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Working group- ARNADIA

CAV - Faenza/Ampelos
CNR-IVV Bari
CNR-IVV Grugliasco
CRA-PAV Roma
CRA-VIT Conegliano
CRSA - Locorotondo
ERSA - FVG

IASMA – San Michele all'Adige
IPAD - Lodi
UNIBO – University of Bologna
UNIMI - University of Milano
UNIPI - University of Pisa
VCR – Vivai Cooperativi Rauscedo

REGIONAL PHYTOSANITARY SERVICES INVOLVED IN THE INTER-LABORATORY RING TEST

SFR Trentino (Laimburg)
SFR Emilia Romagna
SFR Lombardia
SFR Valle d'Aosta
SFR Friuli Venezia Giulia (ERSA)

WHAT'S THE MEANING OF THE WORK DONE?

A protocol validation is the evaluation of a process to determine its fitness for a particular use. A validated assay yields test results that identify the presence of a specific target

The aim of WG has been to produce validated reference protocols allowing for the harmonization of the diagnosis of the selected grapevine viruses. The choice of the protocols to validate has been made taking into consideration their reliability, efficiency, time consuming, cost effective and large scale use

SELECTED TARGETS (VIRUSES)

- Pathological importance
- Diffusion
- Inclusion in phytosanitary rules (EU and Italy)
- Availability of large scale diagnostic tools

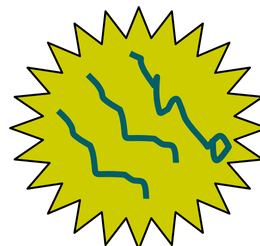
In this view has been selected eight grapevine viruses:

EU:
- GLRaV 1
- GLRaV 3
- GFLV
- GFkV
- ArMV

- GLRaV 2
- GVA
- GVB

METHODS UNDER VALIDATION

VIRUS STRUCTURE



COAT PROTEIN

NUCLEIC ACID (RNA)

ELISA: to detect the viral coat protein

RT-PCR: to detect the viral nucleic acid

SAMPLING

The vegetal matrix to use in all the protocols is the scraped phloem tissue obtained from woody material collected during the winter

Period: all the dormant season

Source of material: lignified shoots one year old

Type of sample: 2 woody portions collected from the basal part of the shoots. The woody samples must be intact and must not show changes due to abiotic or biotic factors

Maintenance of the sample: The plant material must be dry, must be placed in plastic bags to be stored at low temperatures or in a cool place such as to avoid possible dehydration

Traceability of the sample: Each sample must be properly signed on the envelope and on the plant

Shipment of samples: The samples must reach the laboratory within 72 hours, preferably in cool bags

ELISA

Antisera from: Agritest (8), Bioreba (10), Sediag (9)

Viruses: GLRaV 1, 2, 3, GVA, GVB,GFLV, ArMV, GFkV, GLRaV 1+3, ArMV+ GFLV

The tests were conducted following all instructions provided by the Companies for each antiserum

Moreover, comparative tests of extraction were performed for all antisera, using the following methods:

1. Use of plastic bags and homogenizer
2. Use of mortar and pestle with or without liquid nitrogen
3. Use of milling machines

ELISA PLATE SCHEME

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
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MULTIPLEX RT-PCR

Ref. Gambino G. and Gribaudo I. 2006. Phytopathology 96 (11): 1223-1229)

Primers: GLRaV 1, 2, 3, GVA, GVB,GFLV, ArMV, GFkV, 18S ic

The tests were conducted following the above reported protocol with some modifications (ie ArMV primers)

Moreover, also in this case, comparative tests of extraction were performed, using the following methods:

1. **Silica extraction**
2. **CTAB extraction**
3. **McKenzie (1997) +Commercial KIT (RNeasy mini plant Kit – Qiagen)**

SAMPLES UTILIZED IN THE VALIDATION

Phloem tissue obtained from the bark

102 reference samples

- 67 infected (target)
  - 55 varieties
  - 12 rootstocks
- 35 healthy (not target)
  - 17 varieties
  - 18 rootstocks

Plus 20 pool samples composed of 5 plants(1 infected + 4 healthy)

