AGRITEST
PLANT HEALTH MANAGEMENT

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K-29A / K-29B

Flavescence dorée FD-D isolate
ELISA KIT
INSTRUCTIONS FOR USE

Introduction

The intended purpose of the diagnostic kit is the detection of the phytoplasma FD isolate 16SrV-D, responsible for Flavescence dorée (FD) disease, in naturally infected samples of grapevine and *Scaphoideus titanus.* The kit is also suitable for testing experimentally infected samples of *Vicia faba*, and *Euscelidius variegatus.* The kit can be employed in the grapevine certification programs for flavecence doreé disease control.

The kit assay was developed exploiting the patents UIBM n. IT0001429213 and EPO n. EP2918685 owned by Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Italy, and the experimental results of the project EU Horizon 2020 Tropicsafe n° 727459.

Principle of the assay

The method of detection is an enzyme-linked immunosorbent assay (ELISA) based on double antibody sandwich (DAS) by polyclonal antibodies against the recombinant Imp protein specific to FD isolate 16SrV-D. Signal develops by alkaline phosphatase reaction with p-nitro phenyl phosphate.

Specificity and sensitivity

Assay performances refers to the following bibliography:

- Filippin L., Trivellone V., Galetto L., Marzachí C., Elicio V., Angelini E. 2019. Development of an anti-Imp serological assay for the detection of "flavescence dorée" phytoplasmas in grapevine, insect vectors and host plants. *Phytopathogenic Mollicutes* 9(1), 75-76.
- Filippin L., Galetto L., Trivellone V., Elicio V., Marzachí C., Angelini E. (2022). A new ELISA test for monitoring "flavescence dorée" in field samples. Phytopathogenic Mollicutes 12 (1), 59. https://doi.org/10.5958/2249-4677.2022.00024.X

Assay quality control

The positive and the negative controls provided with the kit can be used as references (i) to verify that the assay was carried out correctly, (ii) to check the activity of reagents as prepared for the assay, and (iii) to set the test threshold.

- Positive control vial contains recombinant Imp protein FD isolate 16SrV-D preparation i) freeze-dried, or ii) dehydrated on a solid matrix (cards)
- Negative control vial contains grapevine tissue tested FD isolate 16SrV-D negative in PCR.

See protocol instructions for the control preparations.

Sample preparation

It is recommended to prepare samples the day the assay begins. Please, follow the instructions in the section "Sample preparation"

Testing time

The assay is carried out over 2 days since it requires 1 incubation overnight. Reading of results is made 1-3 hours after adding the substrate.

Storage of kit components

The kit components (antibody reagents and controls) must be used before the expiration date on the vial label and stored at 4°C.

- The reagents (antibodies) are preserved in glycerol.
- reconstituted positive and negative freeze-dried controls: store at -20°C and used no more than 2-3 times.

Components provided with the kit

1. Antibody ELISA reagents
2. Assay controls 1 positive + 1 negative
3. ELISA protocol
4. Lot certificate of analysis

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The following issues are to assist the users of the kits in achieving reliable diagnostic results

Equipment and materials required but not supplied

- ELISA plate reader, 405 filter nm
- thermostatic chamber 37°C
- manual or automatic device for rinsing ELISA plates
- micropipette set and disposable tips (10, 100, 200, 1000 µl)
- ELISA plates 96 wells
- substrate p-nitrophenil phosphate (pNPP),
- ELISA solutions (coating, washing, extraction, conjugate, substrate).

Preparation of ELISA solutions and volume

The ELISA solutions are prepared according to standard ELISA formulation (see section "ELISA solutions formula". The extraction buffer formulation is sample specific (see section "Sample preparation").
The volume calculation for each buffer refers to the consumption of 200 µl/well, or of 20 ml/plate/step (coating, conjugate, substrate) and 60 ml/plate/washing step, while extraction buffer volume depends on sample grind technique.

ELISA reagents preparation and dilutions

Each antibody reagent must be diluted into its ELISA solution before using it. The calculation of the reagent volume to withdrawal from the vial is based on the dilution rate indicated on the label.

Example: dilution **1:500** dilute **40 µl** in 20 ml buffer /plate
dilution **1:250** dilute **80 µl** in 20 ml buffer /plate

Plate lay-out and validation

In order to validate the results, it is recommended to design the plate lay-out including:

- 2 replicate wells of positive control per assay,
- 2 replicate wells of negative control per assay,
- 2 replicate wells of blank (no antigen) per assay

Each sample should be placed into 1 or 2 wells (according to the lab choice or lab conditions).

Reading and interpretation of results

Blank the ELISA reader and read the absorbance values.
The presence or the absence of virus is determined by relating the absorbance of the unknown samples to that of the threshold value. Absorbance greater or lower than the threshold value is, respectively, positive or negative result.
The threshold can be determined as 3 times the mean absorbance value of the negative control.
The mean absorbance value of positive control should be at least equal to the threshold value. If not, the results should be considered uncertain.

