PVY Dormant Tuber Diagnostic Workshop

April 4-6th 2023

Wisconsin Institute of Discovery (WID)
UW Madison Campus 330 N Orchard St. Madison

PVY Dormant Tuber Diagnostic Workshop

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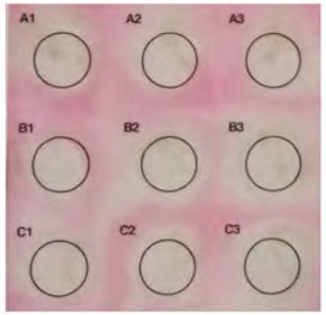
Tuber Tissue Collection – Quick-Reference Guide for Samplers

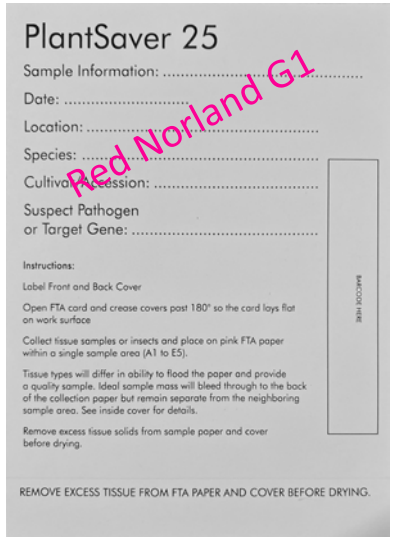


https://youtu.be/NFe2Uq6IOmM
(Spanish translations at 02:46)

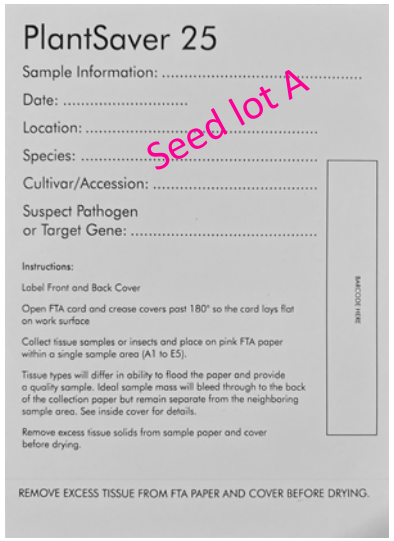
Tissue collection (1)

1 Acquire materials

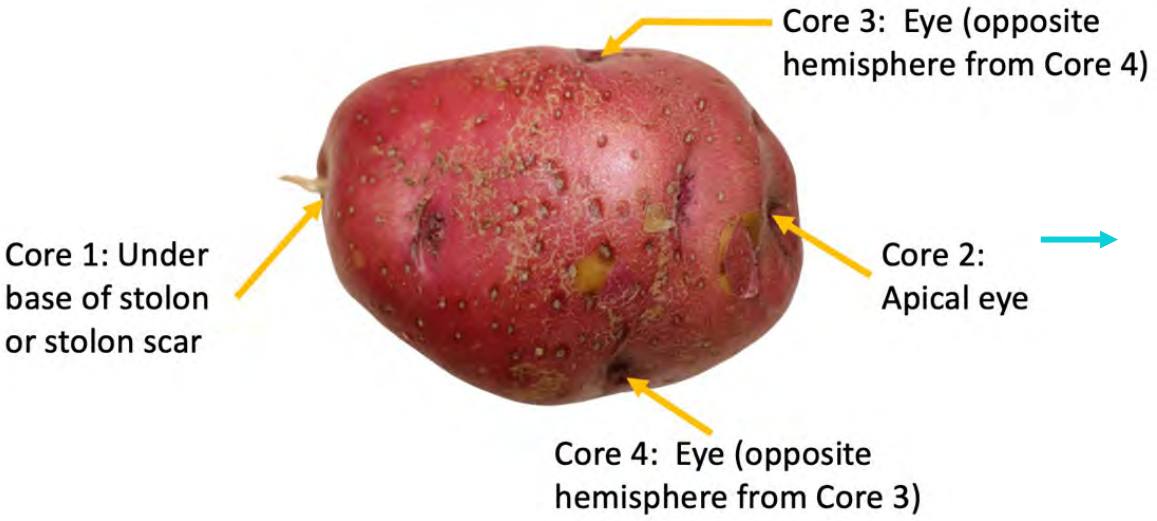
2 Label card with a unique name that will allow you to match card results to a specific seed lot (see examples)

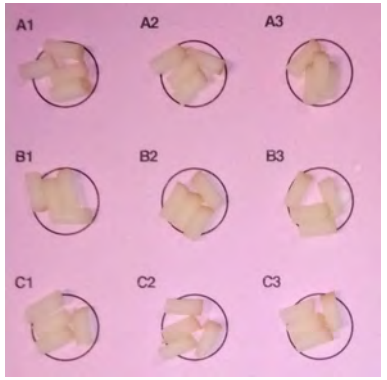


or



3 Collect cores and place in FTA circle





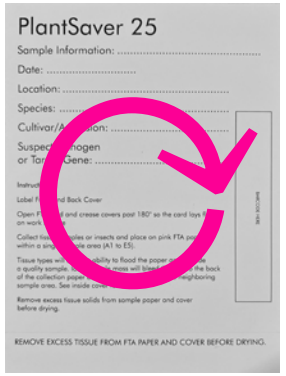
4 Repeat until card is full

Tissue collection (2)

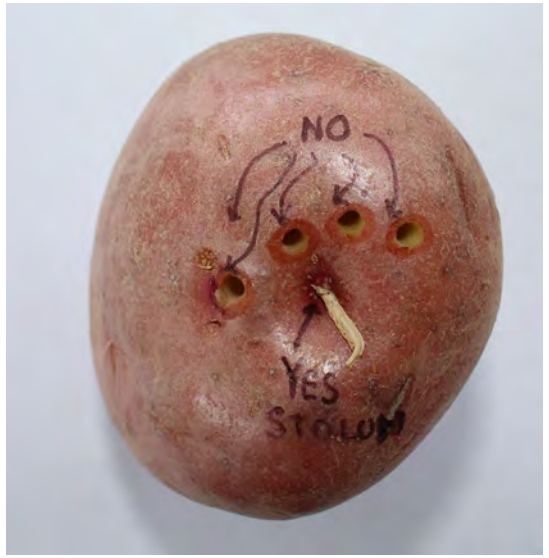
5 Completed cards go to the press

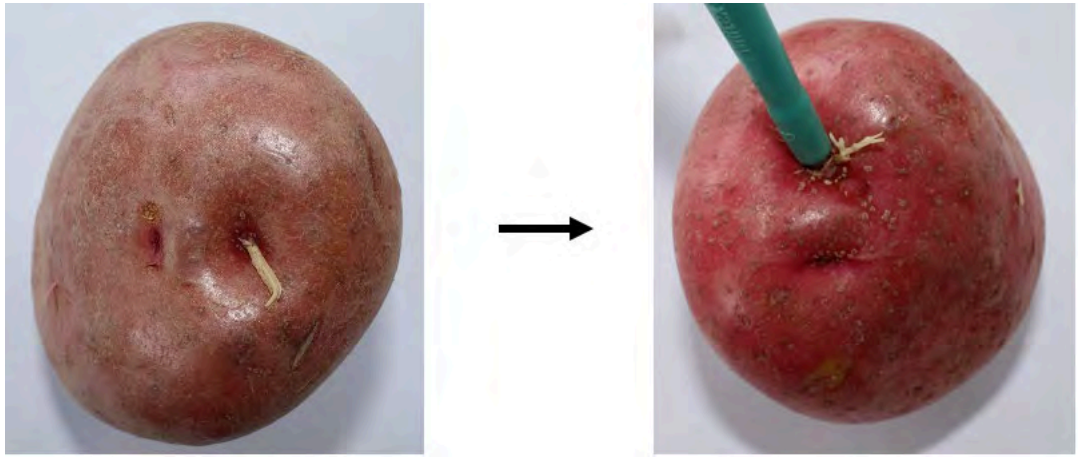
6 Replace tools between cards

7 Repeat with new card and tubers

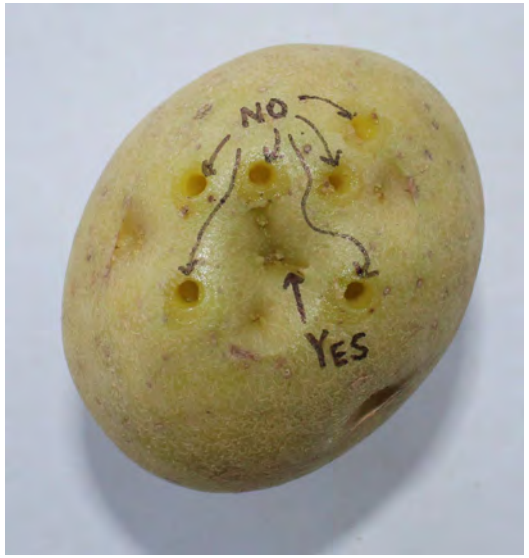


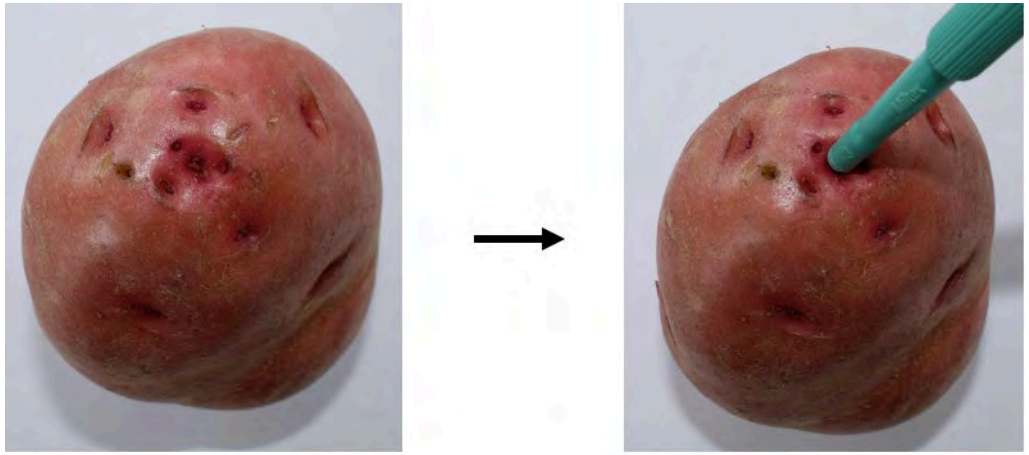
Stolon End





Apical End





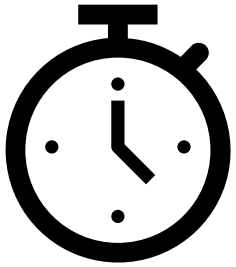
FTA card pressing

1 Create FTA card “sandwich”

Top plate
FTA card
Paper towel
Bottom plate

2 Operate press to maximum capacity

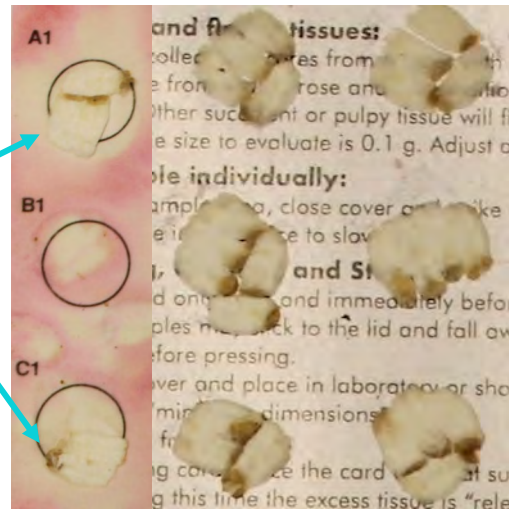
3 Leave card to press for **at least one minute**



FTA card drying

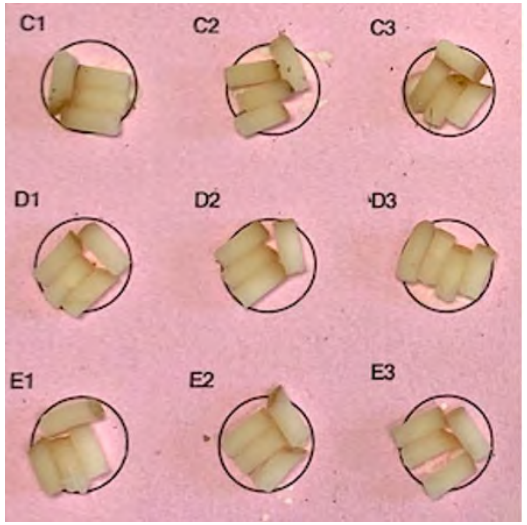
1. Leave card on flat surface to relax for 5 - 15 minutes

2. Remove any remaining tuber tissue (while card still moist)

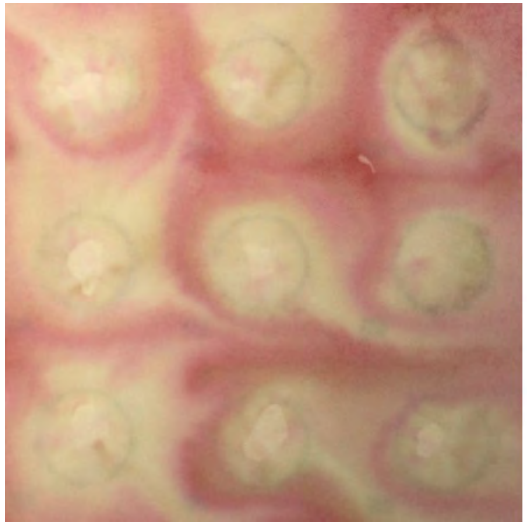


3. Open card and leave to dry (two hours to overnight)

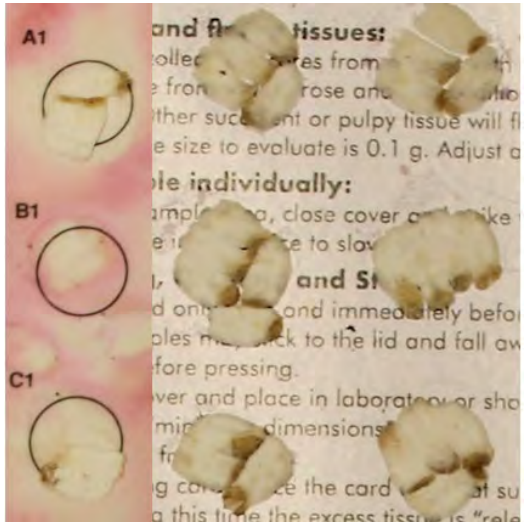
Correct card preparation



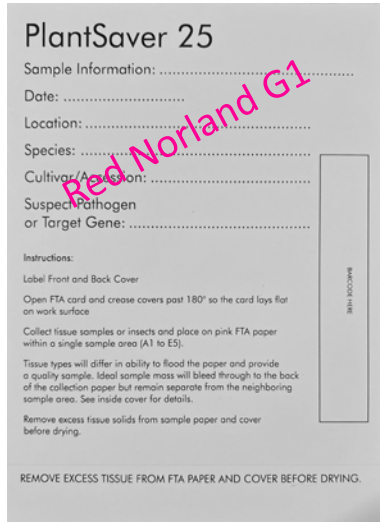
All cores centered in sampling circle



All samples soak through back of card

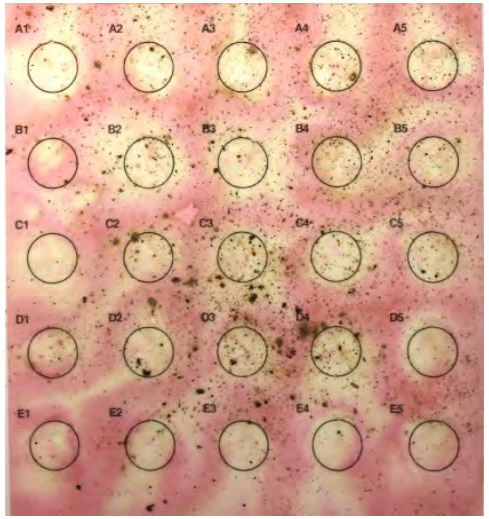


Tissue removed before drying

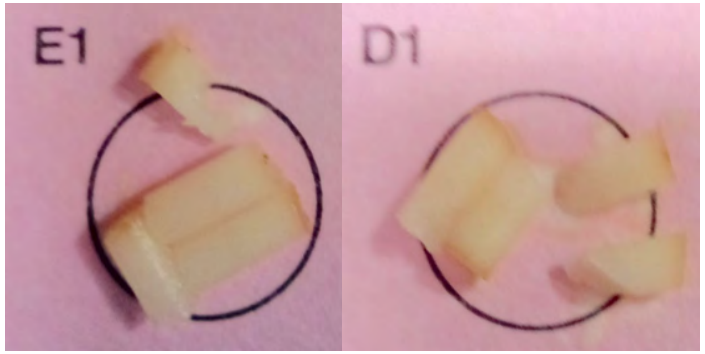


Card is labeled with unique name

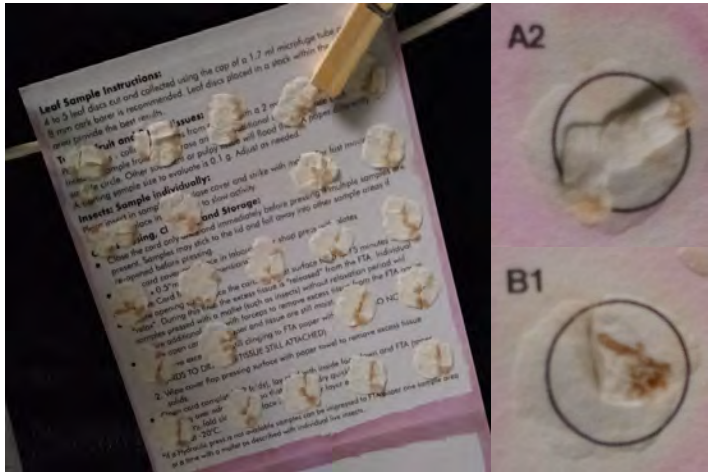
Common errors



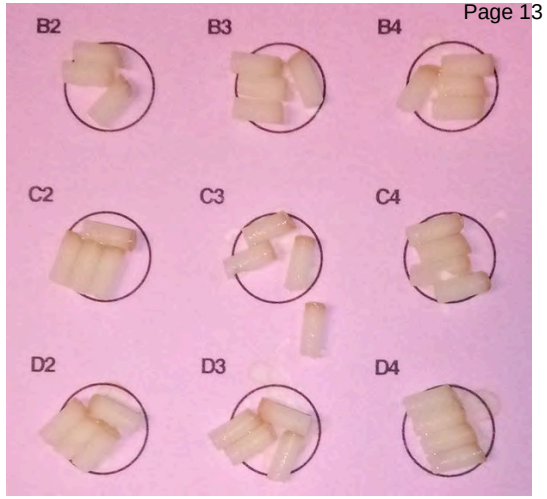
Excessive dirt on cards



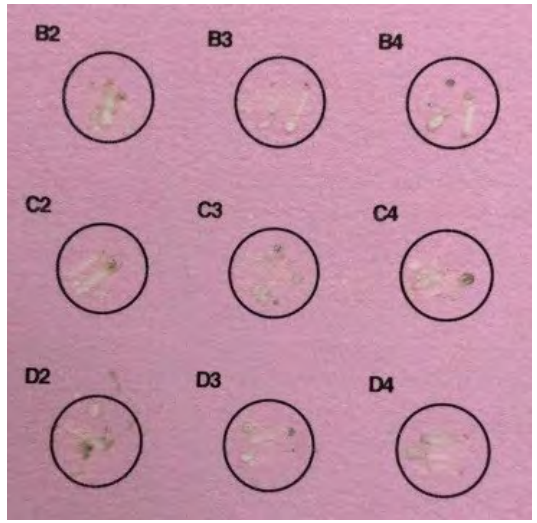
Cores too short



Letting card dry with tissue present



Cores outside sampling circle



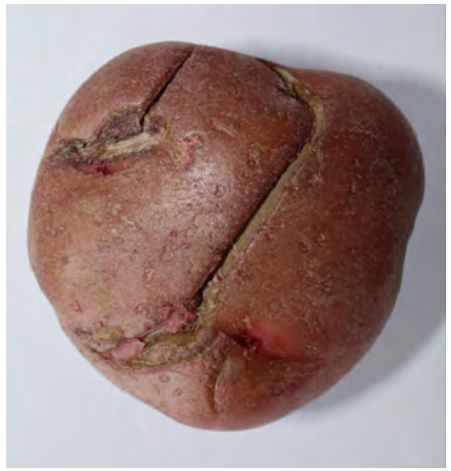
Card not adequately soaked

Incorrect tuber sampling

Core not at stolon

Damaged stolon – do not use!

