecular beacons

13

Nolasco, G., Sequeira, Z., Bonacalza, B., Mendes, C., Torres, V., Sanchez, F. Urgoiti, B., Ponz, F., Febres, V., Cevik, B., Lee, R.F., Niblett, C.L.,(1997) Sensitive CTV diagnosis using immunocapture reverse transcriptional Polymerase chain reaction and exonuclease fluorescent probe assay. Fruits, 52 391-396

Nolasco, G., Sequeira, Z., Sabino, J., F., Febres, V., Cevik, B., Lee, R.F., Niblett, C.L.,(1999) PCR-based detection and strain typing of citrus tristeza virus. Petria 9: 131-135

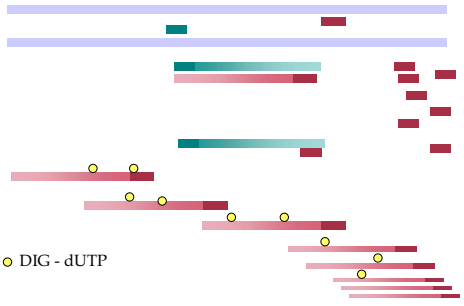
14

Asymmetric PCR - ELISA

Nolasco, G., Sequeira, Z., Soares, C., Mansinho, A., Bailey, A.M., Niblett Asymmetric PCR ELISA: increased sensitivity and reduced costs for the detection of plant viral nucleic acids.
European Journal of Plant Pathology (accepted w/ minor revision)

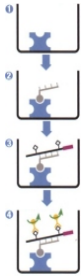
15

Asymmetric PCR



16

Asymmetric PCR ELISA



Coat ELISA plate with Streptavidin

wash

Add the biotinylated capture probe
(broad spectrum probe – 150 CP sequences)

wash

Add the asymmetric RT-PCR Dig labelled product, hibridise.

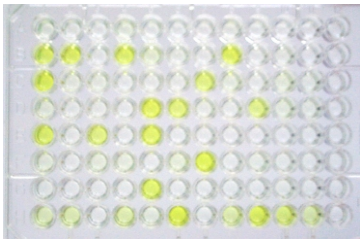
wash

Add the anti-DIG-f(ab')2 alkaline phosphatase conjugate

wash

Add substrate. Absorbance readings at 405 nm.

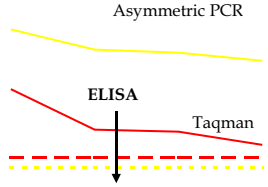
17



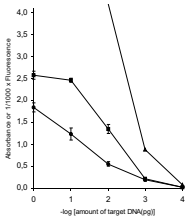
18

Detection in pooled samples

A_{405} x 1000

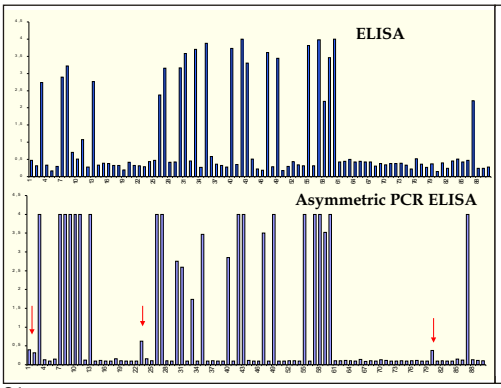


19



Comparison of the fluorescent exonuclease assay (Taqman system, closed circles) and asymmetric PCR ELISA (after 15 min of substrate incubation – closed squares and 1 h - closed triangles; note that at the highest concentrations the absorbance readings at 1h were already higher than 4) for the detection of decreasing amounts of a cloned coat protein gene of CTV isolate B53. Each point represents the mean of three replicates. The values of the mean of three negative controls (non-template) have been subtracted. The positive / negative threshold is represented at its approximated position as the horizontal dashed line for Taqman (threshold = 0.143) and the dotted line for asymmetric PCR ELISA (threshold = 0.014). The values obtained with the lowest amount of target were 0.023 and 0.075 for Taqman and asymmetric PCR ELISA respectively.

20



21

CTV Diagnosis

Objectives

Detection → **High sensitivity, large spectrum**
Typing → **Strain discrimination**

