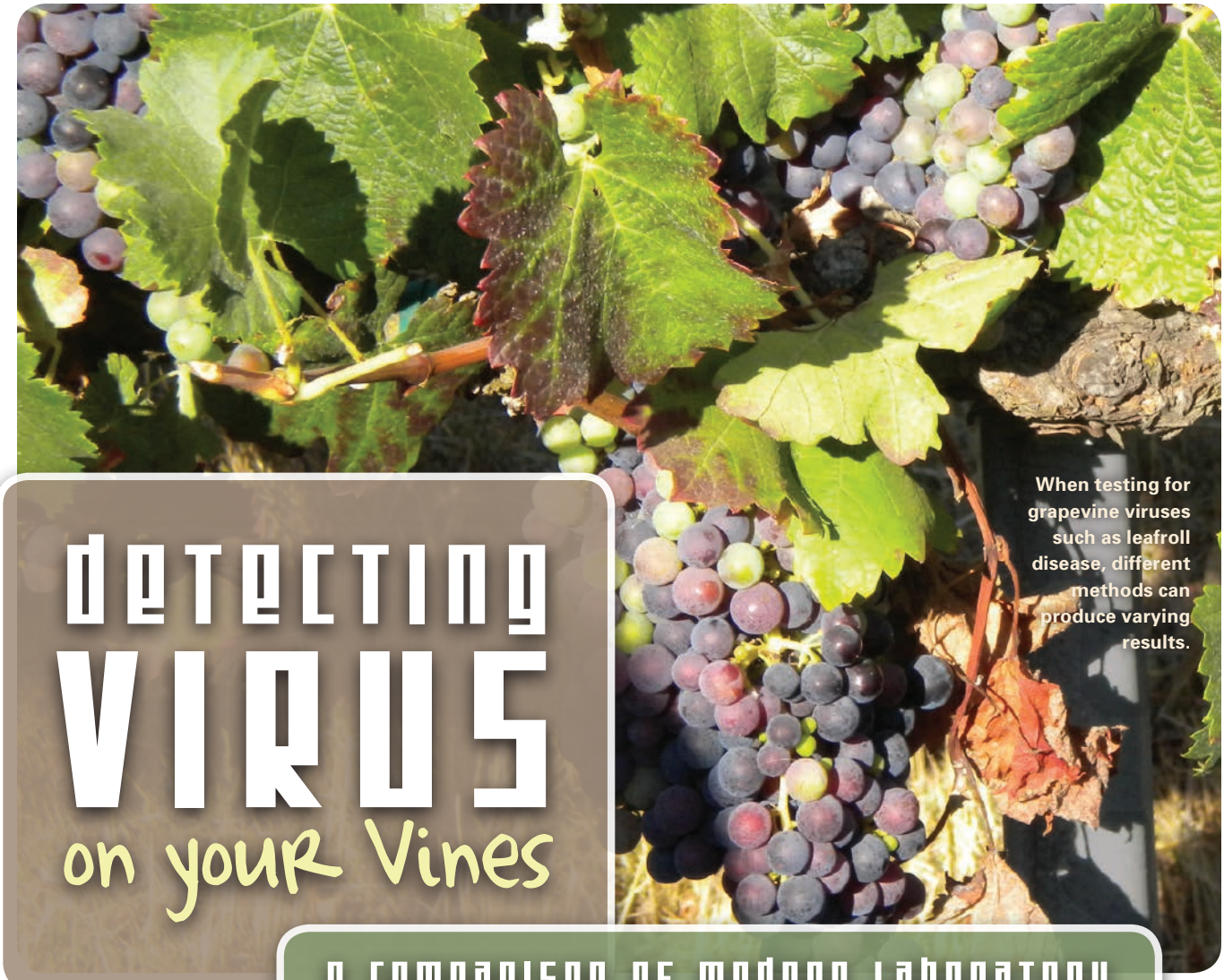

When testing for grapevine viruses such as leafroll disease, different methods can produce varying results.

Detecting VIRUS on your Vines

A comparison of modern laboratory methods and technologies

By Judit Monis, Ph.D.

Each season, thousands of samples are submitted to laboratories to determine whether viruses are present that affect the quality of grapes for wine production. Vineyard professionals wonder why testing results are different when similar samples are submitted to different laboratories. This should not be shocking: Testing methodologies vary from laboratory to laboratory, as do the interpretation of results.

LAB TESTING METHODS

Two methods are standard for the detection of grapevine viruses in a commercial testing setting: ELISA and RT-PCR. ELISA stands for "enzyme-linked immuno-sorbent assay" and RT-PCR is short for "reverse transcription-polymerase chain reaction."