Tuber with skin slip and water loss – do not use!



Damaged tuber – do not use!

Tuber with dry rot and shrunken ends – do not use!

Montana State University Seed Potato Lab
PVY RT qPCR (Immunocapture or RNA) – Short Protocol

1. Immunocapture (IC) Plate

Move Immunocapture plates from the freezer to the refrigerator or bench and allow to come to room temperature and outside condensation to evaporate.

2. Load IC plate with plant tissue

a. Crush plant tissue (leaf, sprout or tuber). Add 1-3 ml of crushing buffer per gram of plant tissue.
b. Transfer 100µL of plant macerate to a well of the coated IC PCR plate.
c. Wrap the loaded plate in plastic wrap. Incubate the macerate-loaded plates overnight at 5C (or 37C for 2-3 hours).
d. Remember to have negative and positives controls in every IC plate!

3. Wash Immunocapture (IC) Plate

e. Following the incubation, dump the sap from the IC plate and wash 3X with PBS Tween and 2X with 2X with water. Slap the plates as dry as possible on a clean paper towel.
f. The dry loaded plates can be processed (RT qPCR) immediately or stored at -20C for several months.

4. IC RT qPCR Cocktail

At this point, any intact PVY particles that were in the tissue sample are adhered to the PVY antibodies coating the PCR well.

Prepare IC RT qPCR reaction master mix and add cocktail to each well of the plate. MSU amplifications are done in a Biorad CFX 96 Thermocycler. This is basically the same cocktail and same amplification parameters that we use to amplify RNA.

RT qPCR Cocktail

Single Probe/Primer Set		
Cocktails – Probe (20 µl Reaction)	**Volume\test (µl /rxn)**	**(# Tests) x (volume)**
NEB Luna Universal Probe 1-step Reaction Mix (2X) – DYE ** (#3005)	10.0 µl	
Water	8.4 µl	
NEB Luna Warm Start Reverse Transcriptase (20X)	0.4 µl	
Forward Primer (10 µM)	0.4 µl	
Reverse Primer (10 µM)	0.4 µl	
Probe (10 µM)	0.4 µl	
Total Volume/well	**20.0 µl**	

RT qPCR Cocktail – Multiplex (two sets of Primer/Probe) (if you want to use more primer sets, adjust the water concentration so that the total volume is 20.0 µl

Multiplex		
Cocktails – Probe (20 µl Reaction)	**Volume\test (µl /rxn)**	**(# Tests) x (volume)**
NEB Luna Universal Probe 1-step Reaction Mix (2X) – DYE ** (#3005)	10.0 µl	
Water	7.2 µl	
NEB Luna Warm Start Reverse Transcriptase (20X)	0.4 µl	
Forward Primer #1 (10 µM)	0.4 µl	
Reverse Primer #1 (10 µM)	0.4 µl	
Probe #1 (10 µM)	0.4 µl	
Forward Primer #2 (10 µM)	0.4 µl	
Reverse Primer #2 (10 µM)	0.4 µl	
Probe #2 (10 µM)	0.4 µl	
Total Volume/well	**20.0 µl**	

5. IC RT qPCR Cocktail Amplification

RT qPCR Amplification Protocol (BioRad CFX 96 – Luna 55 MC)			
1.	Reverse Transcription	55C for 10 minutes	cDNA synthesis
		95C for 5 minutes	Kill Reverse Transcriptase
2.	Amplification	95C for 15 seconds	Melting
		50C for 30 seconds	Annealing/Extension
		Plate Read	
		Repeat 39X	
3.	Melt Curve	65C TO 95C; Increment 0.5C/0.05 Seconds)	Evaluation of Amplicon
		Plate Read	

Immunocapture – IC RT qPCR	
File Name:	
Date:	
Primer:	
Comments:	

RT qPCR Cocktail –Single Primer/Probe Set

Single Probe/Primer Set		
Cocktails – Probe (20 µl Reaction)	**Volume\test (µl /rxn)**	**(# Tests) x (volume)**
NEB Luna Universal Probe 1-step Reaction Mix (2X) – DYE ** (#3005)	10.0 µl	
Water	8.4 µl	
NEB Luna Warm Start Reverse Transcriptase (20X)	0.4 µl	
Forward Primer (10 µM)	0.4 µl	
Reverse Primer (10 µM)	0.4 µl	
Probe (10 µM)	0.4 µl	
Total Volume/well	**20.0 µl**	

IC Setup

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Immunocapture – IC RT qPCR - Multiplex	
File Name:	
Date:	
Primers:	
Comments:	

Multiplex		
Cocktails – Probe (20 µl Reaction)	**Volume\test (µl /rxn)**	**(# Tests) x (volume)**
NEB Luna Universal Probe 1-step Reaction Mix (2X) – DYE ** (#3005)	10.0 µl	
Water	7.2 µl	
NEB Luna Warm Start Reverse Transcriptase (20X)	0.4 µl	
Forward Primer #1 (10 µM)	0.4 µl	
Reverse Primer #1 (10 µM)	0.4 µl	
Probe #1 (10 µM)	0.4 µl	
Forward Primer #2 (10 µM)	0.4 µl	
Reverse Primer #2 (10 µM)	0.4 µl	
Probe #2 (10 µM)	0.4 µl	
Total Volume/well	**20.0 µl**	

IC Setup

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

RT qPCR (multiplex)	
File Name:	
Date:	
Primers:	
Comments:	

Prepare RT qPCR reaction master mix and add cocktail to clean PCR tubes, strips or plates. After the cocktail is aliquoted into the PCR well, you will add the RNA.

Multiplex		
Cocktails – Probe (20 µl Reaction)	**Volume\test (µl /rxn)**	**(# Tests) x (volume)**
NEB Luna Universal Probe 1-step Reaction Mix (2X) – DYE ** (#3005)	10.0 µl	
Water	5.2 µl	
NEB Luna Warm Start Reverse Transcriptase (20X)	0.4 µl	
Forward Primer #1 (10 µM)	0.4 µl	
Reverse Primer #1 (10 µM)	0.4 µl	
Probe #1 (10 µM)	0.4 µl	
Forward Primer #2 (10 µM)	0.4 µl	
Reverse Primer #2 (10 µM)	0.4 µl	
Probe #2 (10 µM)	0.4 µl	
Total Volume/well	**18.0 µl**	

Setup

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
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PVY Direct Tuber Testing

Tuber Sampling

1. Collect 10 tubers from a single lot to create one sample. (Ex: 400 tuber lot contains 40 samples)
2. Take **1 core from the stem and bud end of each of the 10 tubers**. **Additionally take 2 cores from the body of each of the 10 tubers-take these 2 cores from eyes**. Will have a total of 40 cores in a 10-tuber sample.
    a. Cores can be removed from tubers using an **Integra Miltex 4 mm biopsy punch**.
        i. Have several of these on hand as several sterilization steps are required (4-5 punches).
    b. Between each batch of 10 tubers the punch needs to be sterilized.
        i. Place punch in 70% ethanol for a minimum of 1 min.
        ii. Remove and flick it to remove 70% ethanol.
        iii. Transfer to a drying rack and allow to dry. If there is a bit of ethanol still left on punch when ready to use just tap it on clean paper towel to remove excess/
        iv. Remove a single core randomly from the body of a single tuber in the next batch of tubers and discard in trash to finish cleaning the biopsy punch.
        v. Punch is sterilized and ready to be used.
    c. Place 40 cores from 10-tuber sample in a labeled Bioreba or Agdia grinding bag.
        i. Clip multiple sample bags together or apply masking tape to the open side of the bag to ensure the cores stay in the bag.
        ii. Shake cores to the bottom of the grinding bags to ensure they do not shake out.
    d. Samples can be frozen at -20C if not able to process same day as coring.

Sample Processing

1. Place 4.0 ml of General Extraction Buffer (GEB) (or Blotto can be used) in each grinding bag with 20 cores.
    a. To make GEB:

        500 ml GEB: To 500 ml of distilled water with magnetic stirring add:
        10 ml of Tween-20
        16.5 g sample extraction powder

        100 ml GEB: To 100 ml of distilled water with magnetic stirring add:
        2 ml of Tween-20
        3.3 g of sample extraction powder

    b. Powder should be sprinkled continuously, not dumped.
    c. **GEB should be made the same day as processing samples. Make fresh daily**.
    d. Carefully add GEB to mesh bags without contaminating the pipette tip. Change pipette tips as needed.
    e. A repeating pipette or a 5-ml pipette works well to add **4.0** ml of GEB.
2. Macerate the tuber tissue. This can be done with a drill press and special attachment or with a Bioreba hand homogenizer or by hand with a rubber mallet.
3. **Transfer 100 µl of macerated solution from each sample to a pre-coated Immunocapture (IC) PCR plate**
    a. Coating supplies can be purchased from Agdia. Use Biorad PCR plates.
    b. Extract 100 µl macerated liquid sample from outside the mesh and place into IC plates. **It helps to pinch mesh to one side of the bag for easier liquid access.**

c. Using **longer 200 µl tips** helps to reduce contamination risk.

4. Set up controls to include in testing.
    i. Suggested controls are: 2 negative tuber controls, 2 negative tissue culture plantlet controls, 2 positive tuber controls of the following dilutions: 1:1, 1:10 and 1:100, 2 positive tissue culture plantlet control dilution: 1:1, 1:10, 1:100 and 2 buffer control
        1. Not all controls need to be included across every plate
    ii. Place uninfected and infected PVY tuber cores in separate labeled grinding bag, add buffer, macerate and remove macerated liquid solution as needed to fill IC plate(s).
        1. Only the positive tuber sample needs to be diluted 1:1, 1:10, 1:100.
    iii. Place uninfected and infected PVY tissue culture plantlets in separate labeled grinding bag, add buffer, macerate and remove macerated liquid solution as needed to fill IC plate(s).
        1. Only the positive tuber sample needs to be diluted 1:1, 1:10, 1:100.
    iv. Buffer control is GEB.
5. Once all samples are loaded (100 µl per well) each IC plate should be covered with a piece of wet paper towel and placed in an airtight container (ex: Rubbermaid) with a piece of wet paper towel in the bottom of the container.
    a. Plates should be stored at 4C overnight.
    b. Plates can be processed the following day or frozen at -20C until ready for PCR step.

Primer Information

Primer information was pulled out of the following paper: Bright et al. Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan real-time RT-PCR.

Berger Primers:

PVY Forward Primer: 5' GGGTTTAGCGCGTTATGCC 3'
PVY Reverse Primer: 5' TCTTGTGTACTGATGCCACCG 3'
PVY Probe: 5' /HEX/CAGTGAGGGCTAGGGAAGCGCACA/BHQ-1/3'

Primer information was pulled out of the following paper: Singh et al. Optimization of Real-Time RT-PCR Assay and its Comparison with ELISA, Conventional RT-PCR and the Grow-out Test for Large Scale Diagnosis of Potato Virus Y in Dormant Potato Tubers

Note that the Singh Primers do a better job of detecting all strains of PVY.

Singh Primers:

PVY-3 Forward Primer: 5' GGG CTT ATG GTT TGG TGC AT 3'
PVY-1 Reverse Primer: 5' ATA TAC GCT TCT GCA ACA TCT GAG A 3'
PVY Probe: 5' /5YakYel/ TTA GGC AAA TCA TGG CAC AT /3IABkFQ/ 3'

List of Supplies Needed

Supplies for Coring Samples

Integra Miltex 4 mm biopsy Fisher Sci 12460410
PreEmpt RTU Fisher Sci 19039933 spray bottles
RNASE Away Fisher Sci 1475434
95% ethanol
Distilled water
Tri-pour beakers or containers for sterilizing solutions
Papertowel
Gloves-various sizes if needed
Labeled mesh samples bags AGDIA ACC 00930/1000; Bioreba 12x 15 cm 430100b

Supplies for Processing Samples

Integra Miltex 4 mm biopsy/Scoopula
RNASE Away-approx. 400 ml
95% ethanol-approx. 400 ml
Distilled water-approx. 400 ml
Tri-pour beakers-approx. 3
Pipettor tip disposable container
Gloves-varying sizes
2-P200 pipettors
P200 µl filtered tips-longer length is ideal Gilson F1735041
P5000 pipettor

P5000 tips
P1000 pipettor
P1000 µl filtered tips
Centrifuge tubes-1.7 ml
250-ml beakers for GEB and 95% ethanol
GEB made fresh that day AGDIA ACC 00955/016.5
Pre-coated PCR plates
Forceps
Scissors
Infected and uninfected tubers
Infected and uninfected tissue culture plantlets-can make up ahead of time
Tape and markers
Containers to store mesh bag samples during processing and storage
Rubbermaid containers to store IC PCR plates
Plate map-either PCR sheet or ELISA sheet
Drill press/hand homogenizer AGDIA ACC 00900 or Bioreba 400010

Supplies for Setting up PCR plates

PCR sheets with plate map
Pre-mixed master mix-kept on ice
P200 multichannel pipettor
P200 pipettor
P200 filtered tips
1X PBST Buffer
Distilled water
2-Agdia ELISA washing squeeze bottles
Paper towel
96-well cooler block-kept cold
Gloves
Disposable multichannel pipettor basin
Container for tip disposal
Plastic plate cover for IC plate Bio-Rad MSB1001 and others
Roller to adhere the plastic on IC plate (microplate brayer) Fisher has number of different ones

PVY PCR – Berger Primers

Date: _____

Sample ID:_____

_____

_____

_____

Master Mix Formula PCR: ____ Samples: + ____ Controls + ____ Extras = _____

Master Mix Component	Volume per Rxn	Total Volume	Added
2x iTaq Universal Probes Mix	12.5 µl		
iScript Reverse Transcriptase	0.50 µl		
10 µM Forward Primer	0.50 µl		
10 µM Reverse Primer			
10 µM Probe (FAM)			
Water (molecular grade)	11.5 µl		
Sample	NA		
Total Rxn Volume	25.0 µl		

Controls	#
Negative-tuber	2
Negative-plantlet	2
Positive tuber 1:1	2
Positive tuber 1:10	2
Positive tuber 1:100	2
Positive-plantlet	2
Buffer	2

Cycling Parameters (approx. 1 hr)

50°C 10 min
95°C 5 min

95°C 15 sec
52°C 30 sec
X40

Results/Notes:

***Note for this PCR you add 25 µl of the master mix to the washed ELISA/PCR plates and then run the PCR.

Loading Chart

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

PVY PCR-Singh Primers

Date: _____

Sample ID:_____

_____

_____

_____

Master Mix Formula PCR: ____ Samples: + ____ Controls + ____ Extras = _____

Master Mix Component	Volume per Rxn	Total Volume	Added
2x iTaq Universal Probes Mix	12.5 µl		
iScript Reverse Transcriptase	0.50 µl		
10 µM Forward Primer	1 µl		
10 µM Reverse Primer	1 µl		
10 µM Probe (FAM)	0.38 µl		
Water (molecular grade)	9.62 µl		
Sample	NA		
Total Rxn Volume	25.0 µl		

Controls	#
Negative-tuber	2
Negative-plantlet	2
Positive tuber 1:1	2
Positive tuber 1:10	2
Positive tuber 1:100	2
Positive-plantlet	2
Buffer	2

Cycling Parameters (approx. 1 hr)

50°C 15 min
95°C 5 min

95°C 15 sec
60°C 45 sec
X40

Results/Notes:

***Note for this PCR you add 25 µl of the master mix to the washed ELISA/PCR plates and then run the PCR.

Loading Chart

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
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FTA Card Promega Maxwell Extraction Protocol

Updated 3/21/22

This protocol utilizes a guanidine thiocyanate kit called Promega Maxwell RSC miRNA Plasma and Serum Kit (AS1680) and although we have deviated slightly, we are following the Promega Maxwell RSC miRNA Tissue Kit (instruction for AS1460)

Sample Processing

1. This protocol is still being trialed but has worked successfully to extract RNA from positive PVY, PLRV, and TMV infected FTA cards.
2. Add 500 ul of Lysis buffer (Part# MC501C) and 75 ul of Proteinase K to a 2.0 ml centrifuge tube with 25 FTA card punches.
    a. Still trialing the ratio of FTA punches to Lysis buffer/Proteinase K ratio to ensure accurate detection.
3. Vortex tube for 20 seconds to mix.
4. Incubate at room temperature for 10 minutes.
    a. During this time, prepare the Maxwell RSC Cartridge (see steps below)
5. Transfer 500-600 ul of lysate to Well #1 (largest well)
    a. Add 10 ul of blue DNASE 1 Solution to well #4 (yellow regent). Optional.

Promega Maxwell Setup

1. Turn on the Maxwell machine and tablet. Ensure all plugs and connectors are in place.
2. Open the Maxwell door and remove the deck tray.
3. Change gloves before handling Maxwell RSC cartridges, plungers and elution tubes.
4. Place cartridges in the deck tray with Well #1 (largest) facing away from the elution tube. Press down on the cartridge to snap into place.
5. Carefully peel back the plastic from the top of the cartridge(s).
6. Place a plunger into Well #8.
7. Place an empty elution tube (provided) in each position on the deck tray where a sample will be loaded.
    a. Add 60 ul of nuclease-free water to each elution tube.
8. Load samples (see step #5 in sample processing).
9. Carefully snap the deck tray back into the Maxwell.
10. Close the Maxwell door.
11. Select the AS1680 Promega Maxwell RSC miRNA Plasma and Serum run.
    a. Identify which well(s) have samples.
    b. Select start. The run takes approximately 1 hr. and 15 min.
12. Once extraction is complete, remove the elution tubes and proceed with testing.
    a. Dispose of the used cartridges and power off the machine and tablet.

Direct Testing of Potato Tubers and Leaves Using Whatman FTA™ and qPCR

A universal sampling and processing protocol for potato tuber tissue or leaves performed at room temperature to collect nucleic acid samples. The captured nucleic acids are then forwarded to a laboratory and processed as a total nucleic acid extraction. Total nucleic acid is amplified using a 1-step reverse transcription and TaqMan qPCR. This protocol contains further instructions on specific qPCR protocols used after the sampling and extraction process.