Samples list (122) and their sanitary status

No.	Status	No.	Status	No.	Status
1	GLRaV 1 + GLRaV 2 + GLRaV 3 + GVA+ GFkV	46	SANO	91	GFLV, GLRaV 3
2	GVA	47	SANO	92	GLRaV 1-3 e GVA
3	GVB	48	POOL DA 18 (ArMV)	93	GFLV
4	SANO	49	SANO	94	SANO
5	SANO	50	GLRaV 3, GFkV, GFLV	95	ArMV
6	SANO	51	SANO	96	GVA
7	GLRaV3 + GVA	52	sano	97	SANO
8	GFLV	53	POOL DA 44 (GLRaV1)	98	sano
9	GFkV	54	SANO	99	sano
10	POOL DA 42 (GLRaV1, GVA,GFLV)	55	GVA + GFkV +GLRaV-3+GLRaV2	100	SANO
11	GLRaV 3 , GFLV	56	GFLV	101	SANO
12	GVA, GLRAV1, GLRaV3	57	GLRaV3	102	GFLV – GFkV – GLRaV-3
13	ArMV, GFLV, GLRaV1	58	SANO	103	GLRaV 2 (isolato BD)
14	GLRaV-1	59	GVA+GLRaV1+GFLV	104	GFkV, GLRaV3
15	POOL DA 107 (GLRaV1)	60	sano	105	GFKV
16	sano	61	sano	106	GFkV
17	POOL DA 106 (GFKV)	62	Sano	107	GLRaV1
18	ArMV	63	GLRaV 2 + GLRaV 3 + GVA	108	GVA
19	ArMV (GRSPaV)	64	POOL DA 12 (GVA,GLRaV1-3)	109	sano
20	GVA, GRLaV2, GLRaV3	65	SANO	110	SANO
21	GLRaV-3+GVA	66	GLRaV 2, GLRaV 3 GVA	111	GLRaV 3 + GVA
22	GLRaV-1 , GFkV, GFLV	67	SANO	112	GLRaV 3, GVA, GFLV
23	SANO	68	GLRaV 2 (isolato BD)	113	GFKV
24	SANO	69	GFLV – GFkV – GLRaV-3	114	GLRaV 2 (isolato RG)
25	GVA + GFLV	70	GFLV	115	POOL DA 70 (GFLV)
26	GLRaV-1 GLRaV-3 GVA	71	GVA – GFkV – GLRaV-3	116	POOL DA 112 (GLRaV3, GVA, GFLV)
27	POOL DA 93 (GFLV)	72	GFkV+ GLRaV 2 + GLRaV 3 + GVA	117	POOL GFLV
28	GFkV	73	GFkV	118	POOL DA 72 (GFKV, GLRaV2-3,GVA)
29	sano	74	POOL DA 1 (GLRaV1-2-3,GVA,GFkV)	119	POOL DA 95 (ArMV)
30	GLRaV 3	75	ArMV, GFKV	120	POOL DA 25 (GVA,GFLV)
31	GVA	76	GLRaV2 GLRaV3	121	POOL DA 71 (GVA, GFKV,GLRaV3
32	SANO	77	GFkV + GLRaV2	122	POOL DA 113 (GFKV)
33	sano	78	GFLV, GFKV		
34	SANO	79	GLRaV3		
35	ArMV, GFLV, GFKV, GLRaV1, GVA	80	GLRaV1		
36	POOL DA 63 (GLRaV2-3, GVA)	81	SANO		
37	GLRaV 2 + GFkV	82	Sano		
38	POOL DA 66 (GLRaV2, GLRaV3,GVA)	83	SANO POOL		
39	GLRaV-1	84	SANO		
40	ArMV	85	sano		
41	GVA – GFkV – GLRaV-3	86	Sano		
42	GFLV + GLRaV1 + GVA	87	GVA, GRLaV2, GLRaV1		
43	POOL SANO	88	GLRaV2 (isolato BD)		
44	GLRaV1	89	SANO		
45	GLRaV3	90	GLRaV-1 e GVA		