ELISA uses antibodies that bind directly with the virus of interest affixed onto a test plate. After the virus binds, an enzymatic reaction occurs that will develop color in the test well if the sample is positive. ELISA has a broad virus detection capability; however, it is limited to the amount of virus present in the test sample. For example, the virus concentration in the vine sample could be weak due to seasonality, and therefore not be detected. Additionally, ELISA reagents are more dif-

AT A GLANCE

- Testing methodologies and results interpretations vary from laboratory to laboratory.
- The sensitivity of TaqMan and RT-PCR are similar when known virus-infected samples are tested.
- The genetic specificity of TaqMan can be a disadvantage, as it can miss detection of viruses with slight changes (or mutations) in grapevine viruses.
- Two complementary testing methods are recommended for test results assurance.

ficult to produce and good-quality reagents are not available for some of the important grapevine-infecting viruses such as *Grapevine virus* B (GVB).

In recent years, the knowledge of grapevine virus genomic composition has increased markedly, making RT-PCR a powerful technique for detecting grapevine viruses in vines with low-virus concentrations, which ELISA fails to do. The RT-PCR technique works by amplifying (i.e., making multiple copies of) the virus' genetic material extracted from the infected grapevines. The amplification process is repeated many times with each copy, making many more copies of the initial template. After the completion of the final cycle, over a billion copies of the virus are produced.

Because most viruses infecting grapevines are composed of RNA, the viral RNA must first be converted into DNA prior to the amplification step. A couple of primers, short pieces of DNA that specifically bind to each side of the viral RNA, and an enzyme (Taq polymerase) are required for the RT-PCR process. The virus-copied DNA is stained with a UV fluorescent dye and imaged using gel electrophoreses, which separate the products by mass. If a fluorescent band of the correct size is detected, the presence of the virus is confirmed.

The RT-PCR method is laborious and expensive relative to ELISA – the cost of reagents for RT-PCR is roughly 10 times the cost for ELISA – and should be used for the detection of viruses when concentration in the vine is uncertain.

TaqMan is a specific type of PCR (also known as real-time or quantitative PCR). It is named after *Thermus aquaticus*, the bacterium, from which the Taq polymerase was isolated from, and its Pac Man-like chewing nature.

This method is nearly identical to RT-PCR, except that a fluorescent-labeled probe (similar to the primers described above) is included in the PCR reaction. During the amplification process, each DNA copy releases a fluorescent signal from the probe. The total fluorescence is measured during each PCR amplification cycle in real time, hence its name "real-time RT-PCR" (TaqMan is the commercial name for a type of real-time PCR).

Virus presence is concluded if a critical rise in fluorescence with each PCR cycle is detected. TaqMan's primary advantage over RT-PCR is its ability to relatively quantify the concentration of virus rather than a yes or no of virus presence. However, since TaqMan cannot measure the amplified DNA fragment size, one can conclude a false POSITIVE virus presence when, by chance, the fluorescence was caused by an artifact of the PCR process.

TaqMan's other disadvantages compared to RT-PCR include the limitation in the availability of reliable probes for each virus and the significant expense to both develop and run. (The cost is even higher than RT-PCR and probes are not available to detect all known viruses.)

Lastly, while both TaqMan and RT-PCR use two end primers, TaqMan has an additional limitation because the probe must further match the virus template code in yet one more location. This increases the likelihood that TaqMan will miss virus detection in the event of virus mutation and yield a false negative result. This is a very practical concern since viruses in the vineyard have a high frequency of mutation. One can address the mutation issues by using multiple TaqMan probes (one for each mutation), but this fix only works for already characterized mutations. This underscores that, regardless of RT-PCR or TaqMan, the issue of mutation is the critical reason why testing labs should include ELISA to complement their grapevine virus testing program.

While some researchers have stated that TaqMan is more sensitive than RT-PCR, differences in sensitivity are mostly a result of non-optimized RT-PCR procedures, especially in light of the fact that TaqMan and RT-PCR are variations of the same PCR technology.

RESEARCH AND DISCOVERY METHODS

Recently, grapevine viruses have been discovered and characterized using a technique called "deep" or "next generation" sequencing. A "genomic library" – a recombinant copy of all genetic material including grapevine nucleic acid – must be first produced from the individual grapevine (whether it is suspected to be infected or not) to be analyzed.

The technique provides many genetic signatures (short sequences) that are compared using computer algorithms. Because the grapevine genome is known, the host (grapevine) genes can be subtracted from the data so that the software only analyzes sequences that might represent viruses (or other pathogens). The researcher can focus the analysis on practically any organism he or she wishes to study.

The data output looks somewhat like a laundry list of known or related viruses and indicates the number of copies of each present. This method is very expensive (deep sequencing costs $8,000-$17,000 per run) and can only be applied to a few specimens at a time.

Unexpected, yet interesting, results were published in the September 2011 issue of Phytopathology magazine, in which researchers at Missouri State University compared a presumably healthy versus a diseased grapevine. The "healthy" vine was found infected with low copies of certain known viruses while the "diseased" vine yielded many more copies of known viruses, including the groundbreaking discovery of the first DNA virus to infect grapevines (not RNA, as had been found exclusively to date).