Novice Summary: Plant, bacterial, fungal and virus Nucleic Acids are captured and preserved in the Whatman FTA paper matrix when potato tuber or any type plant (or animal) tissue are pressed into the paper. Components integral to the paper aid the shearing of cells; proteinases destroy proteins that would otherwise degrade samples and in general, nucleic acids are stabilized. These cards work universally on all tissue types including leaves, stems, roots, tubers, sprouts and any other part of the potato plant as long as there is enough liquid in the plant cells to flood the paper. Disrupted cell contents are immediately preserved in the paper matrix at room temperature like a dry, Proteinase-K, TRIS-EDTA buffer or RNAlater™ treatment. This collection step can be performed at room temperature without ice, PPE, fume hoods or dangerous chemicals, requires no lab experience for the worker and is not time sensitive to sample degradation. After the cards have been impregnated with sample material, excess materials are picked off, the card dried at room temperature, then frozen to extend the storage period of the samples (we currently do not know how long samples can be stored, manufacturer suggests months to years, current personal experience is one year without loss of fidelity). Later, using a specialized paper punch, samples can be removed from the paper and Nucleic Acid extraction performed as though it were fresh tissue. However, because the sample was disrupted with cells burst into the FTA paper and stabilized in that condition, much of the physical inhibitors to RNA extraction such as tuber starch and plant structural tissues are physically separated when the original tissue sample is pulled away from the paper. The card can be taken from and returned to the freezer for future use. Storability provides a convenient alternative to returning to the original tuber for additional sampling and would be useful when returning to individuals within a pooled extraction to determine actual pathogen incidence. FTA card use is common in criminal and forensic science for the past decade and is becoming more common in veterinary medicine since it removes the time and temperature critical limitations of nucleic acid collection and storage and renders the pathogen noninfectious, safe and legal for transport across borders. This extraction protocol is designed primarily to test for RNA viruses of potato.

Total Nucleic Acid includes both RNA and DNA. For RNA targets, extraction product is cycled in a single reaction well for a Reverse Transcription (RNA to cDNA) reaction followed by qPCR TaqMan amplification and measurement (quantification of DNA or cDNA). qPCR amplification is expressed by a value called Cq (quantification cycles) that represent when the measure of the qPCR amplification fluorescence exceeds background thresholds. The value of this “threshold” is compared to several controls (positive and

negative) and a known virus copy number “standard” to establish presence/absence of the PCR target. This “threshold” is currently at 77 copies a best estimate to match existing ELISA sensitivity of plants grown from these tubers. Final threshold adjustment is expected soon based on 2021 qPCR and ELISA comparisons.

Specific PCR diagnostics included:

Potato Virus Y (PVY)
Direct Detection of Plant Viruses in Potato Tubers Using Real-Time PCR (Chapter) by Neil Boonham , Lynn Laurenson , Rebecca Weekes and Rick Mumford in R. Burns (ed.), Methods in Molecular Biology, Plant Pathology, vol. 508 © Humana Press, a part of Springer Science + Business Media, LLC 2009
DOI: 10.1007/978-1-59745-062-1_19

The PVY Primers outlined in this book chapter are used.
Notes for the Boonham protocol that:

- These are universal PVY primers, but have delayed Cq’s for PVY NE-11 strains.

Potato Mop Top Virus (PMTV) and Tobacco Rattle Virus (TRV) Duplex
Mumford, R. A., Walsh, K., Barker, I., and Boonham, N. 2000. Detection of Potato mop top virus and Tobacco rattle virus using a multiplex realtime fluorescent reverse-transcription polymerase chain reaction assay. Phytopathology 90:448-453.

TUBER COLLECTION, STORAGE AND SHIPPING
(Step 1)

For video summary and tutorial of protocol steps go to:
https://www.youtube.com/watch?v=izWRD_tLpTk

Seed Lots

- 400 tuber samples as per seed certification protocols
- This requires 16 FTA cards (25 tubers per card)
- This results in 16 presence / absence decisions per seed lot.

Screening Fields for PMTV and TRV Samples

- Collect from lower and wetter parts of the field when possible.

Schedules & Timing

- At this time the only guidance we have is at least 2 weeks after complete vine kill.

Tuber Handling

- Grading line clean is good. If unwashed, tubers clean enough to clearly identify stolon and eyes are fine for sampling.

Tuber Storage Conditions

- Dark Storage – especially if PMTV data is of interest.

Sending Tubers –

1. Freshly dug tubers, we would like to get our samples shipped into Ithaca before they have been cooled to long term storage temperatures.
2. Long term storage 38F-42F or as close as you can get to commercial seed storage temperatures.

Ithaca Bound Samples please send to:
Jason Ingram
USDA-ARS
R.W. Holley Center
538 Tower Road
Ithaca, NY 14853
607-255-4596

TUBER TISSUE COLLECTION AND PRESERVATION
(Step 2)
Background

Samples from individual tubers will be pressed into FTA cards using a hydraulic press.

https://www.youtube.com/watch?v=izWRD_tLpTk

FTA Sample Collection: All steps can be completed at room temperature in a work room environment such as a potato grading room.

Tuber: Tubers do not need to be washed, but clean enough to identify eyes and stolon. Grading line clean is good. Cold crisp tubers at 40F do not core as well as room temperature for easy ejection from biopsy tool. 4 cores per tuber substantially increases the chances of correct absence/presence call for pathogen. For 25 tuber pooled samples we use four cores from a 3mm biopsy tool. Maximum tuber mass evaluated that does not overwhelm an individual card sample area (single tuber results) is 0.17g of tuber (4, 2mm cores).

Leaf, sprout or root: Whole leaves, Fresh leaf punches or roots, sprouts placed onto paper and pressed into paper. Leaf tissue is best sampled by pressing a microfuge cap into a stack of 5 leaves and excising 5 discs that can be held at 4C for up to 24 hours, then removed from the cap when skewered with a pipette tip and placed in a sample square in a stack.

Chain of custody of tubers and FTA card are important when temporary labor is used. Confirm tuber punch patterns and sample placement on cards and provide samplers feedback for improvement.

Quality of the impression of tissue to FTA paper is important to nucleic acid capture and yield. A hydraulic press with steel plates consistently provides the best impression and results in material being impregnated with cell contents. FTA paper should be soaked through to the back for maximum capture and preservation, if not see trouble shooting suggestions in on-farm sampling guide.

TUBER TISSUE COLLECTION AND PRESERVATION
(Step 2)
Step By Step

Samples from individual tubers will be pressed into FTA cards using a hydraulic press.

Organize and maintain tubers in Groups of 25 that can be matched to the FTA Card.

- **This provides an opportunity for quality control improvements if there are concerns with the post sampling inspection of FTA cards or tubers.**
- **Allows the best comparison for FTA PCR results vs. other test methods used for comparison when bagged in groups of 25 and post-harvest tested.**

Open FTA card and crease over Backwards so that it will lie flat.

Select Tuber. Use 3 mm dia. biopsy tool. Sample a random location on tuber and discard core to trash. This ensures the tool is clean & empty

Collect 4 cores from the tuber (Figure 1) and place on a single FTA circle (Figure 2 & 3) Making a pile or lying flat is OK. (See Fig
ure 2)

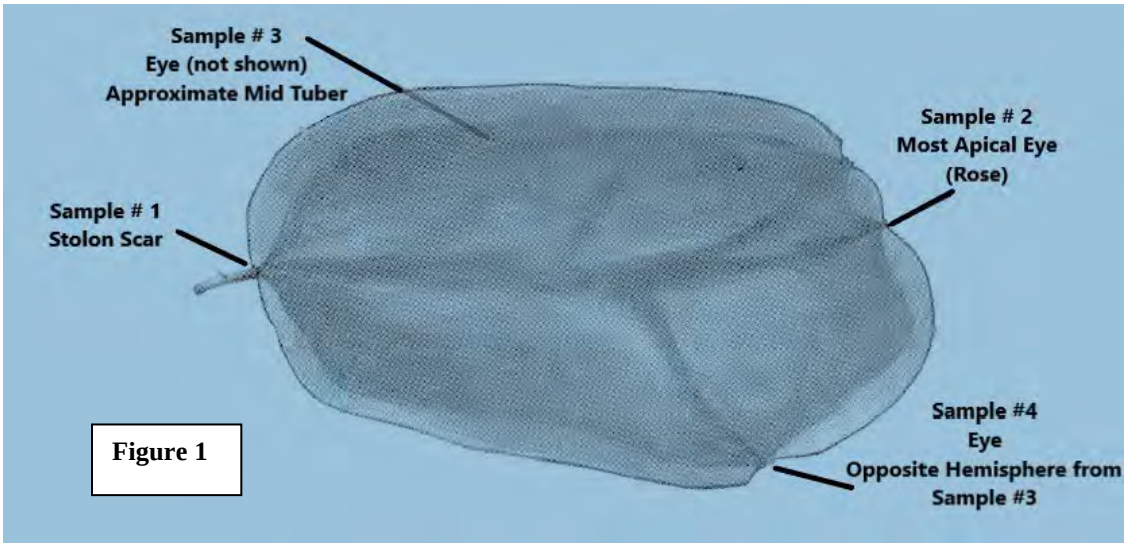


Figure 1

After sampling 25 tubers rinse the Biopsy Tool in 70% ethanol. Place in drying rack (test tube rack with paper towels laid on base) to wick tools dry.

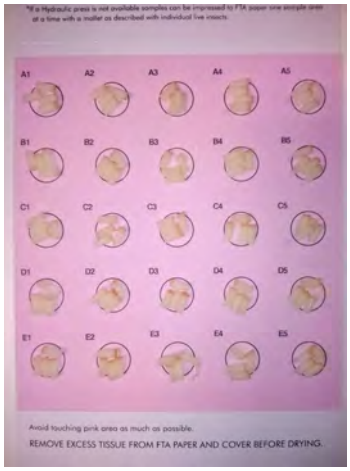


Figure 2

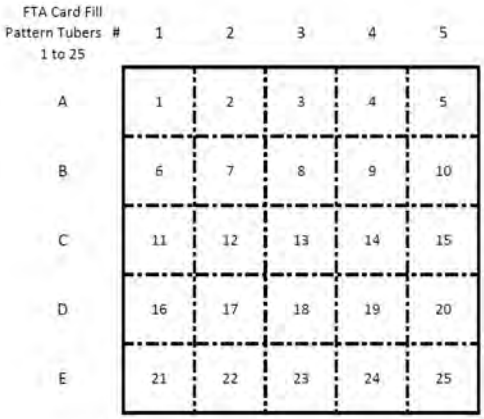


Figure 3

Sample all 25 tubers.

Ensure Press is clean and ready for a new card. (remove previous card)

Place paper towel on bottom plate

Fold FTA Card Lid Closed. (DO NOT RE-OPEN!) Hold closed during move to press so cores do not shift. The lid can only be closed once! Opening again will move everything that was stuck to the lid.

Operate press and squeeze sample card to maximum capability. 12+ Tons pressure, 1 minute in press is enough but leaving it until next card is ready is efficient and acceptable.

Remove Card from press.

Place closed card on flat surface at least 15 minutes to "relax". During this time most tissue releases from the FTA paper and remains stuck only to the cover flap.

Gently open card while paper and tissue are still moist, remove tissue still clinging to FTA paper with forceps. Wipe clean the cover flap with a clean paper towel. (DO NOT ALLOW THEM TO DRY WITH TISSUE STILL ATTACHED)

Open card completely, (2 folds) lay card with inside face down and FTA paper hanging over edge of bench or hang on clothesline so that it can air dry.

When cards are dry, fold them closed and place in the barrier layer envelope provided with the kit. Add desiccant pack if available for improved storage.

Store at -20C.

There could be great variation between sampling precision among workers.

- **Inspect biopsied tubers for correct sampling pattern and correct as needed. This is one reason for matching a bag of 25 tubers to the FTA card.**
- **Inspect Card to ensure core placement is being done conscientiously in the center of the sample square.**

Materials List:

- Hydraulic Press with Press Plates (platens) 6” x 6” or greater, eg. 12 Ton Capacity Model#3850 (Mini-C) Press, Carver Press
- Biopsy Tool, 3mm size with Plunger. #33-32-P/25, Integra; York, PA
- Seed Potato Certification Sample Card, GE, Whatman FTA 25 Sample Custom Designed Card (#WB129358 AA)
    - Or: alternative GE, Whatman FTA Plantsaver sample card (#WB120065) (Common version 4 sample Card)
- 70% Ethanol (RICCA #2546.70-2.5 (10 Liter)
- Additional plastic storage bags and desiccant pouches for FTA card storage, 6” x 8” 6 MIL Minigrip Red Line Reclosable bags
- Forceps (tweezers)
- Paper Towels
- Rinse Container for Ethanol and Biopsy Tools
- Trash Can

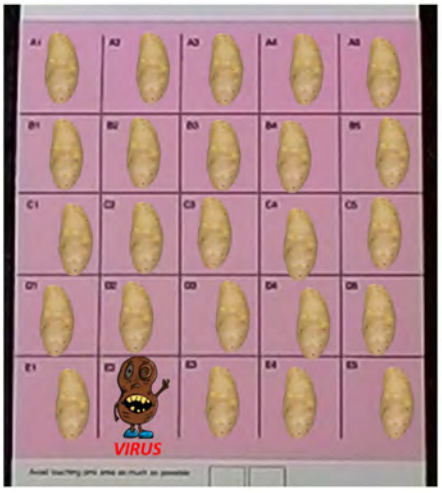


Figure 4

LEAF TISSUE COLLECTION AND PRESERVATION
(Step 2 alternative)

Samples from individual plants will be pressed into FTA cards using a hydraulic press.

Organize Sample leaves into stacks of 5.

Open FTA card and crease over Backwards so that it will lie flat.

Select group of 5 leaves. Use cap of microfuge tube and press through stack of leaves to collect 5 leaf discs. Close cap.

Refrigerate at 4C for up 24 hours

Open cap, skewer discs in cap with pipette tip (Figure 5)

Place stack of discs on FTA sample area. A neat stack will better saturate the paper. (Figure 5)

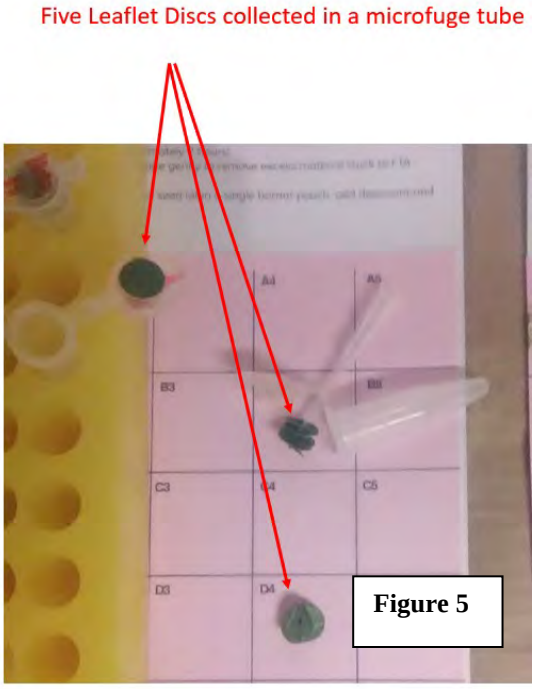


Figure 5

Sample all 25 Leaf Groups

Ensure Press is clean and ready for a new card. (remove previous card)

Place paper towel on press bottom plate

Fold FTA Card Lid Closed. (DO NOT RE-OPEN!) Hold closed during move to press so leaf discs do not shift. The lid can only be closed once! Opening again will move everything that was stuck to the lid.

Operate press and squeeze sample card to maximum capability. 12+ Tons pressure, 1 minute in press is enough but leaving it until next card is ready is efficient and acceptable.

Remove Card from press.

Place closed card on flat surface 15 minutes to "relax". During this time the excess tissue releases from the FTA paper and remains stuck only to the cover flap.

Gently open card while paper and tissue are still moist, remove tissue still clinging to FTA paper with forceps. Wipe clean the cover flap with a clean paper towel. (Figure 6)

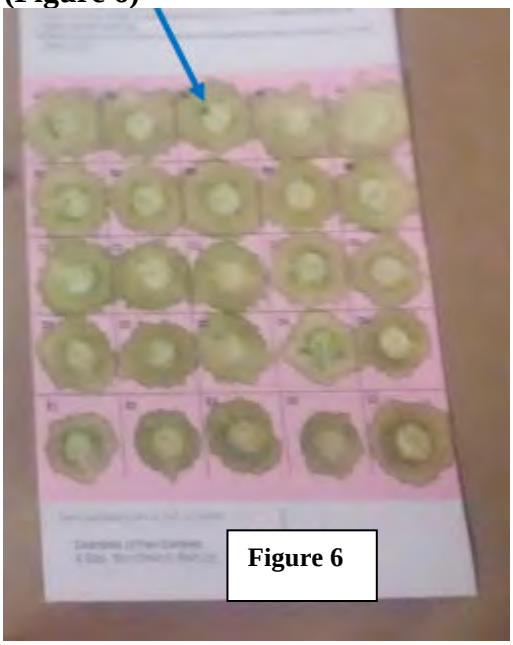


Figure 6

(DO NOT ALLOW THEM TO DRY WITH TISSUE STILL ATTACHED)

Open card completely, (2 folds) lay card with inside face down and FTA paper hanging over edge of bench or hang on clothesline so that it can air dry

When cards are dry, fold them closed and place in the barrier layer envelope provided with the kit. Add desiccant pack if available for improved storage.

Store at -20C.

There could be great variation between sampling precision among workers.

- **Inspect Card to ensure leaf disc placement is being done conscientiously in the center of the sample square.**

Materials List:

- Hydraulic Press with Press Plates (platens) 6” x 6” or greater, 12 Ton Capacity
- Standard 1.7ml Microfuge tube
- Standard pipette tip (10ul is sharpest)
- Seed Potato Certification Sample Card, GE, Whatman FTA 25 Sample Custom Designed Card (#WB129358 AA)
    - Or: alternative GE, Whatman FTA Plantsaver sample card (#WB120065) (Common version 4 sample Card)
- Additional plastic storage bags and desiccant pouches for FTA card storage, 6” x 8” 6 MIL Minigrip Red Line Reclosable bags
- Forceps
- Paper Towels
- Trash Can

FTA PAPER HANDLING AND PROCESSING
(Step 3)
BACKGROUND

Paper discs can be collected any time after the card has dried. Wet or moist cards do not punch well. FTA paper discs can be transferred to the plate or tube intended for the lysis buffer elution step and stored dry as discs in the processing plates or microfuge tubes in the freezer until actual RNA extraction is to be done. Automated Nucleic Acid extraction such as with a Kingfisher is sometimes most efficient to build up a supply of plates with FTA discs and process them all at once. Harris Uni-Core^{TM} puncher can be cleaned by rinsing in 70% ethanol and drying. About 10-25 punch tools are needed to keep constant progress. Wet punches tear rather than cut the paper. This step may be better performed in a cleaner environment than a grading room, but like the tuber sampling there is no need for cold temperatures or speed for fear of sample degradation.

For rapid punching of pooled samples (multiple FTA sample squares in a single nucleic acid extraction well) a Fiskars circular paper punch **(figure 8)** can be modified to accept collection tubes. See materials list for part numbers.

Making a Fiskars punch **(Figure 8)** adapt to microfuge. Equipment needed for the job are a bench vise and a wire wheel on a bench grinder.

- Remove disc collector from Fiskars tool and discard.
- Slowly and by small increments, using various spots on the original punch collection area from where the “collector” was removed, bend inwards over the course of the whole length of that area. Small gradual changes are best. Stop after each bend to test how a microfuge tube fits and then make another adjustment. Tubes should fit firmly to avoid them falling out of the punch.
- When satisfied with bends. Smooth and polish bent area with a wire polishing wheel on a bench grinder to remove burs and rough spots. Those burs will mar microfuge tubes and cause problems.
- Recheck fit and repeat as needed. Wire wheel cleaning often “loosens” the fit and will require additional bending and again polishing afterward.

FTA paper disc size selected is directly related to static electricity problems in disc handling. The larger the disc diameter the less static electricity causes problems with discs floating off or sticking.

FTA Disc cutting with Fiskars requires an air flow hood to exhaust small paper shreds from the work area to eliminate contamination problems.

FTA PAPER HANDLING AND PROCESSING
(Step 3)

To cut and place a "tuber" (three, 1.5mm FTA paper discs) into a sample processing tube. i.e., each tube will contain 75 discs. (25 tubers, complete FTA card)

This page is **ONLY for 25 Tuber pooled samples** (grouped or composite samples) see earlier *conventional* PCR protocols for single sample or 10 sample protocols.