Problems

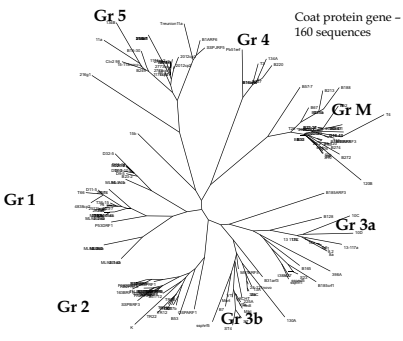
High volume of samples

Low technical skill

→ **Methods that:**

are user-friendly
are cheap
require low degree of manipulation

22



Coat protein gene – 672 nt
160 sequences

23

Gr 1 → Decline on sour orange

Gr 2

Gr 3a

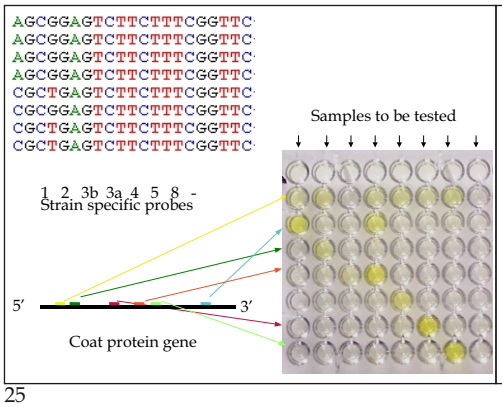
Gr 3b

Gr 4 → Stem-pitting, reduction of fruit size even on tolerant rootstocks

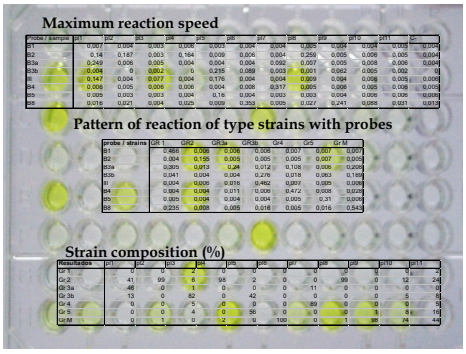
Gr 5 → Stem-pitting, reduction of fruit size even on tolerant rootstocks

Gr M

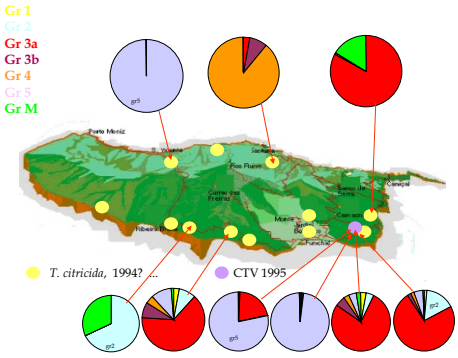
24



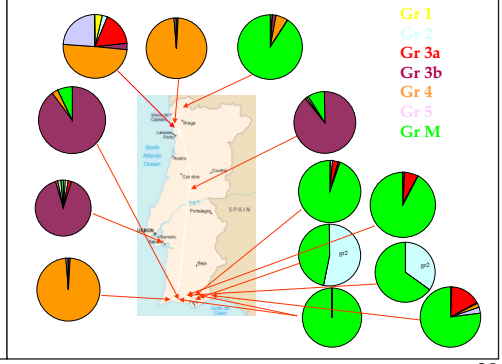
25



26



27



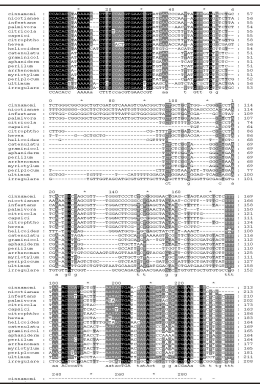
28

Identification to the Species Level of the Plant Pathogens *Phytophthora* and *Pythium* by Using Unique Sequences of the ITS1 Region of Ribosomal DNA as Capture Probes for PCR ELISA

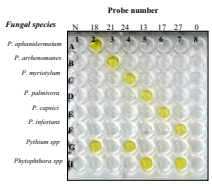
A. M. Bailey[1], D. J. Mitchell[2], K. L. Manjunath[3], G. Nolasco[4] and C. L. Niblett[3]

FEMS Microbiology letters 2002 (in press)

29

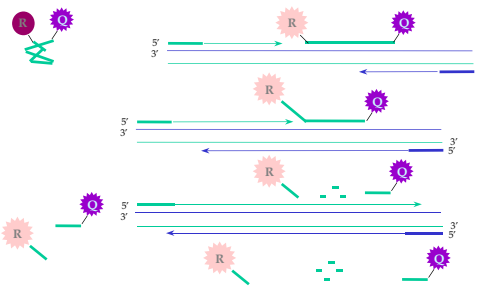


30



PCR ELISA reactions of the DIG-labeled amplified DNA of the ITS1 regions of three species of *Pythium* (*P. aphanidermatum, P. arrhenomanes* and *P. myriotylum*) and three species of *Phytophthora* (*P. palmivora, P. capsici* and *P. infestans*) and mixtures of the DNAs of two *Pythium* species [*P. aphanidermatum* and *P. myriotylum*] and two *Phytophthora* species [*P. palmivora* and *P. infestans*] with their homologous and heterologous capture probes. The DNA samples were placed horizontally in rows A through H. The capture probe numbers are shown above the plate. They were placed vertically in columns 2 through 7. Column N received neither capture probe nor DIG-labeled DNA. Column 0 received the DIG-labeled DNAs, but no capture probes.