While deep sequencing is a powerful research tool, much work remains to characterize these newly discovered viruses. The information can be overwhelming and much like opening a sort of Pandora's Box. Further research will determine which of these new viruses play a role in grapevine disease and if they pose a threat to vineyard health.

COMPARATIVE STUDIES USING TAQMAN

In 2011, Eurofins STA Laboratories teamed up with the UC Davis Foundation Plant Services laboratory to determine if the recent scientific reports on the use of TaqMan for the detection of grapevine viruses could be applied in a commercial lab setting. Our staff visited the FPS laboratory and worked with its staff to process a set of known virus-infected and uninfected grapevine samples.

A series of comparative experiments were run to determine the effects of sample preparation on the detection capabilities of TaqMan. Because each laboratory preparation and testing protocols are unique to its standard operating procedures, sample processing and testing were done using sampling, grinding and extraction methods used in both labs in all possible combinations.

The effectiveness of TaqMan was compared against Health-Check, STA's in-house test panel. The panel includes a proprietary combination of RT-PCR and ELISA for the specific detection of 16 viruses found in California grapevines (Table 1).

Currently, TaqMan is not available for the detection of Grapevine leafroll-associated virus (GLRaV)-6, Grapevine Syrah virus (GSyV)-1 or the Syrah strain of *Rupestris* stem-pitting-associated virus (RSPaV).

We found that both TaqMan and our in-house test panel detected the tested viruses equivalently with the following exceptions: Our in-house panel detected Grapevine fleck virus (GFkV) and GLRaV-4-9 in two out of the nine samples tested in which these viruses were not detected by TaqMan. Additionally, we found no significant difference in virus detection between the different grinding and extracting methods.

Because of time limitations, the round of experiments completed at UC Davis was performed with nine positive and one negative control. Later we expanded the study in our laboratory by running dilution series of more than 30 positive and negative controls to determine if one technique would be better than the other in detecting very low (dilute) concentrations of virus.

This was done by running TaqMan and RT-PCR side by side with serial dilutions of known infected grapevine samples from full viral strength (1:1) to one ten-thousandth viral strength (1:10,000), limited to only those viruses for which TaqMan is developed. We found no significant difference between the virus concentration detection sensitivity for TaqMan and RT-PCR (Figure 1).

Having confirmed the detection sensitivity of both methods as equivalent, we proceeded to the practical goal of this work: How do TaqMan and a full test panel compare in the detection of viruses with "real life" vineyard samples? The essential component to any diagnostic technique is that it must work in the field and not solely in the lab. Therefore, testing many vineyard and nursery samples rather than a limited number of positive or negative controls, is a more effective way to validate a method.

To determine this we used field samples randomly chosen from samples submitted to Eurofins STA Laboratories from clients' plant material collected in the 2011-2012 fall/winter season. Within the group of 251 samples tested, TaqMan failed to detect virus infection in many of the samples compared to the combined RT-PCR and ELISA panel.

Remarkably, TaqMan missed the detection of samples infected with GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-4, GLRaV -5, GFKV, and GVB (Table 2). No difference in detection between the methods was found when testing for GLRaV-2 Red Globe (RG strain, GLRaV-7, GLRaV-9, Grapevine virus A (GVA), and Grapevine virus D (GVD).

The lack of detection of the above viruses by TaqMan can be explained simply by the variation of mutant virus populations present in vineyards. When grapevine viruses multiply they are continuously mutating and are thus prone to minor genetic changes.

TABLE 1: LIST OF GRAPEVINE VIRUSES AND METHODS FOR DETECTION

Virus	ELISA	RT-PCR	TaqMan	Health-Check
GLRaV-1	Yes	Yes	Yes	Yes
GLRaV-2	Yes	Yes	Yes	Yes
GLRaV-2-RG	Yes	Yes	Yes	Yes
GLRaV-3	Yes	Yes	Yes	Yes
GLRaV-4	Yes	Yes	Yes	Yes
GLRaV-5	Yes	Yes	Yes	Yes
GLRaV-6	Yes	Yes	No	Yes
GLRaV-7	Yes	Yes	Yes	Yes
GLRaV-9	Yes	Yes	Yes	Yes
GSyV-1	No	Yes	No	Yes
GVA	Yes	Yes	Yes	Yes
GVB	No	Yes	Yes	Yes
GVD	No	Yes	Yes	Yes
GFkV	Yes	Yes	Yes	Yes
RSPaV	No	Yes	Yes	Yes
RSPaV (Syrah strain)	No	Yes	No	Yes

