give them an excellent starting point. As with the quality manual and associated documents, our goal with the checklist templates is to minimize the time and effort an individual needs to spend developing documents. We understand the value of our diagnostician's time and developing all the STAR-D templates will reduce duplicative efforts.

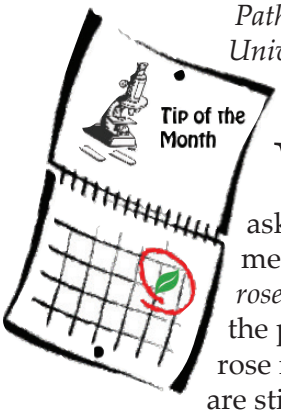
In general, using a checklist ensures that the audit at a minimum will address the requirements to the organization's management system. In addition, checklists help to ensure that an audit is conducted in a systematic and comprehensive manner. After the checklist templates were created the group performed a gap audit at the UFPDC successfully implementing the newly created checklists.

The focus of the STAR-D group this upcoming year will be preparing the trained auditors from the fall 2011 workshop to gain practice in real-world laboratory audits during four scheduled practice audits in NPDN laboratories around the country. Additionally, Quality Management and Auditor Workshops are in the planning stages. None of these activities would be possible without the support of our CPHST partners and financial support from the 2012 Farm Bill.

Diagnostic Updates

How We Test for Rose Rosette in Oklahoma

Jen Olson, Department of Entomology and Plant Pathology, Oklahoma State University



We have had many colleagues ask us to share our method of diagnosing *rose rosette virus* (RRV), the proposed cause of rose rosette disease. We are still working out some of the kinks, but are willing to share our technique. At this point, the test does not *always* work, but we do have good results most of the time. Overall, the results of this assay correlate with our visual diagnosis. This is the method that we use at the Plant Disease and Insect Diagnostic Laboratory at Oklahoma State University. It is likely that you will have to make some adjustments if you choose to use this testing method in your laboratory.

The sample material required for RRV testing is symptomatic shoots. It is best if clients clip at least 6 inch shoots and place them in a sealed plastic bag. If the rose leaves are damp, they should wrap the shoots in a dry paper towel. The plant tissues selected for RNA extraction are the symptomatic leaves and/or floral parts (*Figure 1*).

Figure 1. Ornamental rose with symptoms of rose rosette disease. Photo courtesy of Oklahoma Cooperative Extension Service (OCES).

The method we use is based on the procedure developed by Laney *et al* (1). In this paper, the extraction method calls for a large volume of plant material and a high speed centrifuge. In some cases, we are limited on the quantity of infected plant tissue and this equipment is not readily available in our laboratory. As a result, we decided to try the RNeasy Plant Tissue Kit from Qiagen, Inc. We follow the manufacturer's instructions and we generally use 70 mg of plant tissue for the assay. To date, this method has worked quite well for detection of RRV in our diagnostic laboratory.

Following RNA extraction, we generate cDNA by preparing the master mix shown in Table 1. According to the instructions that are included with the reverse transcriptase enzyme used in our laboratory, following sample preparation, the tubes should be incubated at 37°C for 1 hour. If you use another type of reverse transcriptase, you will need to check the temperature and time requirements for your enzyme. It is expected that other reverse transcriptase enzymes would work well, but these adjustments will need to be made.

For the PCR master mix, we use a 2X ready mix. We have not tried this test with "homemade" master mix because we no longer stock these products for most of our diagnostic testing. We utilize student labor and find that we have fewer mistakes when we use a ready mix as opposed to a homemade master mix where many more ingredients are required. This is our experience and yours may be different.

When we test for RRV, we actually prepare two different master mixes. The first master mix is to test for RRV and uses primers from Laney *et al.* (1). The second master mix uses primers that detect the large subunit of ribulose bisphosphate carboxylase oxygenase (RubiscoL) (2). This compound is found in all plant cells and we use this test to confirm that our RNA extraction was adequate and there are not too many inhibitors in the PCR reaction. We have had cases where we expected a positive for RRV and the PCR test was negative. When we prepared a fresh RNA extraction using less plant material (50 mg), we obtained positive test results for both the RRV and RubiscoL PCR. We suspect that this problem is due to a large amount of inhibitors in the rose plant tissue. By using less plant tissue or diluting the cDNA, you can dilute the inhibitors and lessen their interference with the reaction. We generally do not dilute our cDNA and add it directly to the PCR master mix.