All samples were analyzed in blind

VALIDATION PARAMETERS

For the protocol validation has been calculated the following parameters, according to UNI/CEI/EN ISO/EC 17025 and 16140 – EPPO – Diagnostics PM7/76 and PM7/98:

- **Diagnostic sensitivity:** ability of the method used to detect the presence of the pathogen in the samples surely infected by the pathogen in question - true positive
- **Diagnostic specificity:** ability of the method used to NOT detect the presence of the pathogen in samples not infected by the pathogen in question - true negative
- **Accuracy:** the average of diagnostic sensitivity and specificity
- **Analytical sensitivity:** the smallest amount of infectious entities that can be identified by the diagnostic method
- **Repeatability or accordance:** degree of conformity of the results obtained in replications of the method, made at short intervals of time, using the same reference sample and in the same working conditions i.e. equipment, operator, laboratory
- **Reproducibility or concordance:** degree of conformity of the results obtained using the same method with the same reference samples in different laboratories

PERFORMANCE CRITERIA

Obtained positive / expected positive (positive agreement)	A	C	Obtained positive / expected negative (positive deviation)
Obtained negative / expected positive (negative deviation)	B	D	Obtained negative / expected negative (negative agreement)

% SENSITIVITY: A/(A+B)
% SPECIFICITY: D/(C+D)
% ACCURACY: A + D/(A+B+C+D)

Analytical sensitivity: **the smallest amount of infectious entities that can be identified by diagnostic method (in the case of plant viruses, which cannot be quantified *in vitro*, corresponds to dilution limit of initial extract in which, the used method, is able to identify the pathogen**

For the **REPEATABILITY or ACCORDANCE** has been chosen 4 targets (3 infected and one healthy) and made two dilutions. The samples were analyzed by the same person, with the same reagents, three times on the same day. The values were calculated by checking how many times the same result was repeated regardless of whether they were infected or not

% REPEATABILITY or ACCORDANCE C/N

C = concordant results

N = total samples

For **REPRODUCIBILITY or CONCORDANCE** has been applied the same method of repeatability, only that analyses were performed in different laboratories, using the same reagents, the same protocol and the same standards.

% REPRODUCIBILITY or CONCORDANCE ΣC/ΣN

SC = summation of concordant results for each samples

SN = summation of number of laboratories that analyzed the same sample

ELISA RESULTS EVALUATION

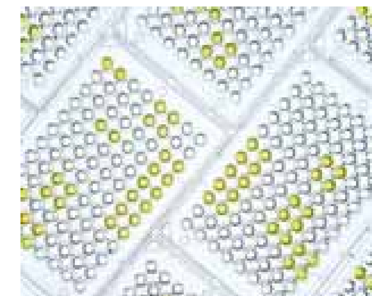
INTERPRETATION OF READINGS WITH PHOTOMETER

Background (A) = average of the values of the absorbance of the negative controls (healthy and blank - max 0.2 OD)
Threshold (B) = A x 2,5 (If this value was greater than or equal to 0.1 OD, otherwise the threshold value will be equal to 0.1 OD).
Reading of sample: ≥ B = positive
Reading of sample: < B = negative

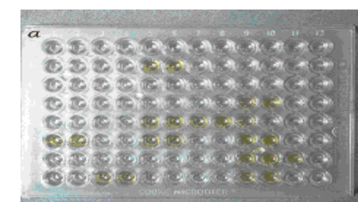
In the event that the two replicas were not both above or below the threshold B, the sample was considered doubtful and analyzed again, using the same homogenate, when stored in the refrigerator within 48 hours of its preparation, otherwise a new extract.