Prepare tubes or plates to receive FTA discs by placing 15ul ddWater into the bottom of the sample tubes. This wets the paper on arrival in the tube and minimizes the effects of static. (wipe rack bottoms and sides, or place on paper towel moistened with staticide if needed for static electricity control) Pre-loading water to tubes is best done in a clean room or master mix room prior to any paper cutting.

Organize Samples relative to final PCR plate map requirements. At this time all samples will be transferred to a 96 well format and handled that way throughout the remaining test steps. (**See Figure 10** plate layout example for ideas)

FTA discs are cut with the Fiskars paper punch (**Figure 8,9)** tool in a fume hood so that loose paper shreds are exhausted out the hood and not overtop the empty or filled sample tubes. In the area adjacent to the cutting area, position one empty rack and one rack populated with microfuge tubes. (The empty rack serves as a destination for the tubes once FTA paper discs are added). **Do not punch over top of tube racks!**

FTA discs cut with an automated BSD system would be much better going directly into a plate and save a lot of labor in liquid transfers. The remainder of this protocol however focuses on individual tubes format with hand punching.

Open a pair of cards. Cut with scissors between B & C; D & E on 1-25 and between A & B; C&D; below E for 26-50. This will provide 6 strips that are all able to be punched quickly with the Fiskars paper punch.

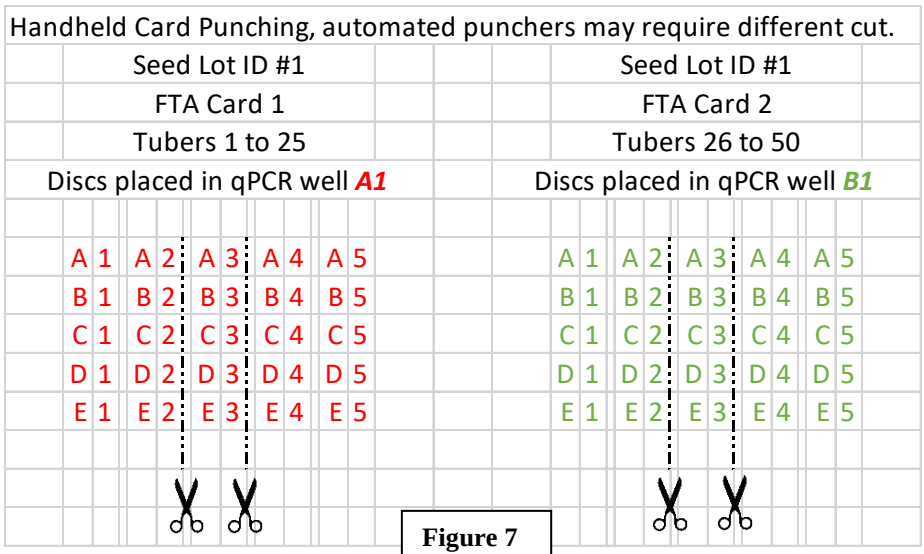


Figure 7

See **Figure10** for depositing samples in 96 well pattern for depositing samples to Wells A1, B1

Remove modified Fiskars 1.5mm punch from drying rack

Insert Microfuge Tube into Fiskars punch.

Punch 3 discs in a triangle pattern within the physical indentation in the FTA paper made by the tissue sample. Punch 3 discs from each of the 25 sample squares on the FTA card. **(Figure 9)**

Operate the punch a few times without paper in it after the last sample is taken, then while still inserted into the puncher, tap the microfuge tube on the work surface several times to ensure all discs have migrated to the bottom of the well.

Remove tube from punch and place in appropriate location of the destination rack. **(Figure 9)**

Rinse punch in 70% ethanol pan, place on rack to air dry.

Repeat to fill all 96 tubes.

Continue on to step 4, **Releasing Nucleic Acids from FTA Paper** *OR Cover plate with adhesive film, ensure tube caps are tight and freeze at -20C.*

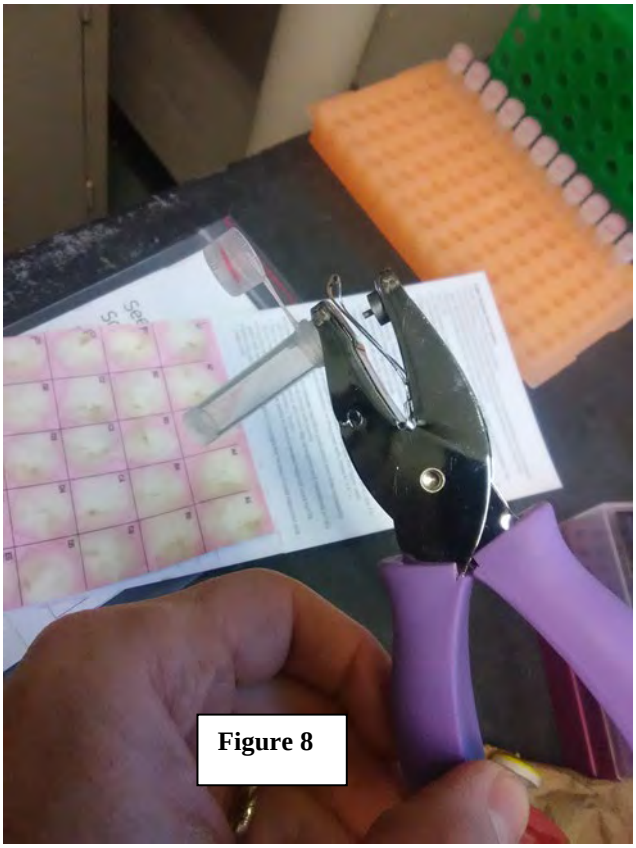


Figure 8

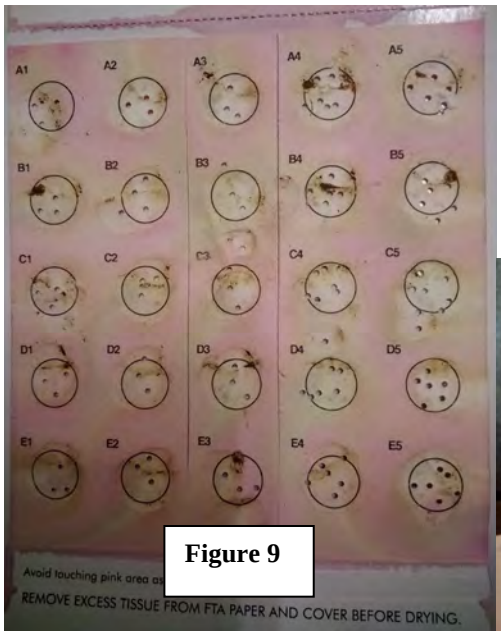


Figure 9

Three, 1.5 mm diameter discs taken from each sample into a tube. Sufficient headspace for multichannel liquid removal “headspace” above settled 75 FTA discs. This would work much better in 96 well format, pending a robot agrees to do the work.

Materials List:
Micrew Tubes #L233226 2ml screw top o-ring

Fume/Exhaust/Laminar flow hood
ddH2O
Fiskars punches, #23507097J ; 2 or 3 needed (1.5mm) 1/16” modified to hold Micrew Tubes
Multichannel Pipettor (10 to 50ul range capable)
Staticide, #2010, ACL Staticide, Chicago, IL.
Harris Uni-Core punch 3.00mm (#WB100078) (for individual 3mm discs if you feel crazy)

RELEASING NUCLEIC ACIDS FROM FTA DISCS (Step 4)

In this step Nucleic Acids bound to FTA paper are eluted into the LYSIS/BINDING Concentrate

DO NOT DILUTE LYSIS/BINDING CONCENTRATE as described in kit instructions.

- Place concentrated LYSIS/BINDING CONCENTRATE in each tube/well. Required volume depends on the number of FTA discs in the tube/well.
    - 75 discs (1.5mm discs in 25-sample pool) = 575ul Concentrate
        - ***All protocols require more lysis/binding concentrate than comes with kit. Order an additional XX ml of concentrate as AM8500 (see below) per 96 well plate***

- Cover plate
    - Deep 96 well plate = Disposable Plate Sealing Film
    - Microfuge Tube = capped

Incubate Sample: (Plates/microfuge tubes may be returned to -20C storage at any point in this incubation and later removed to complete incubation and lysate recovery.

- Place covered plate on shaker table at 600 PRM for 2 hours.
- Remove plate from Shaker. With 8 or 12 row pipette, withdraw 100ul Lysis/Binding Concentrate from a column/row and transfer to a 96 deep well processing plate suitable for the kingfisher magnetic bead extraction.
    - Lysis/Binding Concentrate volumes specifically selected for ability to manage sample removal with a 12-channel pipette without fouling in FTA discs. FTA discs settle in the buffer and are very rarely a problem in tip fouling. Figure 9. *Unless shaking was too aggressive (>600RPM) and pulverized paper.*
- Seal 96 deep well processing plate with disposable plate sealing film and freeze at -20 or move on to Nucleic Acid extraction. **(Step 5)**

Materials:
Disposable Plate Sealing Film for use on 96 well plate (Thermal Seal STR-THER-PLT, Excel Scientific)
Lysis/Binding Concentrate, #AM8500 Applied Biosystems (***CALCULATE NEEDED VOLUME WHEN ORDERING EXTRACTION KITS USED IN STEP 5)***
8 or 12 Row multichannel pipette 100ul to 1000ul capacity
96 Deep Well processing plate, Thermo, # 95040450
Shaker Table 200-600 RPM Range

NUCLEIC ACID EXTRACTION WITH MAGNETIC BEADS & KINGFISHER (Step 5)

Background

We perform nucleic acid extraction and 1-Step TaqMan qPCR (Steps 5 & 6) as one continuous workflow to avoid a freeze thaw cycle on the RNA.

Procedure Details:

- **Volumes are given for a single complete 96 well plate.**
- **25 minutes set up for Kingfisher wash plates, enough plates for up to three machine runs can be safely performed at once.**
- **28 Minutes is actual run time for a plate in the Kingfisher**
- **Product immediately processed in the qPCR Reaction (step 6).**
    - **See full protocol for hints with multiple plate runs, we typically run three sample plates (488 samples) for extraction and qPCR (steps 5 & 6) in about 3 hours that includes a single set up period for the Kingfisher**

MagMAX 96-Viral RNA Isolation Kit # AM1836, AMB1836-5, with Nucleic Acid sourced from FTA paper.

This protocol is based on the ThermoFisher MagMAX 96-Viral RNA Isolation, Kit # AM1836, AMB1836-5 kit for total RNA extraction from "Tissue Sample" protocol (AM1836M & A27828) optimized for "nearly cell free" samples and uses punched FTA paper discs as the "tissue source" in a 96 well format. Expect 20 minute set up for buffers. Two idle hours with FTA paper sample discs in the kit lysis buffer (Guanadine isothiocyanate-based solution, on a shaker table.

- Manual magnetic block extraction 2.5 hours for manual RNA extraction using 12 row multichannel system and 96 well magnetic block for transfers etc. until RNA is in the freezer.
- The Kingfisher robot reduces extraction procedure to 30 minutes. Use manufacturer's Kingfisher Bindit software program (*MagMAX.Pathogen_STDVolume.bdz*) to run the machine for this protocol with a minor change described below.

Procedural notes:
All steps (except last, remove nucleic acid from kingfisher) are performed at room temperature.

The RNA bead kit works based on affinity of magnetic beads to RNA that is chaotropic (salt concentrations) dependent. Similarity in bead dispersion or clumping across wells indicates consistency, and most likely that all is well. Variability across wells often indicates remnant solvent from previous wash steps in some wells and inaccurate salt concentration from carryover contamination.

First attempts to complete this protocol should be made with barrier tip pipettes. Aspiration of magnetic bead wash solutions are performed with 200 or 300ul pipette tips that are RNAse/DNAse free. This manual extraction protocol has been successfully completed with generic non-filtered (non-barrier) pipette tips due to economy. Just pipette slowly and carefully. Stock many, it is an obscene number of tips to complete this procedure for a 96 well plate.

NUCLEIC ACID EXTRACTION WITH MAGNETIC BEADS & KINGFISHER
(Step 5)

Step – by - Step

We perform nucleic acid extraction and Reverse Transcription (Steps 5 & 6) as one continuous workflow to avoid a freeze thaw cycle on the RNA.

Procedure Details:

- **Volumes are given for a single complete 96 well plate.**
- **11" x 17" office paper is kept in factory 500 sheet bundles and placed on bench surfaces to prevent kingfisher and PCR plate contact with other surfaces, then disposed of after the experiment. This keeps the kingfisher and thermal cyclers clean.**
- **25 minutes set up for Kingfisher wash plates, enough plates for up to three machine runs can be safely performed at once in this time.**
- **28 Minutes is actual run time for a plate in the Kingfisher**
- **Product immediately processed in the qPCR (step 6).**

Materials Referenced are from Ambion MagMAX^{TM}-96 Viral RNA Isolation Kit
Prep Materials:

- Thaw Lysis Binding enhancer on ice.
- Vortex Magnetic beads to assure they are well suspended
- Add ethanol and isopropanol to Wash Buffers 1 & 2 respectively per manufacturer's instructions
- (*Never ever add the suggested isopropanol to the LYSIS/BINDING Concentrate)*
- Thaw qPCR reagents on ice (keep enzymes in freezer until use).
- Open 6 standard 96 well Kingfisher plates
- Label Plates:
    - Wash 1
    - Wash 1
    - Wash 2
    - Wash 2
    - Tip
    - Elution Plate (along with sample ID information desired)
        - Efficiency Tip - label only the Column 12 side of the plate that will always face out of the Kingfisher carousel.

Plate Preparations:

- Tip Plate – Place *Deep Well* Comb tip cover in "Tip" plate
- Load Kingfisher plates with buffers using multichannel pipettor with stepper feature if available.
    - Pour estimated need for a buffer into a reagent reservoir, pipette to fill plates, then pour buffer remaining in reagent reservoir back into source bottle. (Work clean!)

- Elution Plate – 90ul per well
- Wash 1 – 150ul per well
- Wash 2 – 150ul per well

(see full protocol when making multiple runs per day for additional time and labor-saving steps related to filling all plates at one time for a half-day's set of runs.)

Thaw 96 well processing plate from **Step 4** containing 100ul aliquot of Lysis/Binding concentrate and eluted nucleic acids from FTA discs.

- Add 100 ul isopropanol to each well of plate containing with Lysis/Binding Concentrate and eluted nucleic acids.

- Place on shaker 200 RPM until bead addition.

Mix in 8 row reagent reservoir tray (less waste than 12 row reservoir).

- 1060 ul thawed Lysis Binding Enhancer (*10ul/well*)
- 1060 ul Recently vortexed Magnetic Bead suspension (*10ul*/*well*).
- Mix by pipetting up and down.

Dispense 20*ul* into all wells. Use 8 row step pipettor to draw enough to fill a plate and then dispense into all wells in the 12 plate columns without pipette tip changes *and without plate contact*. Bead suspension is viscous and loss due to plastic adhesion is great if tips are changed. Individual workers will need to experiment with what works for them and the amount of extra material they need to mix. (10% overage for a single plate and 5% overage when mixing for multiple plates is a good start)

Cover sample plate with disposable plate sealing film if it will not be immediately placed in Kingfisher. Store at room temperature. (e.g. multiple plate runs being prepared at once)

Select Program on Kingfisher Menu and place each plate in the carousel when the machine requests them during program set up.

- Use manufacturer's pre-programmed Kingfisher BindIt™ software program (*MagMAX.Pathogen_STDVolume.bdz*) to run the machine for this protocol with minor changes.
  - Adjust Sample Plate Starting Volume to 220ul
  - Adjust Elution Volume to 90ul
  - Adjust Temperature
    - -Elution Step: Mixing/Heating: Heating Temperature: to 65C
- BindIt™ Software to download, modify and save programs can be loaded from disc provided with Kingfisher materials.

Remove Elution plate from Kingfisher immediately after completion and place on ice

- Aliquot 5ul elution buffer to qPCR plate pre-loaded with qPCR Reaction Mix. (PCR plate is best preloaded).

- Seal Kingfisher total nucleic acid elution plates with disposable film (good sealing at -80) and place at minus 80C.

Multiple plate extractions for the Kingfisher Extractions:
When three thermal cyclers are available, we have run 3 plates in series in about 3 hours total from setup to having RNA in the thermal cycler for the reverse transcription (Steps 5 & 6)

- Fill all Elution and wash plates in one procedure, plates filled can be stacked to prevent evaporative loss.
- The top plate in the stack, typically the last of the three elution plates to be run is sealed with disposable plate sealing film.
- Master Mix are typically mixed while the kingfisher is running the first plate extraction.
- As each elution plate containing nucleic acids is removed from the kingfisher, a new plate run is set up immediately for extraction.
- The elution plate removed from the kingfisher is maintained on ice and as soon as possible.

Go to lunch, you just processed 6600 potatoes

Materials Needed:
MagMAX 96-Viral RNA Isolation Kit #AM1836 or AMB1836-5 (5 plate kit Discount)
100% Isopropanol (2-Propanol, Fisher# A416-500)
100% Ethanol (Fisher # BP2818-500)
Kingfisher Flex™ ***Deep Well Magnet and covers needed***
BindIt™ Software to download and modify Kingfisher programs
96 Standard Plates, Thermo #97002540
96 Deep Well Plates Thermo, # 95040450
Kingfisher TIP comb deep well 96 #97002534
Multichannel loading reservoirs:

25ml split 4/8, 25ml Reservoir with Divider # RESE-2000 (Sterile) Diversified Biotech; Dedham, MA
25ml 12 channel 25ml Reservoir # RESE-3000 (Sterile) Diversified Biotech; Dedham, MA
50ml 12 channel 50ml Reservoir # RESE-5000 (Sterile) Diversified Biotech; Dedham, MA

Foil Plate seal for Elution plate (clear seals do not hold well at -80) #AFS-25 Excelscientific.

1-Step qPCR
REVERSE TRANSCRIPTION & TaqMan qPCR
(Step 6)

Converting RNA to cDNA with pathogen specific primers followed by qPCR amplification and measurement

Be sure all regents are fully thawed (on ice) before using.

All steps performed ON ICE. Return stock reagents to the freezer ASAP.

We perform the RT-qPCR (Step 6) as the final step in the nucleic acid extraction (Step 5) Preferring to avoid a freeze/thaw cycle which has potential to degrade RNA.
Preparing the PCR plates prior to aliquoting nucleic acid from elution plate cuts down reagent volumes required and improves consistency when nucleic acid eluate can be washed in the pipettes using the Reaction mix as carrier.

Use the Direct Tuber Necrotic Virus Testing sample tracking and PCR reaction volume calculator in Excel **MASTER PVY qPCR calculator Boonham rev4-14-21** or XXXX provided with this protocol to calculate the reagent volumes needed for the samples.