At this point, we have not tried a

Table 1. Preparation of master mix for cDNA synthesis

Reagent	Vol. for 1 Reaction (ul)	____ reactions*	Final Conc.
RNase free water	7.0		
5X buffer	5.0		1X
dNTP mix (2 mM stock)	5.0		400 uM
Random hexamers (5 uM)	2.5		0.5 uM
Reverse transcriptase (200 U/ul)	0.5		100 units
Aliquot volume	20.0		
Template RNA	5.0		

**Multiply by the number of tubes needed plus 1-2 (10-15%) more than needed to allow for waste while pipetting.*

multiplex where we mix the RRV and RubiscoL primers in one tube. Instead, each master mix is placed in a separate tube. The PCR mix and primer sequences are shown in Tables 2 and 3 respectively. It is satisfactory to put the RRV and RubiscoL tubes into the same thermal cycler machine and use the same cycling parameters. The primer sets have only a 1˚C difference in annealing temperature, so we have used the lower temperature of 53˚C. The cycling parameters used by our laboratory are 94˚C for 2 min, 35 cycles of 94˚C for 30s, 53˚C for 10s, and 72˚ for 10s, with a final extension at 72˚C for 10 minutes. Following amplification, we visualize our PCR products on a 1.5% agarose gel.

When we first started running this test, we felt that we were running into inhibition problems that interfered with the PCR. We have since added a small amount of bovine serum albumin (BSA) to our reactions and this seems to help with the inhibition problem. If you attempt to follow this procedure, we suggest that you include this compound.

For those diagnosticians who are interested in having samples tested for RRV, but do not wish to run this procedure, we are willing to accept samples for RRV diagnostic testing from out-of-state. Please contact the PDIDL in advance so that we can send you our PPQ526 permit that includes instructions for properly packaging samples. Our prices are subject to change, but our current fee is $25 per sample for RRV testing. We do require prepayment for testing of out-of-state samples and checks should be made out to Oklahoma State University.

Table 2. Preparation of master mix for PCR

Reagent	Vol. for 1 Reaction (ul)	____ reactions*	Final Conc.
2X ready mix (we use Econotaq Plux Green 2X Master Mix by Lucigen Corp)	12.5		1X
PCR grade water	4.75		
Forward primer **(5 or 2 uM)	2.5		0.5 or 0.2 uM
Reverse primer (5 or 2 uM)	2.5		0.5 or 1.2 uM
BSA (10 mg/ml)	0.25		0.1 mg/ml
Aliquot volume	22.5		
Template cDNA	2.5		

**Multiply by the number of tubes needed plus 1-2 (10-15%) more than needed to allow for waste while pipetting.*

***Primers for RRV are used at 5 uM stock and 0.5 uM final concentration. Primers for RubiscoL are used at 2 uM stock and 0.2 uM final concentration.*

Table 3. Primers pairs and expected band size for RRV testing

Primer name	Primer Sequence	Expected band size	Source
RRV for	CAGAATGAACCATAGATGTC	319	Laney et al.
RRV rev	AATGGTCTGCTCGAGATT		
RbcL-C705	CATCATCTTTGGTAAAATCAAGTCCA	171	Nassuth et al.
RbcL-H535	CTTTCCAAGGCCCGCCTCA		

We encourage others to try this method in your laboratories and let us know how it works. If you have questions or comments, please contact jen.olson@okstate.edu or (405) 744–9961.

1. Laney, A. G., Keller, K. E., Martin, R. R. and Tzanetakis, I. E. 2011. A discovery 70 years in the making: characterization of the Rose rosette virus. J. Gen. Virology. 92:1727–1732.
2. Nassuth, A., Pollari, E., Helmeczy, K.,Stewart, S., and Kofalvi, S. 2000. Improved RNA extraction and one-tube RT-PCR assay for simultaneous detection of control plant RNA plus several viruses in plant extracts. J Vir Meth. 90:37–49.

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The Plant Diagnostic Lab Experience

Linnea G. Skoglund, Plant Disease Diagnostician, Montana State University and Tamla Blunt, Plant Disease Diagnostician, Colorado State University

Workshop: Nucleic Acid-based Pathogen Detection

Paul Vincelli, Department of Plant Pathology, University of Kentucky

A hands-on workshop for applied plant pathologists on nucleic acid-based pathogen detection will be held at the University of Kentucky in Lexington. The workshop will begin on Tuesday, January 22, 2013, with introductory lectures and lab activities suited for those with little PCR experience. All participants—beginners and experienced alike—will attend from Wednesday morning, January 23, 2013, through mid-day Friday, January 25, 2013, during which time participants will design, execute, and interpret three real-time PCR experiments (SYBR® Green and Taqman® assays). Presentations and discussions will include basic theory of real-time PCR, experimental controls, PCR inhibition, use of PCR kits, verifying amplicon identity, arrays, minimizing contamination, troubleshooting, sequencing (direct vs. cloning), and selecting fluorophores. Activities and discussions will be included on primer design, interpreting BLAST searches and the use of curated genomics databases. The topic of quantification will be covered but not in depth. Registration will be $250 and $300 for Wednesday-Friday and Tuesday-Friday, respectively. For more information, contact Paul Vincelli (pvincell@uky.edu).