ELISA - OBTAINED RESULTS

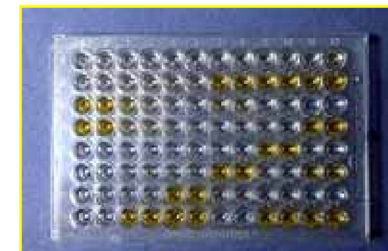
	AGRITEST							
Parameter	GLRaV1	GLRaV2	GLRaV3	GFKV	ArMV	GFLV	GVA	GVB
Sensitivity	89	86	81	85	64	75	74	86
Specificity	97	100	100	100	85	96	100	100
Accurancy	93	90	84	88	74	80	80	92
Repeatability	100	94	100	100	100	100	95	100
Reproducibility	92	88	98	92	94	87	92	100
Sensitivity pool	85	32	66	72	64	65	73	nt
Specificity pool	100	95	94	100	94	100	100	nt
Accurancy pool	89	59	75	84	81	79	81	nt
Repeatability pool	100	94	100	100	100	100	95	nt



	BIOREBA								
Parameter	GLRaV1	GLRaV2	GLRaV3	GFKV	ArMV	GFLV	GVA	GLRaV 1+3	ArMV+GFLV
Sensitivity	94	67	90	90	48	82	45	84	88
Specificity	100	100	100	100	95	92	100	100	62
Accurancy	96	77	92	92	71	84	58	90	82
Repeatability	100	92	100	100	100	100	100	100	100
Reproducibility	91	82	91	86	93	91	73	92	93
Sensitivity pool	61	38	97	47	42	90	30	81	79
Specificity pool	100	100	100	100	100	93	75	94	62
Accurancy pool	73	64	98	70	76	91	43	85	73
Repeatability pool	100	86	94	100	100	86	86	100	86



	SEDIAG							
Parameter	GLRaV1	GLRaV2	GLRaV3	GFKV	ArMV	GFLV	GVA	ArMV+GFLV
Sensitivity	96	87	97	30	50	77	87	67
Specificity	100	100	100	100	96	92	96	67
Accurancy	98	91	97	46	72	81	89	67
Repeatability	100	92	100	95	100	100	100	96
Reproducibility*	92	82	92	88	96	91	88	74
Sensitivity pool	77	21	100	42	43	79	82	47
Specificity pool	100	95	93	100	97	94	100	67
Accurancy pool	84	53	98	67	74	85	87	53
Repeatability pool	100	92	100	95	100	100	100	83



ELISA – Some considerations arising from the analysis of the results of different parameters

Extraction methods

Method 1: Use of plastic bags and homogenizer
Method 2: Use of mortar and pestle with or without liquid nitrogen
Method 3: Use of milling machines

Method 1 resulted less sensitive (5-8%) of method 2 and 3 in detection of GFLV, ArMV and (2-4%) GVA.
Method 2 and 3 resulted equivalent between them

Time reading of the results

It was not possible to establish an optimum time for reading regardless viruses and antisera. It seems to be absolutely dependent only from the laboratory.

Rootstocks

No difference highlighted for GLRaV 1, 2, 3 and GFkV between the samples of European varieties and rootstocks. Small and not always statistically significant differences (in negative for rootstocks) for ArMV, GVA and GFLV. No difference between the different Companies for the same antiserum,