- Place clean multichannel loading reservoir on ice and add
    - water
    - Master Mix
    - Taqman Primers
        - See excel spreadsheet calculator for volumes.
- Rock reservoir side to side to mix reagents and fully wet the pickup groove.
- Wash pipette tips several times to continue mixing reservoir and saturate binding to tips. Place 20*ul* of the mixture in each PCR well. *(this is best done before Nucleic acid is placed in wells so pipette tips need not be changed, otherwise shrink calculations must increase dramatically)*
- Add Samples - 5ul nucleic sample (remove tips A11, G11, H11 tips from rack). Deposit eluate into Master Mix in well, cycle/wash pipette once.
- Add CONTROLS - 5 ul of the following controls to the specific PCR plate wells. Deposit Control into Master Mix in well, cycle/wash pipette once
    - Well A11 - No template Control (Poly A Carrier w/o gBLOCK (1mg/ml))
    - Wells G11, H11 - Threshold Cq Standard (gBLOCK) Dilution
    - All other controls are extraction plate controls and already transferred with nucleic acid in previous step.
- Cover Plate
- Spin plates so reagents are in well bottom.
- Place in thermal cycler (protocol is below and works for PVY and PMTV/TRV)

96 well Plate Loading Map

FTA Card 1	FTA Card 9	FTA Card 1	FTA Card 9	FTA Card 1	FTA Card 9	FTA Card 1	FTA Card 9	FTA Card 1	FTA Card 9	FTA Card 1	NTC PolyA PCR	A
FTA Card 2	FTA Card 10	FTA Card 2	FTA Card 10	FTA Card 2	FTA Card 10	FTA Card 2	FTA Card 10	FTA Card 2	FTA Card 10	FTA Card 2	Healthy 1 extract	B
FTA Card 3	FTA Card 11	FTA Card 3	FTA Card 11	FTA Card 3	FTA Card 11	FTA Card 3	FTA Card 11	FTA Card 3	FTA Card 11	FTA Card 3	Healthy 2 extract	C
FTA Card 4	FTA Card 12	FTA Card 4	FTA Card 12	FTA Card 4	FTA Card 12	FTA Card 4	FTA Card 12	FTA Card 4	FTA Card 12	FTA Card 4	Water extract	D
FTA Card 5	FTA Card 13	FTA Card 5	FTA Card 13	FTA Card 5	FTA Card 13	FTA Card 5	FTA Card 13	FTA Card 5	FTA Card 13	FTA Card 5	PVY+ extract	E
FTA Card 6	FTA Card 14	FTA Card 6	FTA Card 14	FTA Card 6	FTA Card 14	FTA Card 6	FTA Card 14	FTA Card 6	FTA Card 14	FTA Card 6	PMTV / TRV+ Extract	F
FTA Card 7	FTA Card 15	FTA Card 7	FTA Card 15	FTA Card 7	FTA Card 15	FTA Card 7	FTA Card 15	FTA Card 7	FTA Card 15	FTA Card 7	77 Copies PCR	G
FTA Card 8	FTA Card 16	FTA Card 8	FTA Card 16	FTA Card 8	FTA Card 16	FTA Card 8	FTA Card 16	FTA Card 8	FTA Card 16	FTA Card 8	77 Copies PCR	H
1	2	3	4	5	6	7	8	9	10	11	12	
Seed Lot 1	Seed Lot 1	Seed Lot 2	Seed Lot 2	Seed Lot 3	Seed Lot 3	Seed Lot 4	Seed Lot 4	Seed Lot 5	Seed Lot 5	Seed Lot 6	No Transfer from Kingfisher Plate	

Figure 10

Thermal Cycler Settings (generic) for:
PVY TaqMan, Boonham 2009
&
PMTV & TRV Duplex TaqMan, Mumford 2000

Step	Thermal Cycler	Time	Purpose	FAM - Channel 1	VIC - Channel 2	TX Red - Channel 3
PVY				PVY	Available?	Inhibition Control?
PMTV /TRV Duplex				PMTV	TRV	Inhibition Control
1	48.0C	30 Minutes	Reverse Transcription			
2	95.0C	10 Minutes	Denaturing template DNA/cDNA			
3	Read and Repeat 40 Cycles		Amplification and Quantification			
4	95.0C	15 sec	Denaturing			
5	60C	60 sec	Anneal and Extension			
Return to step 3 (39 times)						

Figure 11

Molecular Reagents:

Poly-A - Sigma (catalog number P9403-25MG)

Poly-A Prepare as 4mg in 4ml water (0.004g) in 4 ml Nuclease free water in a 5 ml tube.

For eventual gBLOCK dilutions –

- Aliquot 10 tubes with 180ul (1 to 10),
- Aliquot small 20 ul volumes for daily use in qPCR as “no template Control template”
- remainder Keep as Stock Poly-A in aliquots needed for later use.

gBLOCK
Standard for both qPCR protocols includes genome target and overlap for PVY, PMTV and TRV in this protocol based on consensus sequence for US isolates in GenBank (not exact primer match in some cases) To best simulate real world isolate results. All primers match F and R-compliment *(See gBLOCK) supplement files for details on construction and dilutions.*

TGGAGATCCGATTGACCAGAATAATGAGTGCTGAAGGTTCACCGGTGGATGT
AATTGAGCCAGGGGGCCAGGAACTTATTTCCATGCAAGTGCATGGAGAAAGT
CTAACCGTCATAAATGCCGGCACCGACTCTCCCACAGTGATCAGATCCGCGT
CCTTGTCGACGCGGCACCGTAGTTCTGGTATAGAAATCGGTTCTTTTGCAGTG
GATAAAGTGCTGCCTTACGTGTTCTTGGTCATGAGACGTGTAGTATTGGTCAT
GCTAACAAGTTGCGAAAGCAAGTTGCTGACATGGTCGGTGTCACACGTAGGT
GTGCGGAAAATAATTGTGGATGGTTTGTCTGTATTATTATTAACGATTTTACT
TTTGATGTGTACATACGACATAGGAGAAACTGAGATGCCAACTGTGATGAAT
GGGCTTATGGTTTGGTGCATTGAAAATGGAACCTCGCCAAATGTCAACGGAG
TTTGGGTTATGATGGATGGGAATGAACAAGTTGAGTACCCGTTGAAACCAAT
CGTTGAGAATGCAAAACCAACCCTTAGGCAAATCATGGCACATTTCTCAGAT
GTTGCAGAAGCGTATATAGAAATGCGCAACAAAAAGGAACCATATATGCCAC

1-Step qPCR Mix reagents

Product	Concentrations	Part#
RNase/DNase Free H2O (Promega)		P1195
TBD - Inhibition Control Primer/Probe Assay		?X
TaqMan™ Fast Virus 1-Step Master Mix	2X	#4444432

PVY TaqMan, Boonham 2009

PVY 411F	411	GGGCTTATGGTTTGGTGCA
PVY 477R	477	CCGTCATAACCCAAACTCCG
PVY Probe (FAM)		TGAAAATGGAACCTCGCCAAATGTCA

Custom TaqMan "Gene Expression Assays
PVY - Applied Biosystems Assay ID# AP9HN6A
Stock Concentration 20xAssay=900nM

- Conversion to 25ul rxn/0.5ul=360nM
- Actual Assay Concentrations Used - 25ul rxn/0.4125ul = 300nM

TBD- Taqman Inhibition Control COX gene Primer/Probe (TX-Red) or CY5, from potato endogenous control gene (COX) cytochromoe oxidase (Boonham 2009). This would be a different channel than FAM/VIC so it would triplex with the current PMTV/TRV Duplex. If I could go back in time I would have developed this with a master mix without ROX so I could use channel 3 (TX RED) for the plant gene extractions control on both Biorad-QFX I have here and the AB7500. Bottom line reality is that we will need Taqman probes that work across at least 3 of the different channels, however we need to take note that the channel wavelength thresholds are slightly different by thermal cycler manufacturer but can be enough to be a nuisance.

RNA free Water	13.34 ul
Taqman PVY (FAM) (300nM conc.)	0.41ul
Fast Virus 1-Step Master Mix	6.25 ul
Master Mix (each PCR well)	20 ul
Add	
MagMaxx Eluate	5 ul
Tot. Volume/rxn	25 ul

PMTV & TRV Duplex TaqMan, Mumford 2000

PMTV-1948F	1948	GTGATCAGATCCGCGTCCTT
PMTV-2017R	2017	CCACTGCAAAAGAACCGATTTC
PMTV-1970 (FAM)		ACCAGAACTACGGTGCCGCGTCG
TRV-1466F	1466	CATGCTAACAAATTGCGAAAGC
TRV-1553R	1533	TACAGACAAACCATCCACAATTATTTT
TRV-1489 (VIC)		ACGTGTGACACCAACCATGTCAGCAACT

Custom TaqMan "Gene Expression Assays
PMTV - FAM- Applied Biosystems Assay ID# APAAFHZ
TRV - VIC - Applied Biosystems Assay ID# APCE93X

Stock Concentration 20xAssay=900nM

- Conversion to 25ul rxn/0.5ul=360nM
- Actual Assay Concentrations Used - 25ul rxn/0.4125ul = 300nM

TBD - Taqman Inhibition Control COX gene Primer/Probe (TX-Red) or CY5,

RNA free Water	12.93ul
Taqman PMTV (FAM)	0.41ul
Taqman TRV (VIC) (300nM conc.)	0.41ul
Fast Virus 1-Step Master Mix	6.25 ul
Master Mix (each PCR well)	20 ul
Add	
MagMaxx Eluate	5 ul
Tot. Volume/rxn	25 ul

OPEN AND RUN EXCEL CALCULATOR FILE FOR REQUIRED VOLUMES AND INFORMATION FOR MIXING 96 WELL PLATE HANDLING VOLUMES. IMAGE BELOW IS AN EXAMPLE OF THE CALCULATOR. Enter the number of samples in the yellow box. (The calculator increases the needed volume by variable % for loss due to reagent adhesion in tubes and pipette tips. This increase is suitable for a small number of samples. The amount increase needed will vary by worker technique and disposable plastics used.)

Materials Needed:

96 well qPCR plates – BIORAD HSP9601White Shell, clear well 200 ul (Ithaca Specific)
#4444432 TaqMan™ Fast Virus 1-Step Master Mix 1 x 1ml Applied Biosystems
RNAse/DNAse free Water, Promega #P1195
TaqMan Assay aliquot (Aliquot on arrival for contamination control)
Thermalcycler BIORAD C1000 CFX Touch
PCR plate seal films MSB1001 Microseal 'B' Film adhesive
Multichannel loading reservoirs:
25ml split 4/8, 25ml Reservoir with Divider # RESE-2000 (Sterile) Diversified Biotech; Dedham, MA

REVERSE TRANSCRIPTION & PCR:
Example RT-PCR reagent mix calculator for a complete 96 well plate.

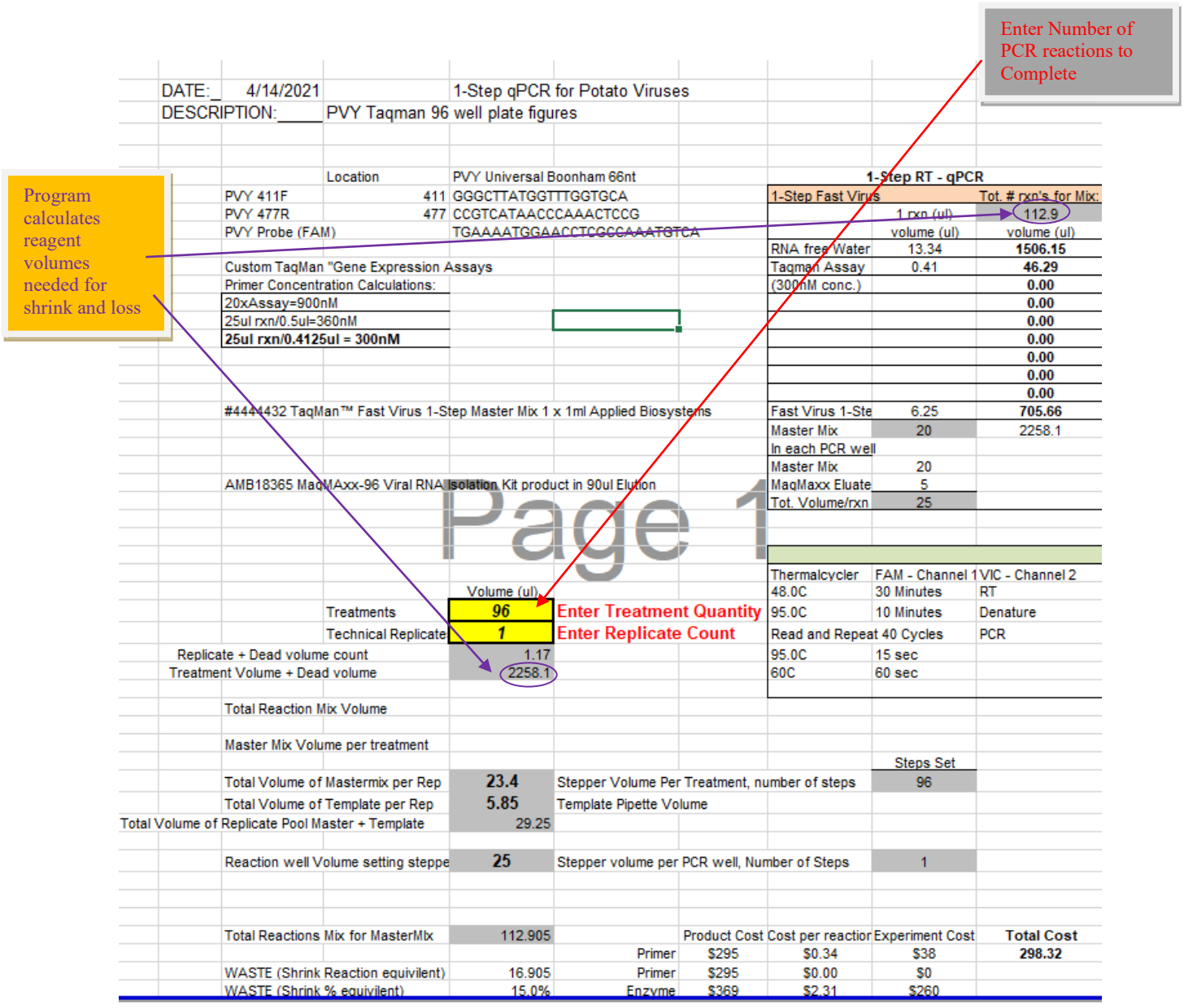


Figure 12

Curve Analysis
(Step 7)

Interpret Cq values based on baseline threshold adjustments and Cq "Positive" threshold template

PVY TaqMan, Boonham 2009

- Find Final RFU value for positive control extraction sample. Calculate 10% RFU and manually set as baseline threshold for qPCR analysis software.
    - Channel – FAM (PVY)
- Determine Cq value of (FAM Channel) Known virus copy standard. *(Currently 77 Copies, subject to change)*
    - Sample Cq > Standard = Negative Result (Pathogen absent)
    - Sample Cq , Standard = Positive Result (Pathogen Present)
- *TBD - Inhibition Control Curves (Potato COX Gene with Tex-Red/CY5) – All similar? What measure is "similar"*

PMTV & TRV Duplex TaqMan, Mumford 2000

- Find Final RFU value for positive control extraction sample. Calculate 10% RFU and manually set as baseline threshold for qPCR analysis software.
    - For each Channel – FAM (PMTV)
    - For each Channel – VIC (TRV)
- Determine Cq value of (FAM or VIC Channel) Known virus copy standard. *(Currently 77 Copies, subject to change)*
    - Sample Cq > Standard = Negative Result (Pathogen absent)
    - Sample Cq , Standard = Positive Result (Pathogen Present)
- *TBD - Inhibition Control Curves (Potato COX Gene with Tex-Red/CY5) – All similar? What measure is "similar"*

Reporting
(Step 8)

Current reporting includes excel file with each row designated by a single FTA card (25 tubers) and 16 rows (16 cards) representing a seed lot. E.g. (See **Figure 10**, Plate Columns 1 & 2 for Seed Lot #1 FTA card 1 to 8 and 9 to 16)

Then converted to a modeled PVY% estimate. **Figure 13**

Review needed for 2022 increased sample volume and tracking.

Current:

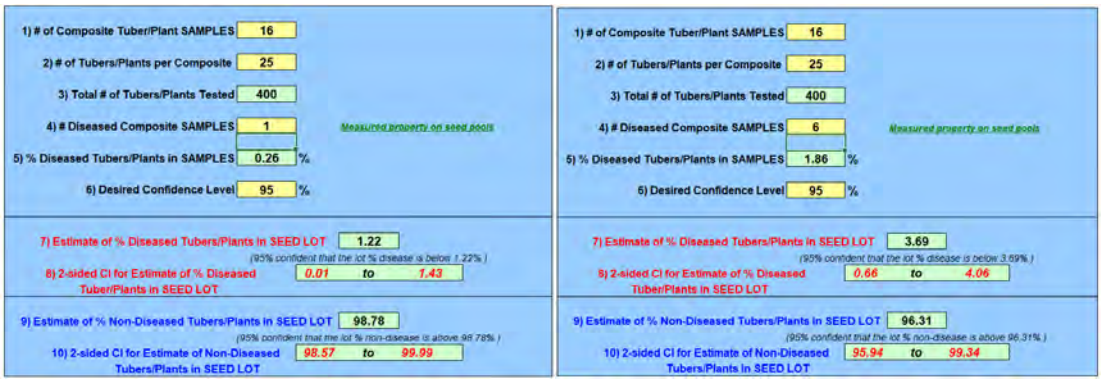


Figure 13

References:

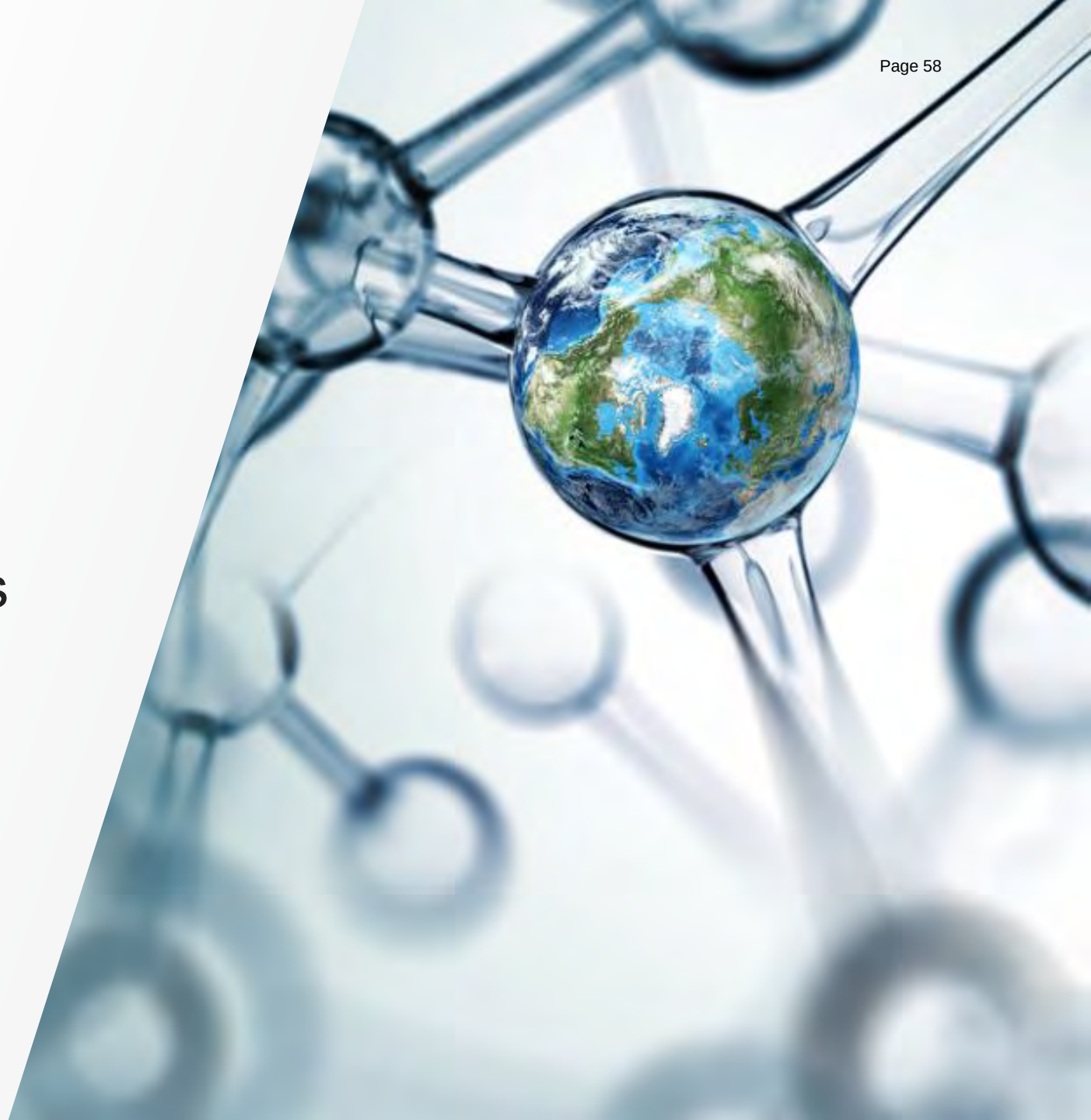
Neil Boonham , Lynn Laurenson, Rebecca Weekes and Rick Mumford & R. Burns (editor), Direct Detection of Plant Viruses in Potato Tubers Using Real-Time PCR (Chapter) in Methods in Molecular Biology, Plant Pathology, vol. 508 Humana Press DOI: 10.1007/978-1-59745-062-1_19

Mumford, R. A., Walsh, K., Barker, I., and Boonham, N. 2000. Detection of Potato mop top virus and Tobacco rattle virus using a multiplex realtime fluorescent reverse-transcription polymerase chain reaction assay. Phytopathology 90:448-453.