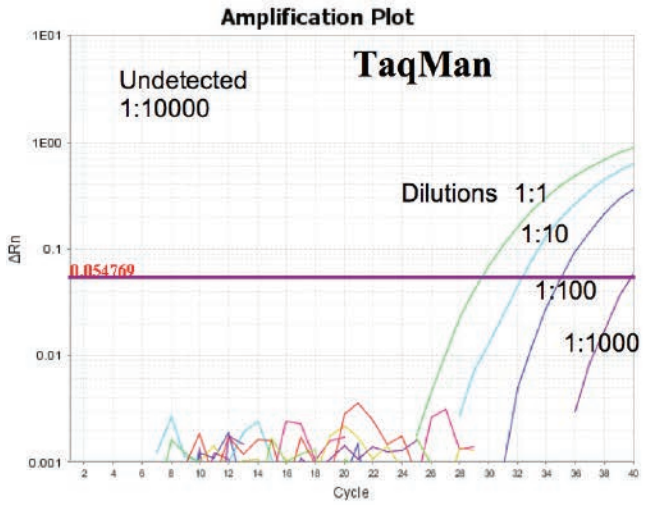

Figure 1a

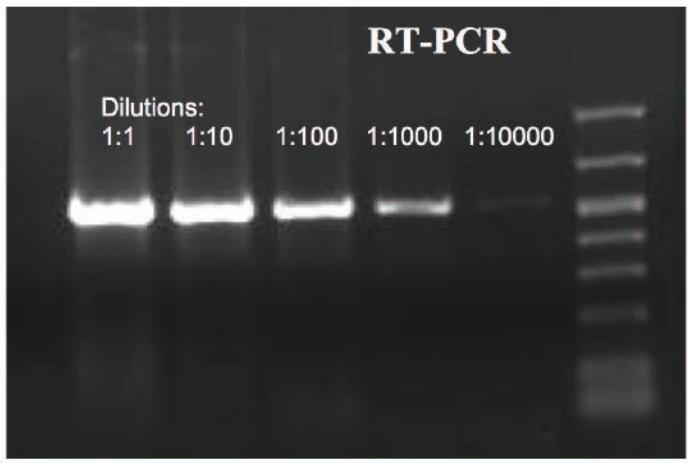

Figure 1b

This shows TaqMan and RT-PCR comparative results of the dilution series of a sample infected with GLRaV-3. The left panel shows a chart plotting the fluorescent light intensity versus the number of PCR thermal cycles (TaqMan amplification plot). Each of the curves represents the amplification of each of the serial dilutions. (Note that the 1:1 curve appears much earlier than the more diluted treatments.) The line in the plot (threshold line) is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The curve observed at the very right of the chart (1:1000) appears to be at the limit of detection with a CT (the cycle at which the sample reaches the threshold line) value of 39 out of 40. The right panel shows the photograph of a gel where the same sample was analyzed with RT-PCR. Each band represents amplified DNA fragments for each dilution treatment. Note that both TaqMan and RT-PCR equally detected virus for dilutions of 1:1 down to 1:1,000. RT-PCR shows a faint band at the 1:10,000 dilution treatment; the same diluted treatment was not detected by TaqMan.

As an example, several strains of Grapevine leafroll associated virus-3 are known to infect vineyards in single or mixed populations. Because TaqMan uses probes that are very specific to genetic sequences and can only detect the exact match, its specificity causes the probe to miss slight genetic changes in the targeted viruses.

The increased detection of Eurofins STA HealthCheck Panel relative to TaqMan, in addition to not requiring extra TaqMan probes, is that the panel uses two complementary detection techniques – ELISA with broad-spectrum detection, and RT-PCR with specific and sensitive detection capabilities – instead of relying on one test method alone.

TABLE 2: DETECTION OF INFECTED SAMPLES BY HEALTHCHECK AND TAQMAN

Virus Tested	TaqMan® Detected	HealthCheck™ Detected	Percentage TaqMan® Missed
GLRaV-1	12	14	14%
GLRaV-2	23	25	8%
GLRaV-2-RG	2	2	0%
GLRaV-3	20	30	33%
GLRaV-4	3	6	50%
GLRaV-5	4	6	33%
GLRaV-7	2	2	0%
GVA	13	13	0%
GVB	15	19	21%
GVD	0	0	0%
GFkV	16	24	33%
RSPaV	NT	120	ND

As we increase our knowledge on the biology of grapevine viruses and continue to characterize known and new viruses, each of the test methodologies discussed in this article will play an important role in vine sanitation on foundation blocks and commercial vineyards. While some might claim that one method is better than another, the best approach is to use many complementary methods in combination.

This author's experience finds that it is detrimental to rely solely on one detection method for the production of clean planting stock or vineyard disease management. More than one complementary detection method, namely ELISA and one of the PCR methods described here, should be used for accurate and sensitive pathogen detection. ■

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Comments? Please e-mail us at feedback@vwm-online.com.