Pool samples

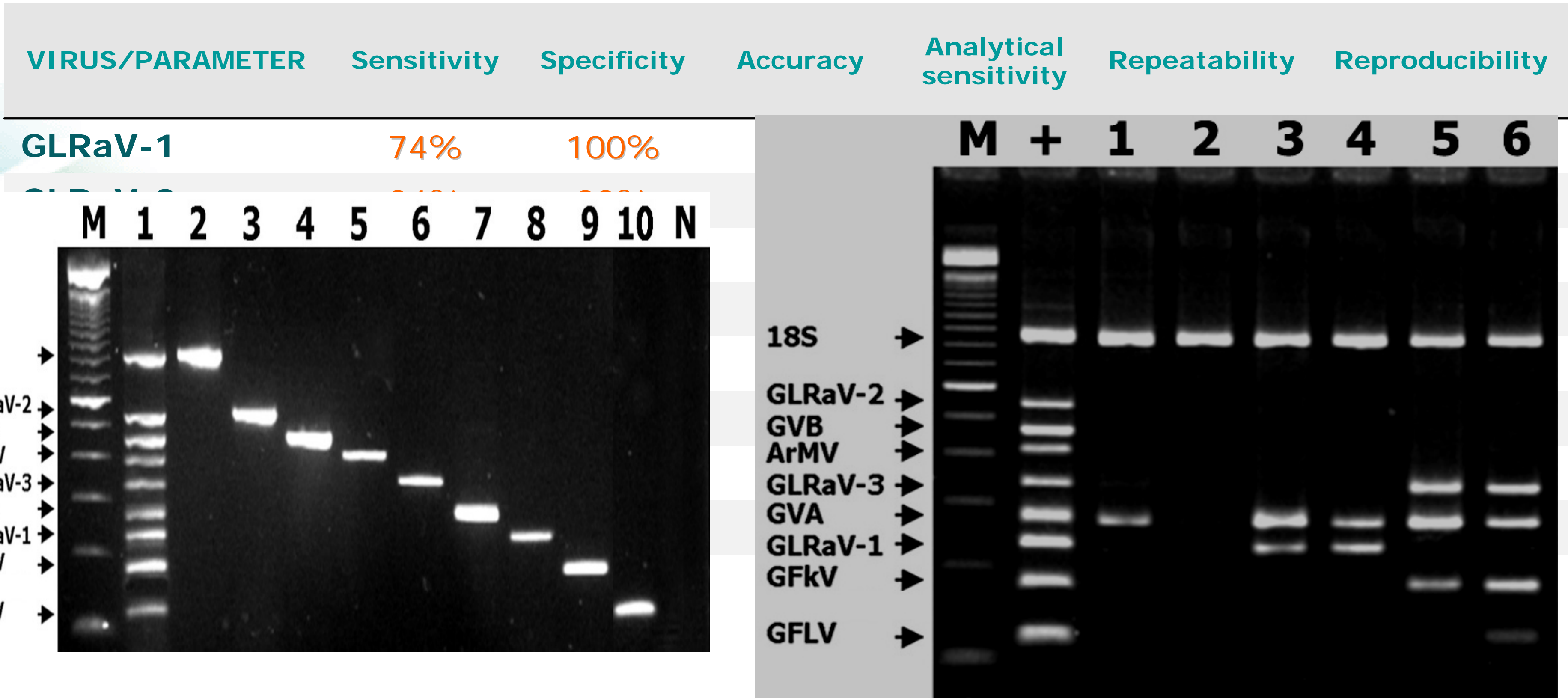
Generally good results by the pool samples. Surely the accuracy was found to be lower (10-15 percentage points) for GLRaV 1, GLRaV 2 and GFkV compared to individual samples. No statistically significant difference for others.

Antisera comparison

All Antisera behaved absolutely equivalent in the diagnosis of GLRaV 1, 2, 3, GFLV, ArMV. Only two antisera (GFkV of Sediag and GVA of Bioreba) have given results less valid than the respective antisera of other Companies. Good results were obtained by mixed antisera (GLRaV 1 + 3 and GFLV + ArMV) by Bioreba, while mixed antiserum GFLV + ArMV by Sediag proved less performant.

MULTIPLEX RT-PCR - OBTAINED RESULTS

VIRUS/PARAMETER	Sensitivity	Specificity	Accuracy	Analytical sensitivity	Repeatability	Reproducibility
GLRaV-1	74%	100%				



MULTIPLEX RT-PCR

results evaluation with regards to:

Extraction methods

Method 1: Silica extraction
Method 2: CTAB extraction
Method 3: McKenzie (1997) +Commercial KIT (RNeasy mini plant Kit – Qiagen)

THE THREE METHODS resulted equivalent among them, we suggest the use of the METHOD 3 since is foresees the use of a commercial KIT, giving more assurances about the standardization of the methodology