KingFisher – Magnetic Bead Purification of Biological Samples

Alex Durkin
Sample Prep Technical Sales Specialist
Christian Kis
Field Application Scientist

The world leader in serving science



Multiple Applications

The KingFisher Apex instrument supports a variety of applications with a wide range of validated protocols from trusted reagents.

- Applied Biosystems™ MagMAX™ kits and reagents
- Invitrogen™ Dynabeads™ magnetic beads
- Thermo Scientific™ Pierce™ magnetic agarose beads
- Thermo Scientific™ SMART Digest™ kits

Find out more at
thermofisher.com/kingfisherapplications

DNA

- gDNA
- Cell-free DNA
- Viral/pathogen
- Microbiome
- FFPE
- PCR cleanup

RNA

- Total RNA
- miRNA
- mRNA
- FFPE
- Microbiome
- Viral/pathogen

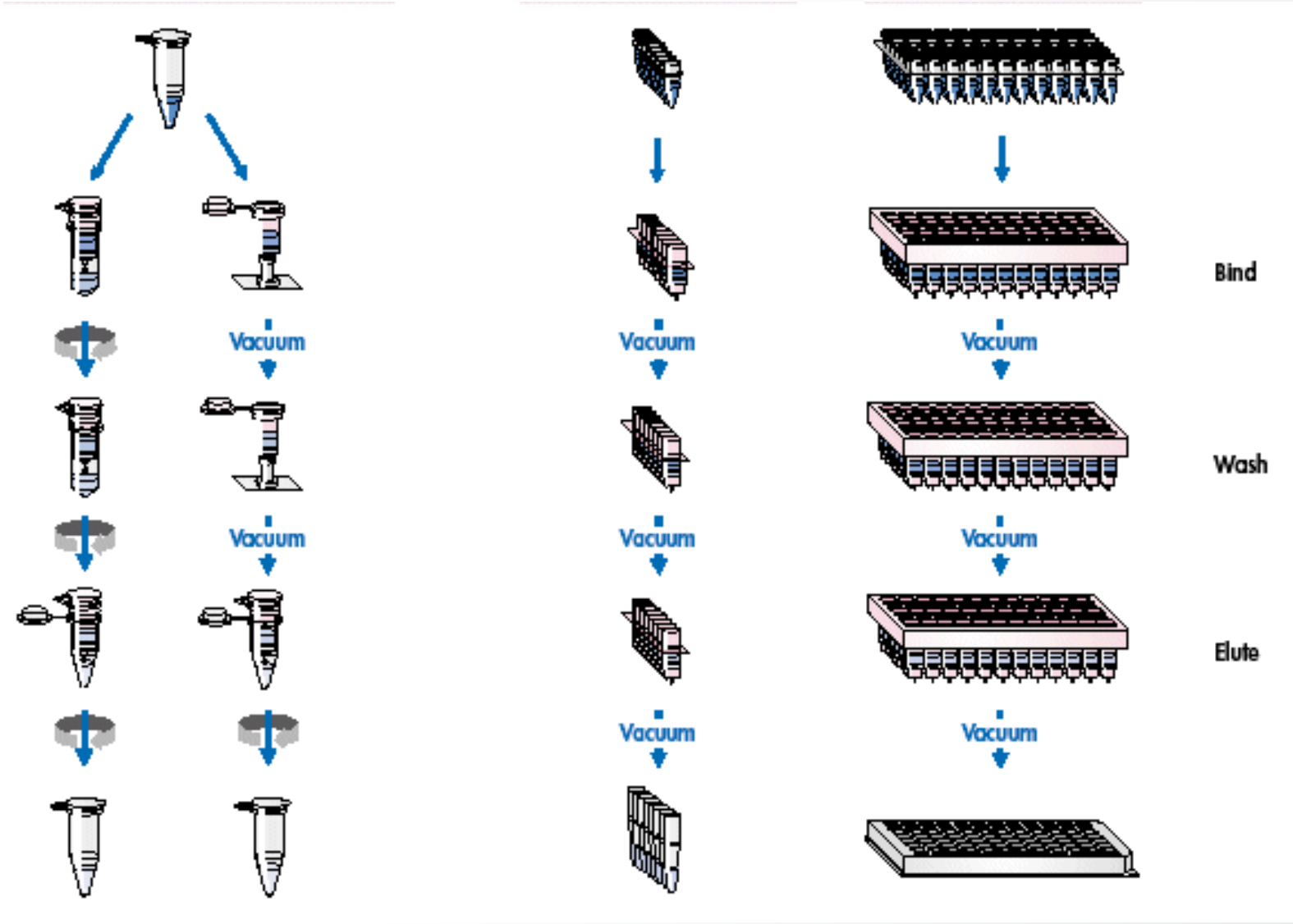
PROTEINS

- Immunoprecipitation
- Protein purification
- Antibody purification
- Phage display
- Mass spec

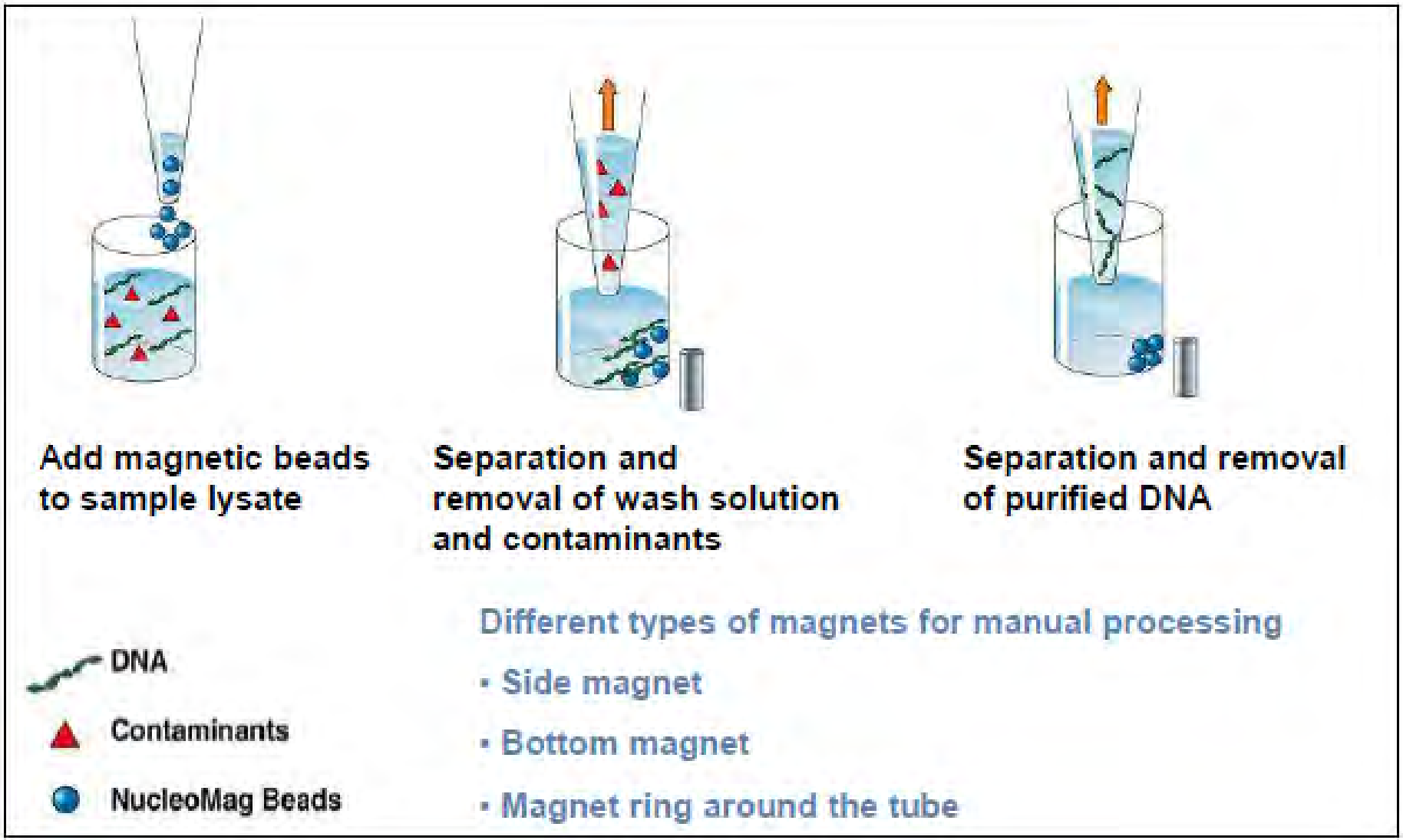
CELLS

- Exosomes
- T cells
- Primary cells
- Cell lines

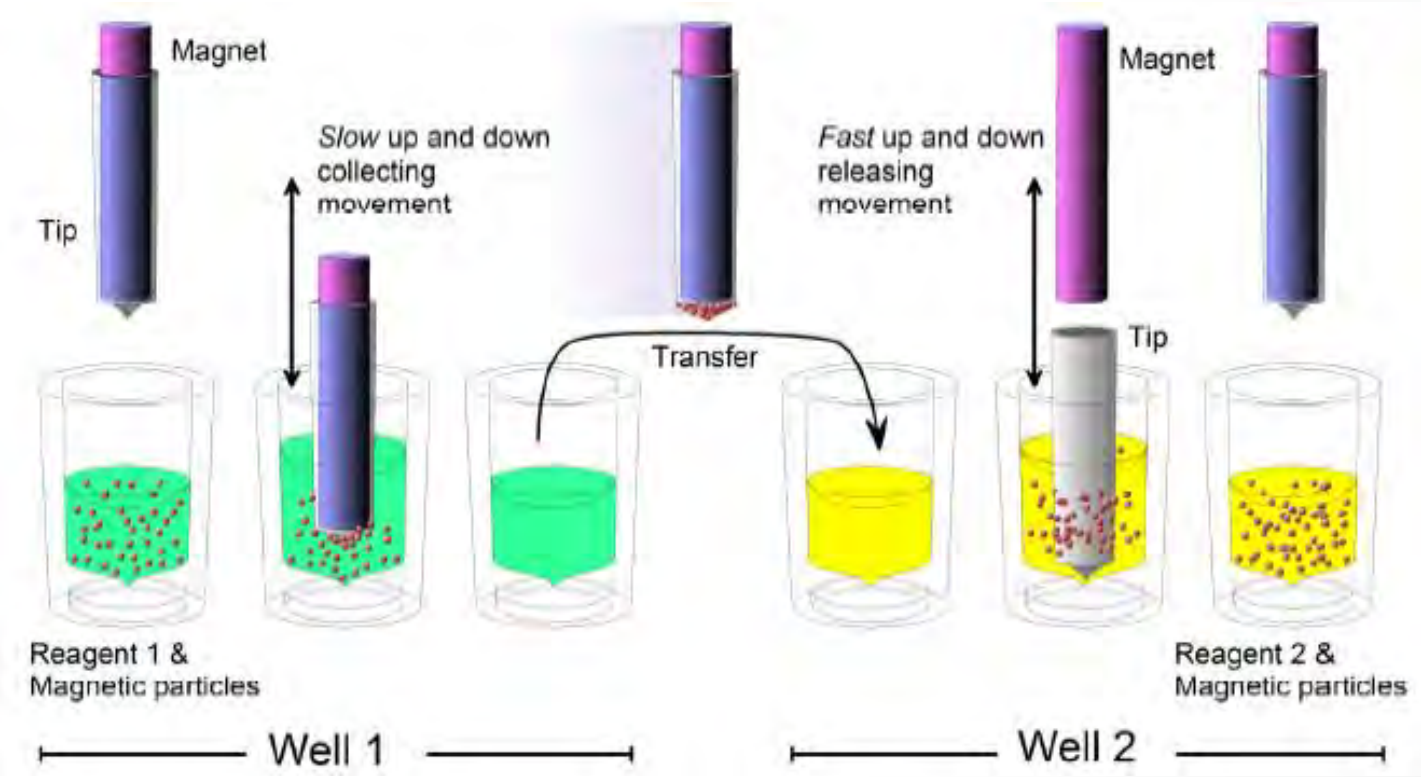
Column Based Purification



Manual Magnetic Bead Processing



KingFisher – Inverse Magnetic Particle Processing



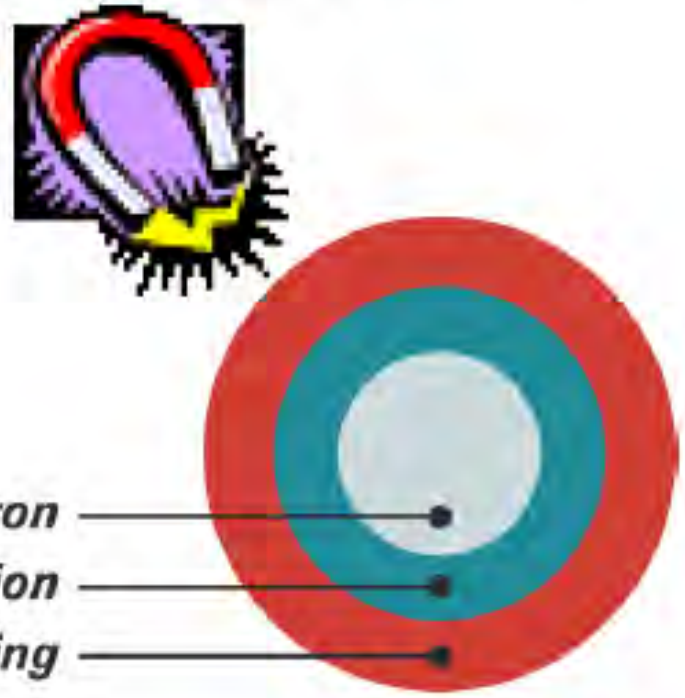
Move the magnetic beads instead of the liquids.

Plates with samples and reagents are pre-loaded into the instrument.

Moves magnetic particles instead of liquids, resulting in superior purification and high quality end product, with contaminants left behind.

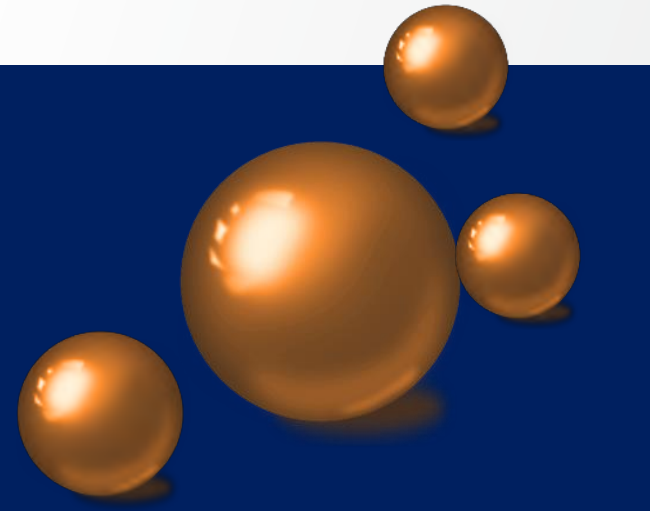
Superparamagnetic Polymer Beads

- Exhibit magnetic properties only when placed within a magnetic field and show no residual magnetism when removed from this field.
- Polymer bead coating protects the target from toxic exposure to iron
- Optimal bead size is 0.5 – 10 µm
- Coatings are specific for targets
- Possible to use custom made magnetic particles



Advantages of Magnetic Beads

- Large surface = Efficient nucleic acid binding
- Better binding and washing efficiency than with filter based methods
- Can be used for samples with different viscosities
- Easy to automate
- Suitable for low to high throughput applications



Magnetic Beads and Automation

Advantages vs. vacuum filtration

- Less or no foam and aerosols formation
- Can be used for samples with different viscosities
- Low risk of contamination
- No liquid transfer
- Up-scaling und down-scaling are possible
- Easier for automation
- Higher concentrated eluted DNA

History of KingFisher purification systems

1999 — KingFisher launched

Automation for magnetic bead extractions: 24 samples/run (20–200 µL) (no heating/cooling)

2003 — KingFisher mL launched

Automation for larger sample volumes: 15 samples/run (50–1,000 µL) (no heating/cooling)

2005 — KingFisher 96 launched

Automation for high throughput: 96 samples/run (20–5,000 µL)

2008 — KingFisher Flex launched

Automation for even larger sample volumes: 24 deep-well format; 24–96 samples/run (20–5,000 µL)

2011 — KingFisher Duo launched

Automation for low/ medium/varied throughput: script upload via USB, downloadable run logs from instrument; 6–24 samples/run (30–5,000 µL)

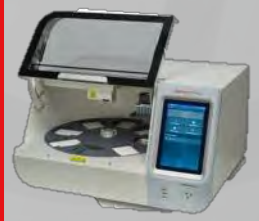
2015 — KingFisher Duo Prime with UV lamp launched

Automation for low/ medium/varied throughput: new elution strip caps released; 6–24 samples/run (30–5,000 µL)

2016 — KingFisher Presto launched

Automation for high throughput with direction integration with liquid handlers: 24–96 samples/run (50–5,000 µL)

2021 — KingFisher Apex launch



Automation for high throughput, cloud-based, barcode scanner, touchscreen interface: 24–96 samples/run (50–5,000 µL)

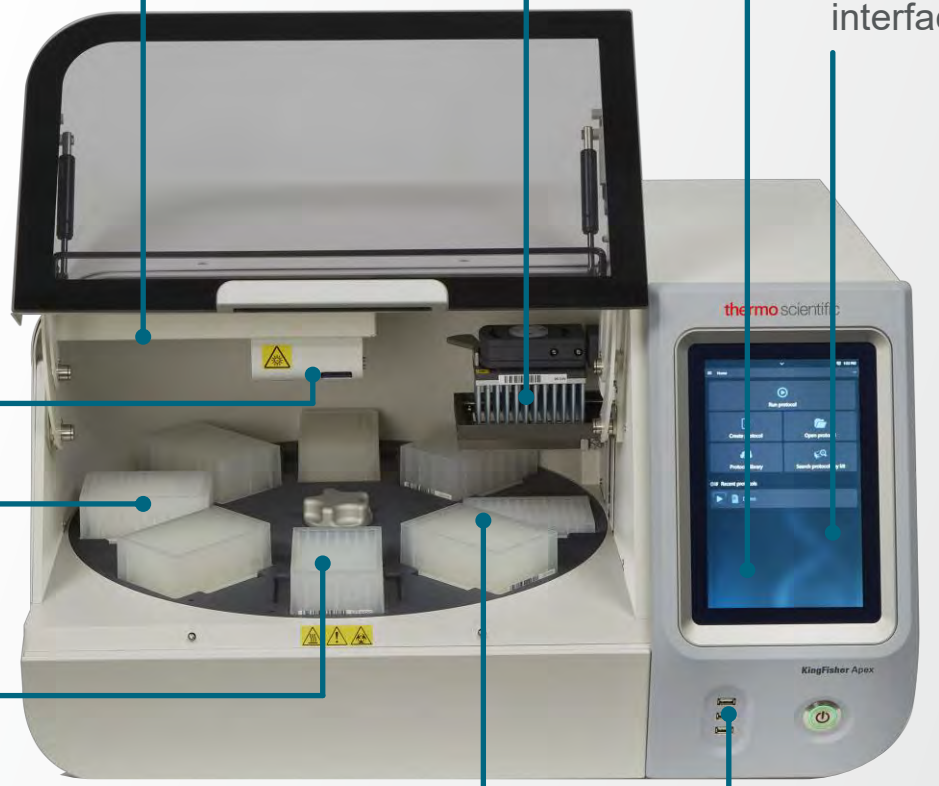
KingFisher Flex and KingFisher Presto now have GPLE "For Laboratory Use" classification

To date, ~14,000 KingFisher instruments being used for research

Introducing KingFisher Apex | New product features

The pinnacle of automated purification for DNA, RNA, Proteins and Cells

Building on decades of product value and excellence from Thermo Scientific™ KingFisher™ systems, we've combined unparalleled instrument capabilities with complete touchscreen-based control to deliver unrivaled flexibility and performance, so sample prep can be simple.