31



Polymerase **C**hain **R**eaction

32

Protocols

CTV detection by Immunocature Reverse Transcriptional-Polymerase Chain Reaction (IC/RT-PCR)

1. Coat 0.2 ml PCR tubes with CTV specific antibodies as for ELISA. (e.g. use 50 µl of a 1:1000 dilution of polyclonal antibodies in carbonate buffer to each tube/well). Incubate plates overnigth at 4 ºC or alternatively 3 - 4 hours at 37ºC.
2. Wash tubes by flooding with PBS-Tween (3 x 3 minutes). Finally empty the tubes/plate by manual shaking. Use immediately or keep frozen (up to several weeks).
3. Prepare citrus extract as for ELISA. (grind at 1/10 -1/20 (w/v) using carborundum or a mechanical grinder). Clarify the extracts by centrifuging at 5000 g for 5 min. Keep on ice until use. Extracts may be kept frozen for several months.
4. Add 50 µl of plant extract to each coated tube/well. Incubate for immunocapture for 3-4 hours at room temperature or at 4ºC overnight.
5. Wash tubes twice with PBS-Tween and one last time with sterile distilled water (3 minutes each). Empty the tubes by manual shaking.
6. Add 50 µl of reverse transcriptional - polymerase chain reaction mix (asymmetric, with Dig-dUTP) to each tube.
7. Close the tubes, put on the thermocycler and run the following program:

    1. 38ºC for 45 min.
    2. 94ºC for 2 min.
    3. 92ºC for 30 s
    4. 52 ºC for 30 s
    5. 72 ºC for 45 s.Repeat steps 3 to 5 for 50 cycles.*
    6. 72 ºC for 5 min.

(*)- note the higher number of cycles that compensates for the lower yield of asymmetric PCR.

Composition of RT-PCR mixtures

RT-PCR mix (50 µl)	**1 sample**	**1.1 x n samples**
H_20 (MiliQ)	34.9	
Stock solution 1-10	14.6	
RT-PCR Enzyme mix	0.5	

Stock solution 1-10

	1 sample
PCR Buffer (without Mg) 10×	5
$MgCl_2$ (25 mM)	8
Dig labelling mix	0.5
Primer 1 (10µM) (forward)	0.1
Primer 10 (10µM) (reverse)	1

RT-PCR enzyme mix	**1 sample**
RNA guard (Pharmacia 27-0815 – 01, 27.2 U/µl)	0.14
Transcriptase reverse (Perkin Elmer N808-0018 50 U/µl)	0.16
Taq polymerase (MBI Fermentas, 5 U/µl)	0.20

Dig Labelling Mix

H2O	82.1 µl
dATP, dCTP, dGTP (100 mM)	2.0 µl/each
dTTP (100 mM)	1.9 µl
Dig-11-dUTP (Roche 1093088, 1 mM)	10.0 µl

Primer	**Sequences**	**Position in CP gene**
CTV 1	(5' to 3') ATG GAC GAC GAA ACA AAG AA	1
CTV 10	(5' to 3') ATC AAC GTG TGT TGA ATT TCC	653

Other Solutions

Coating buffer, pH 9.6	
$NaHCO_3$	2.93 g/l
Na_2CO_3	1.59 g/l
NaN_3	0.2 g/l
PBS 1x pH 7.4	
NaCl	8 g/l
KH_2PO_4	0.2 g/l
Na_2HPO_4	1.15 g/l
KCl	0.2 g/l
NaN_3	0.2 g/l

Stock solution can be prepared 10x concentrated and kept at 4ºC.

PBS-Tween: PBS containing 0.05 % Tween 20. Keep at 4ºC

CTV Extraction Buffer
PBS 1x
PVP 2%
0.05% Tween 20

Colorimetric detection of IC/RT-PCR amplified products.

1. Streptavidin coating of ELISA plate: add 100 µl per well of Streptavidin (Roche 1721666, 1 mg /ml in PBS) diluted 1:100 in coating buffer. Incubate overnight 4ºC or 1.30 h at 37ºC
2. Wash 3 3 minutes with PBS-Tween
3. Add biotinylated capture probe 1:1000 in hybridization buffer (100 µl/well). Incubate for 30 minutes at 37ºC
4. Wash 3 3 minutes with PBS-Tween.
5. Add to each well 65 l of hybridization buffer and 35 µl of IC/RT-PCR Dig-labeled asymmetric product from each sample. Incubate for 90 minutes at 37ºC
6. Wash 3 3 minutes with PBS-Tween
7. Add anti-DIG-F(ab')$_2$, Roche 1 093 274 conjugated alkaline phosphatase (1:1000 from a 150U/200 µl stock) in ELISA conjugate buffer, 100 µl/well. Incubate for 30 min at 37 ºC
8. Wash 3 3 minutes with PBS-Tween
9. Add substrate (p-nitrophenyl phosphate) 1 mg/ml in ELISA substrate buffer, 100 µl/well.