MOLECULAR vs ELISA

Virus	Diagnostic protocol	Sensitivity	Specificity	Accuracy	Analytical sensitivity	Repeatability	Reproducibility
ArMV	*Multiplex RT-PCR*	92 %	99 %	98 %	10^{-2}	100%	100 %
	ELISA – A/B/S	64/48/50%	85/95/96%	74/72/72%	10^{-2}	100%	95%
GFLV	*Multiplex RT-PCR/*	68 %	100%	90 %	10^{-3}	100%	76%
	ELISA – A/B/S	75/82/77%	96/92/92%	80/84/81%	10^{-2}	100%	90%
GFkV	*Multiplex RT-PCR*	95%	95%	95%	10^{-2}	100%	95%
	ELISA – A/B/S	90/90/30%	100%	92/92/46%	10^{-1}	98%	88%
GVA	*Multiplex RT-PCR*	96 %	99 %	98 %	10^{-2}	100%	94 %
	ELISA – A/B/S	77/45/87%	100/100/96%	83/58/89%	10^{-1}	98%	82%
GVB	*Multiplex RT-PCR*	100%	100%	100%	10^{-2}	100%	100%
	ELISA – A/B/S	86%	100%	92%	10^{0} (2^{-2})	100%	85%
GLRaV 1	*Multiplex RT-PCR*	74 %	100 %	94 %	10^{-2}	100%	70 %
	ELISA – A/B/S	89/94/96%	100%	93/96/980%	10^{-2}	100%	92%
GLRaV 2	*Multiplex RT-PCR*	84%	98%	85%	10^{-2}	95%	83%
	ELISA – A/B/S	86/67/87%	100%	93/96/98%	10^{0} (2^{-2})	93%	84%
GLRaV 3	*Multiplex RT-PCR*	100 %	93 %	95 %	10^{-3}	100%	100 %
	ELISA – A/B/S	81/90/97%	100%	84/92/97%	10^{-3}	100%	94%
ELISA		81/75/77%	98/99/97%	87/86/86	10^{-1}	99%	89%
MULTIPLEX RT-PCR		89%	98%	95%	10^{-2}	100%	90%

REFERENCE SAMPLES COLLECTION

Ref.	Variety/origin	Sanitary status
1	Sagrantino	GLRaV 1
2	Sagrantino	GLRaV 3
3	P4/K-S	sano
4	P6/K-S	sano
5	Pinot nero	GLRaV 1
6	Gold Traminer	GLRaV 3
7	Gold Traminer	GFLV
8	Traminer 921 vm	GFLV
9	Pinot nero 189	ArMV
10	Muller Th. 8013	ArMV
11	Traminer 920 vm	GFLV
12	1103 Paulsen P.38	sano
13	Pizzutella 2	GVA, GFkV,GLRaV 3
14	161/049	GFKV
15	157/11	GFKV
16	camp. 31637	GVB
17	camp. 31635	GVB
20	85 ALB 027	GLRaV 2
21	66 MLI 63 P8	GLRaV 2
22	ELISA 17/2007	GLRaV 3,GVA, GVB
23	ELISA 28/2007	GLRaV 3,GVA, GVB
24	Piedirosso 4-19-019	sano
25	Piedirosso 4-19-034	sano
26	Scimiscià	GLRaV 1
27	Pecorello	GLRaV 2
28	Berla Grossa	ArMV
29	Nebbiolo 185	GFkV
30	Moscato 30	GVA
31	Albarossa	GVB
32	varieta europea	GLRav 1, GFLV, ArMV
33	Riesling	ArMV, GFKV
34	Sangiovese Ceppo G2	GFkV
35	Neg 8	sano
36	Neg 13	sano
37	2/9/4 riparia Scribner	GLRaV 2, GLRaV3, GVA
38	142/19/4 Terra Promessa	GLRav 1, GFLV, GVA
39	145/20/3 Red Globe	GLRaV 2
40	151/14/5 Cereza	GLRaV 3, GVA
41	1/7/2 Riparia Baron	GFLV
42	152/11/2 Corazon de Angel	GVA
43	4/9/2 Vitis Coignetiae	GLRaV 2, GFkV
44	147/19/1 Madelaine Vialette	GLRaV 3

CONCLUSION

- For the first time are available harmonized and validated reference diagnostic protocols for the main grapevine viruses
- The efficiency and robustness of the protocols has been proved using a large number of samples in a high number of labs
- For the first time a reference samples collection (targets and not targets) has been established
- The use of these diagnostic tolls will improve the quality of grapevine germplasm for collections, for mobilization or for sanitary selection purposes

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