Taking the best features of KingFisher instruments and improving upon them

Enhanced Performance

Achieve peak performance for purifying DNA, RNA, proteins, or cells, now with even greater flexibility for fast and reproducible results with almost any application.

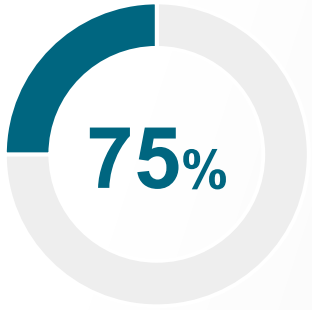
- Elute in lower volumes (10 µL) for demanding downstream applications
- Purify 24 or 96 samples in 25–65 min
- Control heating and cooling to maintain sample integrity
- Elute in storage tubes and revisit samples later
- Safeguard against contamination with 2 UV lights

Sample prep as it should be—fast, flexible, consistent*

Average time required using our automated solutions

Average time for manual sample prep

gDNA from blood



less touch time

cfDNA

less touch time

RNA

less touch time

* Based on actual extraction times in lab

Touchscreen Control

A modern touchscreen display and intuitive interface put you in control of the instrument, making it easy to operate and reducing errors

Easily write or edit protocols directly on the instrument

Quickly access recent protocols, validated protocol libraries, and other instrument settings

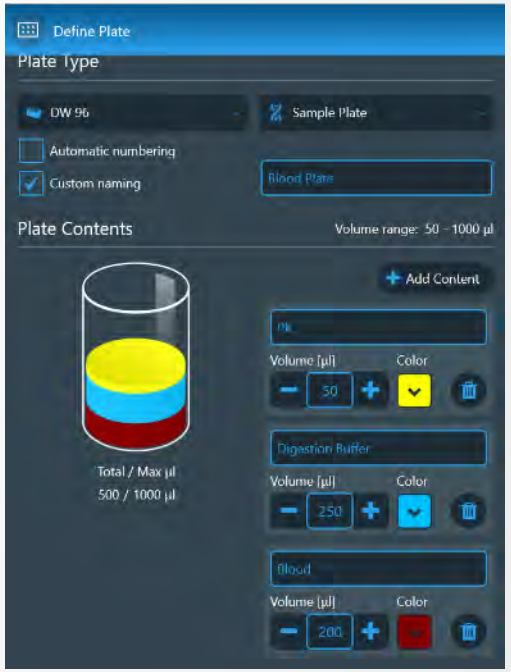
Rely on guided visuals for easy plate loading, protocol changes, and instrument setup

No computer needed

Customize Protocols

Easily edit or write protocols directly on the instrument. It allows customization of every step in your protocol—bind, wash, and elute

- Create protocol
- Open protocol
- Download protocol
- Search protocol by kit



Quickly optimize any step in your protocol directly from the touchscreen

Instrument-defined limits reduce errors in any protocol modifications

Visual guides make it simple to edit or customize protocols

Option to work from your PC with Thermo Scientific™ BindIx™ Software

Cloud and Network Connection

Connect the instrument to the cloud for even greater access and control, or use a network connection

Conveniently save, transfer, or download protocols from (virtually) anywhere using a cloud library, local area network (LAN) file locations, USB, or by scanning the bar code from kits

Run reports

Generate run reports in PDF or XML format for ease of run tracking and LIMS incorporation

Protocol report
MagMAX_cfNA_APEX_Rebind_v1
7/30/2019 7:45:47 AM 1/4

General info

Protocol information

Protocol name: MagMAX_cfNA_APEX_Rebind_v1
Kit name: MagMAX Cell-Free Total Nucleic Acid Isolation Kit
Description: MagMAX Cell-Free Total Nucleic Acid Isolation Kit - Rebinding protocol
FOR APEX VERIFICATION

Run information

Execution started: 7/30/2019 7:12:25 AM

Instrument parameters

Instrument name:
Instrument type: KingFisher Apex
Firmware version: 0.1.35
Instrument serial number: 714-P00021

Software parameters

Software version: 1.0.2
PC or device: Device

Protocol info

Configuration

Magnets
96 DW
Heating blocks

Plate and reagent info

Tip Plate:
Type: DW 96
Plate barcode: -
Name | Volume [µl] | Lot information
- | - | -

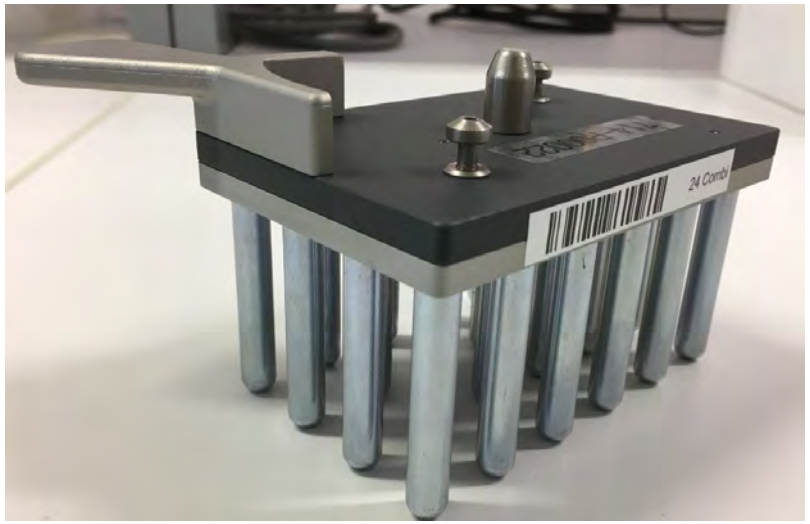
Rebind
Type: DW 96
Plate barcode: -
Name | Volume [µl] | Lot information

Magnet heads

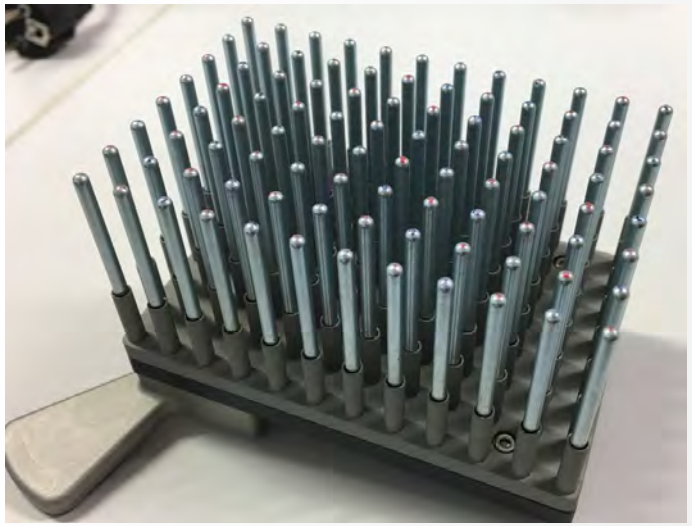
- 4 different magnet head options available
  - 96 DW – flat end magnets
  - 96 Combi – round end magnets
  - 24 Combi – round end magnets
  - 96 PCR

5400910	KingFisher Apex with 96 PCR head
5400930	KingFisher Apex with 96 DW head
5400920	KingFisher Apex with 96 Combi head
5400940	KingFisher Apex with 24 Combi head

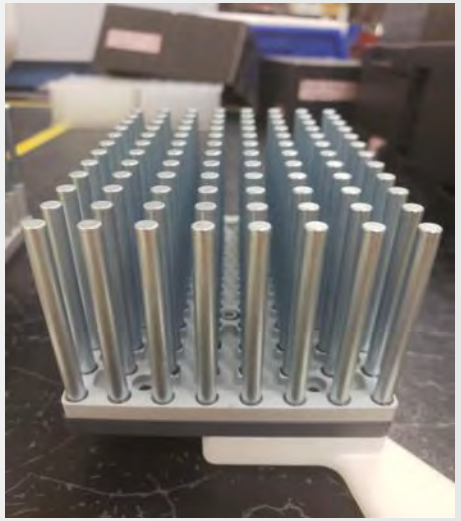
24 Combi



96 Combi



96DW



Low volumes

- Use of low volumes made routine
  - Consentrated samples in the end of the run
  - Ready for downstream application

Plate	Tip comb	Minimum volume (µl) KF Apex	Maximum volume (µl) KF Apex
96 DW	96DW / 96Combi	50 / **15**	1000 / 1000
96 Standard	96DW / 96Combi	50 / **15**	150 / **200**
96 Standard	96KF	20	200
96 PCR	96PCR	**10**	**80**
96 Standard	96PCR	20	200
96 Storage tube	96Combi	30	200
24 DW	24DW / 24Combi	200 / **30**	5000 / 5000
24 Storage tube	24 Combi	200	1000

Note ! SW allows exceed volume range for 96DW tip comb (20 µl) and 24 DW tip comb (50 µl)

Compatibility

- Magnet head & tip comb

	96 DW magnet head	96 Combi magnet head	24 Combi magnet head
96 DW tip comb	x	x	
96 Combi tip comb		x	
24 DW tip comb			x
24 Combi tip comb			x

Efficient lysis

- P

	96 DW tip comb	96 Combi tip comb	24 DW tip comb	24 Combi tip comb
96 DW plate	x	x		
96 KF plate	x	x		
24DW plate			x	x
96 storate tube		x		
24 storage tube				x

Dual Magnets and Multiple Formats

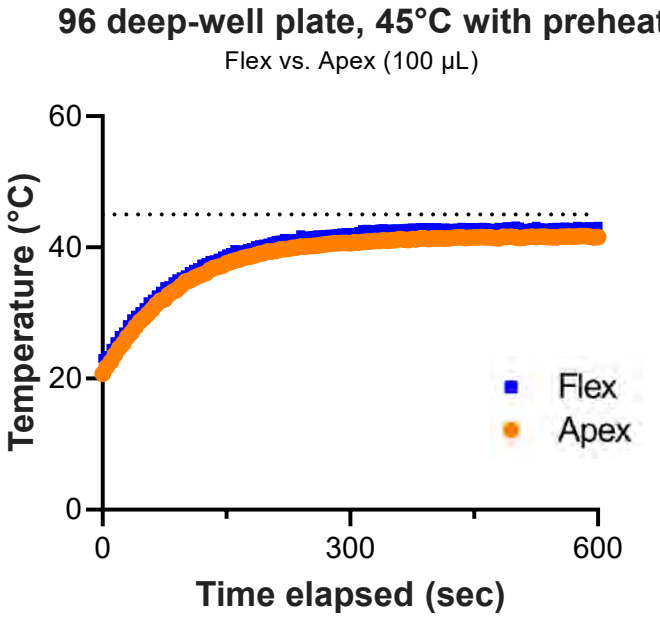
Install two magnetic heads

The KingFisher Apex instrument intelligently selects the correct magnetic head based on your protocol needs. Work with small to large volume ranges by choosing from 24-well, 96-well, or PCR formats with corresponding magnetic heads and plates.

On-Deck Incubation

Heat blocks recessed below deck until needed, tight control of sample temperature

Heating and cooling
(4°C–100°C)



Bar-coded and Optimized Plastics

Thought it was just a plastic plate?

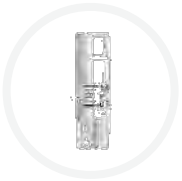
Think again. Achieve optimal performance, reduce errors, and increase traceability with plastics specially designed for KingFisher instruments.

Specialized tip combs and well design enable lower elution volumes, storage tube elution, and heating and cooling consistency

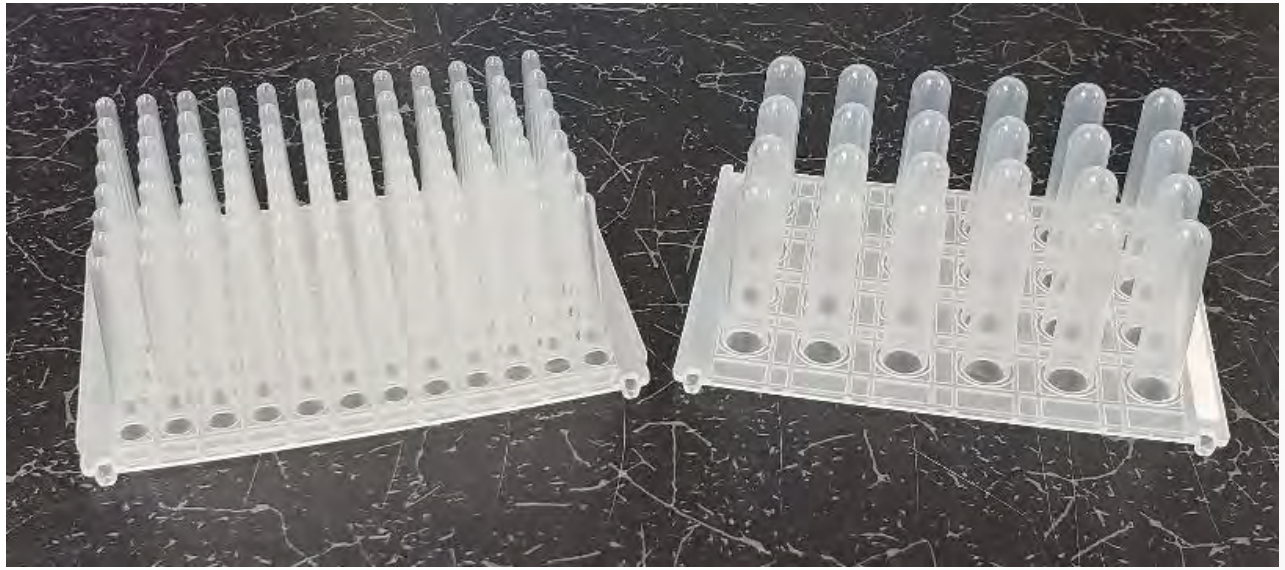
---

Lot-specific bar codes confirm proper plate position during loading or may be used for lot tracking and documentation

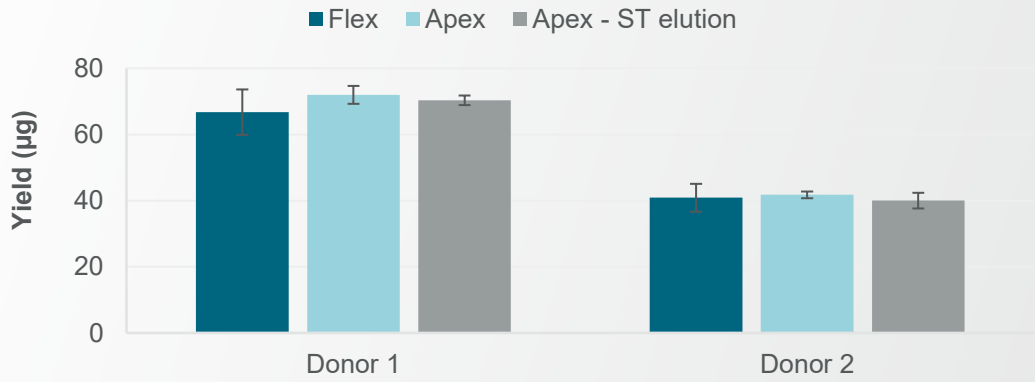
Storage Tube Elution



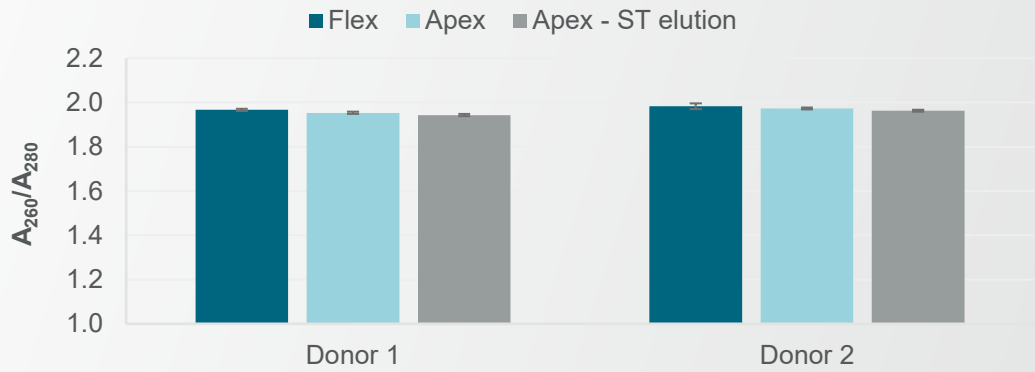
Option to elute in screwcap storage tubes
(96 or 24 format)



Total yield
2 mL whole blood



Purity (A_{260}/A_{280})
2 mL whole blood

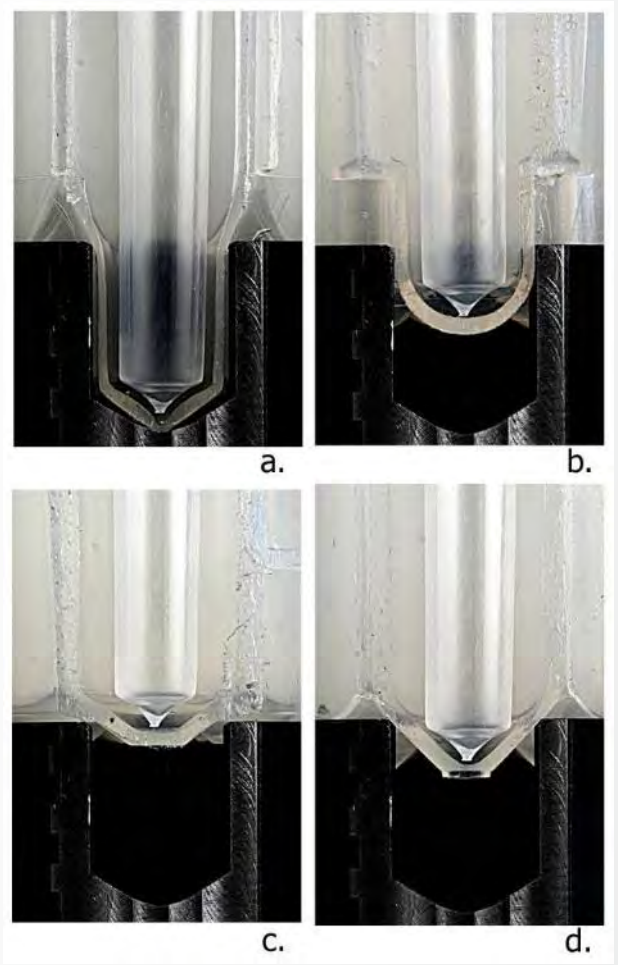


KingFisher Flex Plastics

- Specially designed KingFisher microplates, tubes and tip combs for optimal mixing and isolation of target molecules
- Made of polypropylene
- Low binding of biomolecules
- Enables >99% (KingFisher) and >95% (KF mL & KingFisher Flex) recovery of magnetic particles