   Read the plate in an ELISA reader at OD 405 nm

   Determine negative / positive threshold as for ELISA and analyze the results.

Biotinylated probe sequence (stock 200 µM)
CTV ABC (5' to 3') Biotin-AAA AAA CTG ATA GCG ATG AAC GAT GTG CGT CA

Solutions

ELISA coating buffer, pH 9.6	
$NaHCO_3$	2.93 g/l
Na_2CO_3	1.59 g/l
NaN_3	0.2 g/l

PBS pH 7.4

NaCl	8 g/l
KH_2PO_4	0.2 g/l
Na_2HPO_4	1.15 g/l
KCl	0.2 g/l
NaN_3	0.2 g/l

PBS– Tween
PBS plus 0.5 ml Tween 20 per litre

Hybridization Buffer

n-laurylsarcosine	**1 g**
20x SSPE (see below)	**250 ml**
5 M NaCl	**100 ml**

Complete the volume to 1l with double-distilled water

20x SSPE pH7.4

NaCl	175.3 g/l
NaH_2PO_4, H_2O	27.6 g/l
EDTA	7.4 g/l (or 40 ml of 500 mM stock)

Adjust the pH to 7.4 with NaOH

ELISA Substrate Buffer pH 9.8	1 litre
Diethanolamine	97 ml
NaN3	0.2 g

Adjust pH to 9.8 with concentrated HCl

ELISA conjugate Buffer

PBS 10x	100 ml
Tween20	500 µl
PVP-40	20 g
BSA	2 g

Add water to 1l.

Typing of CTV strains in an array of strain specific probes.

1. Set up a PCR reaction for the reamplification of the previously amplified products that will be typed. The reamplification will be done with internal primers 42-43.

PCR mix (50µl)	**1 sample**	**1.1 x n samples**
H_20 (MiliQ)	30.7	
Stock solution 42-43	19.1	
Taq DNA polymerase (MBI Fermentas, 5 U/ µl)	0.2	

2. Distribute the PCR mix on the tubes and add 1 µl of each IC/RT-PCR to be typed.

3. Close the tubes, put on the thermocycler and run the following program:

    1. 94ºC for 2 min.
    2. 92ºC for 30 s
    3. 52 ºC for 30 s
    4. 72 ºC for 30 s. Repeat steps 2 to 4 for 50 cycles.
    5. 72 ºC for 5 min.

Stock solution 42-43

		1 sample
PCR Buffer (without Mg) 10×		5
$MgCl_2$ (25 mM)		8
Dig labelling mix		5
Primer 42 (10µM)	(reverse)	0.1
Primer 43 (10µM)	(forward)	

Dig Labelling Mix

H_2O	82.1 µl
dATP, dCTP, dGTP (100 mM)	2 µl/each
dTTP (100 mM)	1.9 µl
Dig-11-dUTP (Roche 1 093 088, 1 mM)	10 µl

Primer	**Sequences**	**Position in CP**
CTV42	(5' to 3') CTC AAA TTG CGR TTC TGT CT	454
CTV43	(5' to 3') ATG TTG TTG CNG CNG AGT C	59

Colorimetric detection of the CTV strains.

1. **Streptavidin coating of ELISA plate: add 100 µl per well of Streptavidin (Roche 1721666, 1 mg /ml in PBS) diluted 1:100 in coating buffer. Incubate overnight at 4°C or 1h30 at 37°C**
2. Wash 3 3 minutes with PBS-Tween
3. Add each of the biotinylated strain specific probes to each row of the plate (use each row for one probe); 1:1000 in hybridization buffer (100 µl/well). Incubate for 30 minutes at 37°C
4. Wash 3 3 minutes with PBS-Tween.
5. Mix 45 µl of PCR product with 855 µl of hybridization buffer. Distribute 100 µl per well of each column (use one column for each sample). Incubate for 90 minutes at 37°C
6. Wash 3 3 minutes with PBS-Tween
7. Add anti-DIG-F(ab')$_2$, Roche 1093274 conjugated alkaline phosphatase (1:1000 from a 150U/200 µl stock) in ELISA coating buffer, 100 µl/well. Incubate for 30 min at 37 °C
8. Wash 3 3 minutes with PBS-Tween
9. Add substrate (p-nitrophenyl phosphate) 1 mg/ml in ELISA substrate buffer, 100 µl/well.

Read the plate in an ELISA reader at OD 405 nm at 15 min intervals up to 1h30.

Compare the values with a set of values obtained with specific clones.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
B1												
B2												
B3a												
B3b												
III												
B4												
B5												
B8												