Precautions and good practice

In order to obtain reliable results a strict observation of the instructions and a skillful technique are required.
It is recommended to

- check for quality of buffers (purity, contamination, pH, etc...)
- be accurate in dispensing, withdrawing and washing steps
- replace micropipette tips when different vials or samples are used
- avoid substrate contamination by using clean labware
- avoid using reagents remaining from other kit

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SAMPLE PREPARATION

1. Grapevine

Sampling season	from June to early October
Sampling	symptomatic leaves
Sample storage	short term (4-5 days) at 4°C
	Long term at -20°C
Sample preparation	excise leaf main veins and cut them in small pieces
	Extraction buffer: TRIS-HCl, pH 8.2
	Amount per test 1: 10 (w:v)
	Extraction method: manual, grinding into mortar
	Suggested number of extractions per samples: 2

2. Vicia faba

Sampling season	any
Sampling and storage	see grapevine
Sample preparation	see grapevine
	Extraction buffer: ELISA general, PVP, PBS, pH 7.4

3. Scaphoideus titanus and Euscelidius variegatus

Sampling season	from June to early October
Sampling	adult insect collected in the field alive or dead
Sample storage	room temperature / +4°C, from the field to the lab
	ethanol 99% at -20°C (long term)
Sample preparation	1 insect per test, or grouping 10 insects of experimentally infected E. variegatus
	Extraction buffer: PBS, pH 7.4
	Amount per test: individual insect in 0.450 ml of extraction buffer
	Extraction method: pestle vortexed in the vial for a few seconds

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ELISA PROTOCOL

Double Antibody Sandwich - DAS

❑ **1 CAPTURE ANTIBODY**

- dilute specific antibody reagent **(blue code)** in coating buffer at the dilution read on the label
- dispense 200 µl per well
- incubate 2 hrs at 37°C
- wash the plate 3 times with washing buffer at room temperature, 3 minutes soaking each time

❑ **2 SAMPLE DISTRIBUTION**

- prepare samples and controls as described in the kit instructions
- dispense 200 µl per well according to the designed plate lay-out
- **incubate over night at 4°C**.
- wash the plate 3 times with washing buffer at room temperature, 3 minutes soaking each time

❑ **3 CONJUGATE ANTIBODY**

- dilute the specific conjugate antibody **(green code)** in conjugate buffer at the dilution read on the label
- dispense 200 µl per well
- incubate 2 hrs at 37°C
- wash the plate 3 times with washing buffer at room temperature, 3 minutes soaking each time

❑ **4 SUBSTRATE**

- dilute p-nitrophenil phosphate in substrate buffer at 1 mg/ml
- dispense 200 µl per well
- incubate at room temperature following signal development for 2-3 hours

❑ **5 RESULTS**

- read the absorbance of the wells by the ELISA reader filter 405 nm, after blank setting
- validate and interpret the assay outcomes against the OD readings of positive, negative and buffer controls
- signal development can be stopped by dispensing 50 µl per well of sodium hydroxide 3 M

NOTE: protocol was validated by using the high binding ELISA plate, Nunc maxisorp

ASSAY CONTROL PREPARATION

A- Positive control CARD

The positive control card consists of a preparation of recombinant Imp-FD-D protein dehydrated on a solid matrix (card)
The supplied cards are packaged in a disposable vial to be stored at +4°C.
Start the positive control preparation 10-15 min before the use.

1. Transfer **1 card** into the vial for positive control preparation, then add **500 µl PBS**
2. shake and vortex 2-3 times for few minutes
3. withdraw 200 µl as for the protocol requirement. The exceeded volumes, if any, should be discharged.
4. discharge the exhausted card according to your lab safety procedures

B -Positive and negative controls FREEZE-DRIED

The freeze-dried controls, positive and/or negative, are supplied into a glass vial. Reconstitute the content by adding distilled water as described in the vial label. Store reconstitute positive at -20°C, reconstitute negative at -20°C or +4°C.

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ELISA SOLUTIONS FORMULA

The Agritest ELISA kits are validated by using the ELISA solutions and extraction buffers according the here below formula:

Solution ELISA	Component	Quantity/1 litro	Note
Coating pH 9,6			
	Na_2CO_3	1,59 g	In distilled water pH 9,6
	$NaHCO_3$	2,93 g	
Extraction Grapevine pH 8,2			
	TRIS-HCl	37,2 g	In distilled water pH 8,2
	TRIS-base	32 g	
	NaCl	8 g	
	PVP MW 24000	20 g	
	PEG MW 6000	10 g	
	Tween 20	0,5 ml	
Extraction Vicia faba			
	PVP MW 24000	20 g	In PBS pH 7,4
	Tween 20	0,5 ml	
Extraction S. titanus and E. variegatus			
	PBS pH 7,4		
Conjugate pH 7,4			
	PVP MW 24000	20 g	in PBS pH 7,4
	BSA	2,0 g	
	Tween 20	0,5 ml	
Substrate pH 9,8			
	Dietanolammina	97 ml	In distilled water
	$MgCl_2$	0,095 g	Bring to pH 9,8 in HCl
Washing pH 7,4			
	Tween 20	0,5 ml	in PBS pH 7,4
PBS pH 7,4			
	NaCl	8 g	In distilled water
	KH_2PO_4	0,2 g	Bring to pH 7,4
	Na_2HPO_4	1,15 g	
	KCl	0,2 g	