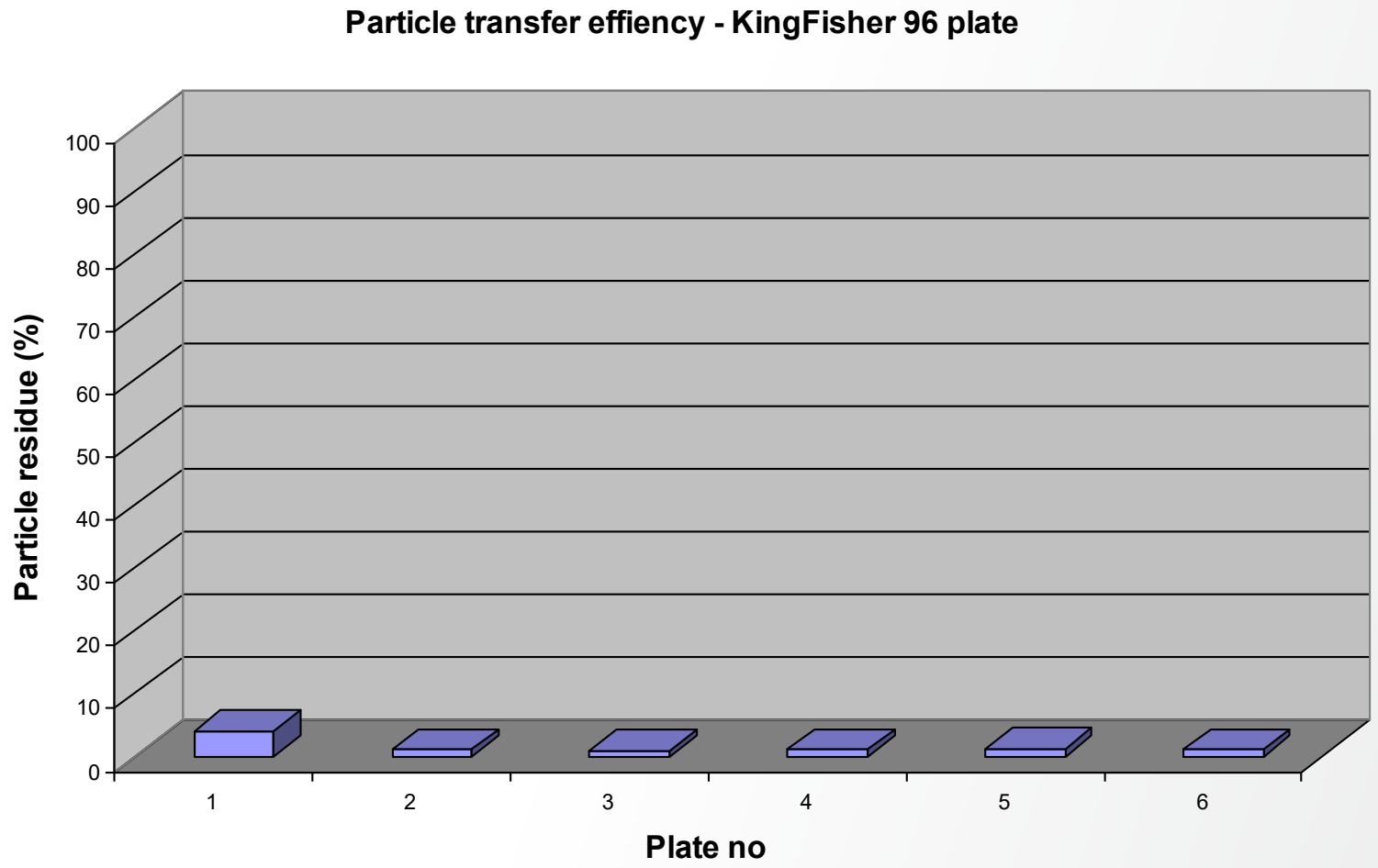
A Word About KingFisher 96 DeepWell Plates

- **Specially designed to fit precisely** to the tip comb and heating block shapes.
- Liquid volumes & tip movements are **optimized according to well bottom height**.
- Plate shape **allows maximal heating efficiency** in the heating block.
- **Other deepwell plates may cause unexpected mixing issues** due to the different well volume and bottom height.
- If other deepwell plates are used, the heating block may raise the bottom height of the plate. Unsuitable mixing, a plate stuck to the Tip Comb, or even damage to the KF Flex head assembly can result!



Sectional view of the Microtiter deep well 96 plate (a), deep well 96 plates of other manufacturers (b, c, d) and the KingFisher Flex 96 deep well heating block

High Recovery of Particles: 95-99% step to step



Multiple applications

The KingFisher Apex instrument supports a variety of applications with a wide range of validated protocols from trusted reagents.

- Applied Biosystems™ MagMAX™ kits and reagents
- Invitrogen™ Dynabeads™ magnetic beads
- Thermo Scientific™ Pierce™ magnetic agarose beads
- Thermo Scientific™ SMART Digest™ kits

Find out more at
thermofisher.com/kingfisherapplications

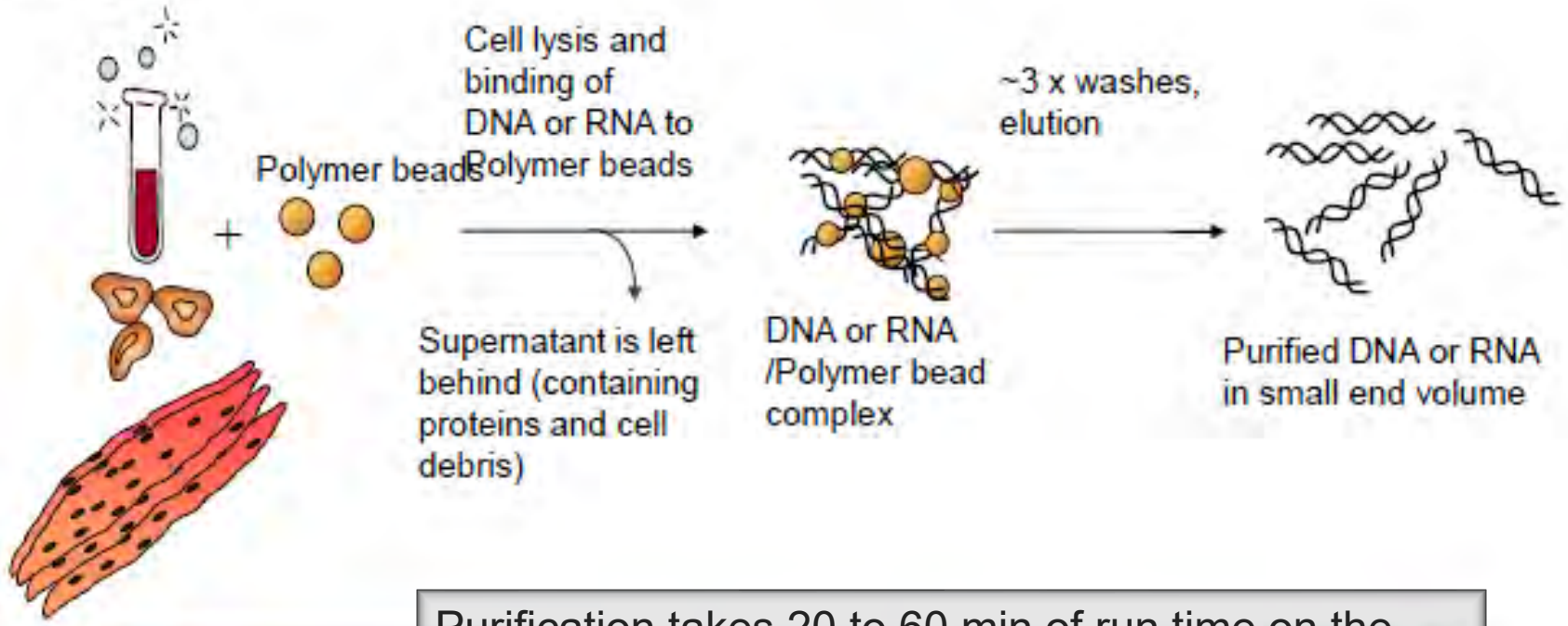
DNA
- gDNA
- Cell-free DNA
- Viral/pathogen
- Microbiome
- FFPE
- PCR cleanup

RNA
- Total RNA
- miRNA
- mRNA
- FFPE
- Microbiome
- Viral/pathogen

PROTEINS
- Immunoprecipitation
- Protein purification
- Antibody purification
- Phage display
- Mass spec

CELLS
- Exosomes
- T cells
- Primary cells
- Cell lines

Purification Workflow in the KingFisher

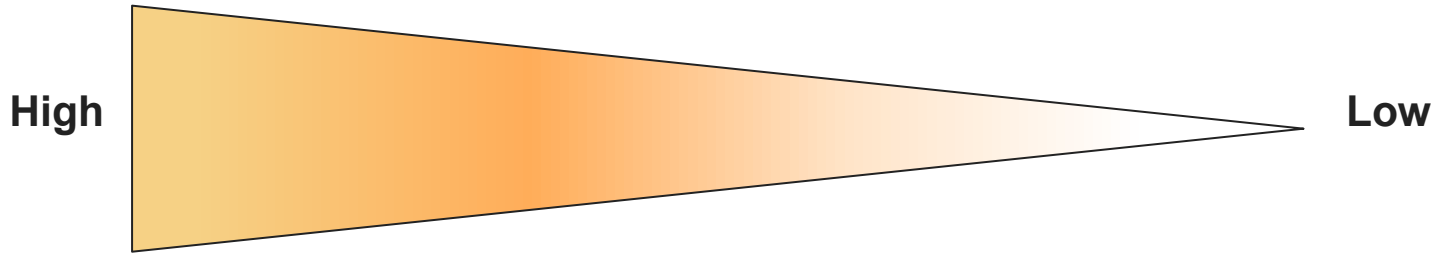


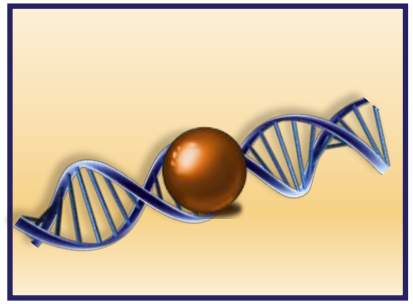
Purification takes 20 to 60 min of run time on the Kingfisher instruments depending on kit used, sample type and nucleic acid being purified

DNA/ RNA Capture By KingFisher Magnetic Beads

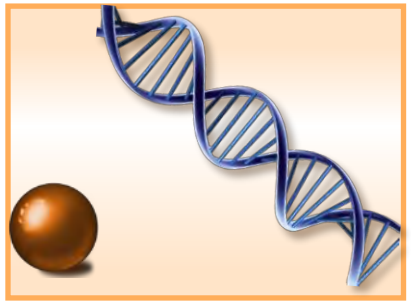
- KingFisher Magnetic Beads are polymer beads
- Buffers include chaotropic salt which cause DNA to bind to polymer beads

Salt concentration





DNA **binds** to magnetic particles



DNA **detaches** from magnetic particles

Thermofisher Sample Prep Kits for Nucleic Acid

Sample Type	Total RNA (excluding small RNA)	True Total RNA (miRNA and large)	mRNA (polyA selected)	genomic DNA	Bacterial/Fungal NA	Viral NA	plasmid DNA	PCR Product
Blood	MagMAX for Stabilized Blood, Tempus (4451893) MagMAX for Stabilized Blood, Paxgene (4451894) MagMAX mirVana (A27828)	MagMAX mirVana (A27828)	Dynabeads mRNA Direct (610-11) (610-21)	MagMAX DNA Ultra 2.0 (A36570)	MagMAX Viral/Pathogen Ultra (A42356)	MagMAX Viral/Pathogen (A42352)		
Plasma/Serum		MagMAX Cell-Free Total Nucleic Acid Kit (A36716)	Dynabeads mRNA Direct (610-11) (610-21)	MagMAX Cell Free DNA Kit (A29319)				
Tissue (non-plant)	MagMAX mirVana (A27828)	MagMAX mirVana (A27828)	Dynabeads mRNA Direct (610-11) (610-	MagMAX DNA Ultra 2.0 (A36570)				
FFPE		MagMAX FFPE Ultra (A31881)		MagMAX FFPE Ulltra (A31881)				
Cell Culture	MagMAX mirVana (A27828)	MagMAX mirVana (A27828)	Dynabeads mRNA Direct (610-11) (610-21)	MagMAX DNA Ultra 2.0 (A36570)	MagMAX Viral/Pathogen Ultra (A42356)	MagMAX Viral/Pathogen (A42352)	MagJet Plasmid DNA (K2791)	
Saliva Buccal Swab Other swabs				MagMAX DNA Ultra 2.0 (A36570)				
Plant Tissue	MagMAX Plant RNA (A33784)		Dynabeads mRNA Direct (610-11) (610-21)	MagMAX Plant DNA (A32549)				
Feces				MagMAX Microbiome (A42357 or A42358)	MagMAX Microbiome (A42357 or A42358)			
Clean-Up			Dynabeads mRNA Purification (610-06)					MagJet Clean Up Kit (K2821)

Fast Purification Using the MagMAX Microbiome Ultra Nucleic Acid Isolation Kit

15 min

35 min

Purified total nucleic acid

Hands-on processing

Automated isolation

Bead-beating

Bind

Wash

Elute

DNA and RNA in same eluate

Sample to purified total nucleic acid:

50 minutes

Microbiome Total Nucleic Acid Isolation

KingFisher Apex vs. Flex instrument

Performance is equivalent

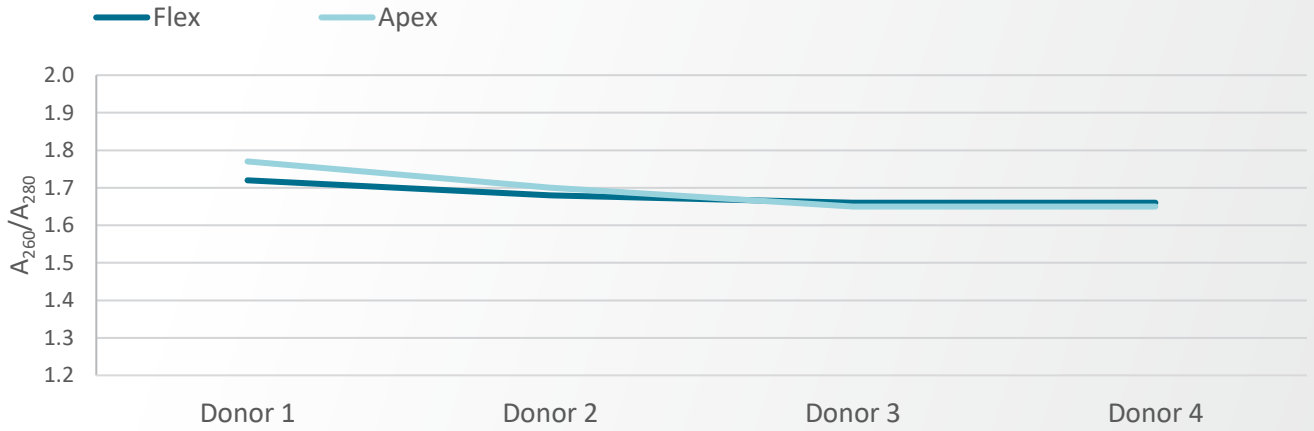
4 human donors,
100 µg fecal input

Applied Biosystems™ MagMAX™ Microbiome Ultra Nucleic Acid Isolation Kit

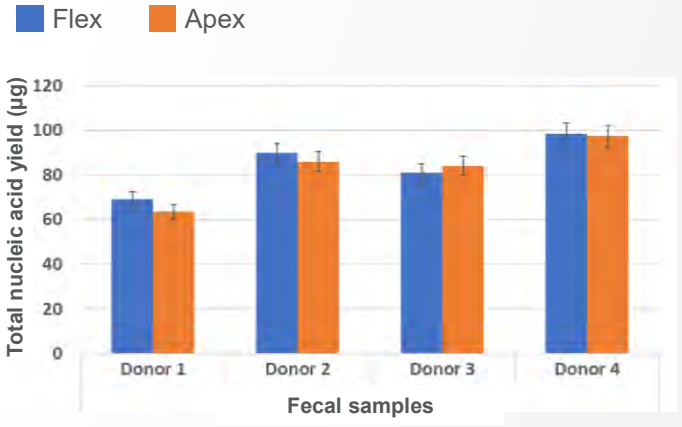
KingFisher Apex instrument

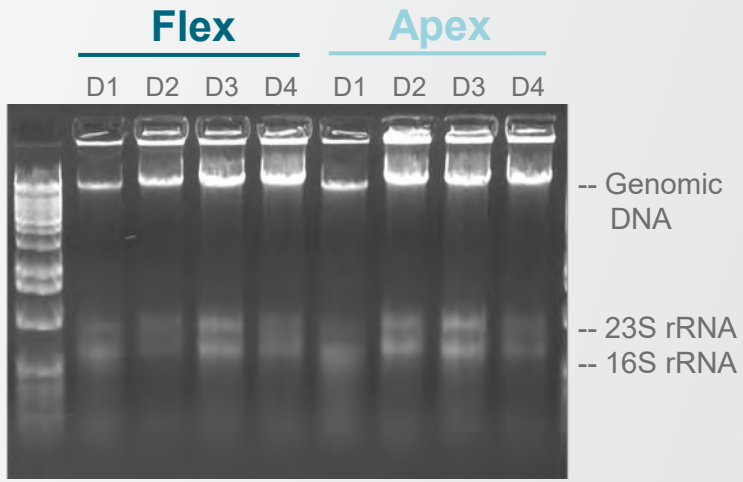
KingFisher Flex instrument

Nucleic acid purity



Total yield (µg)





1% agarose EtBr gel (2 µg per lane)

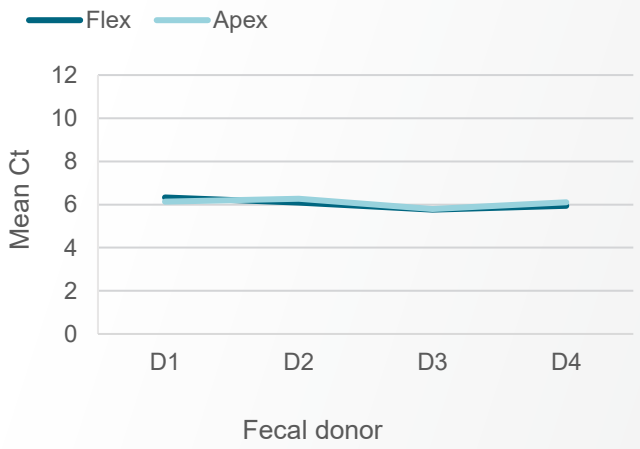
Comparison of qPCR Results

Comparable C_t values on KingFisher Apex and Flex instruments

High-quality nucleic acid, no inhibition

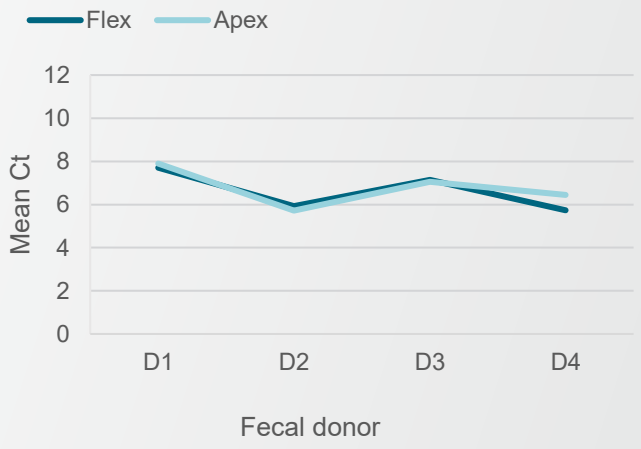
Bacteroidetes

(Gram-negative)



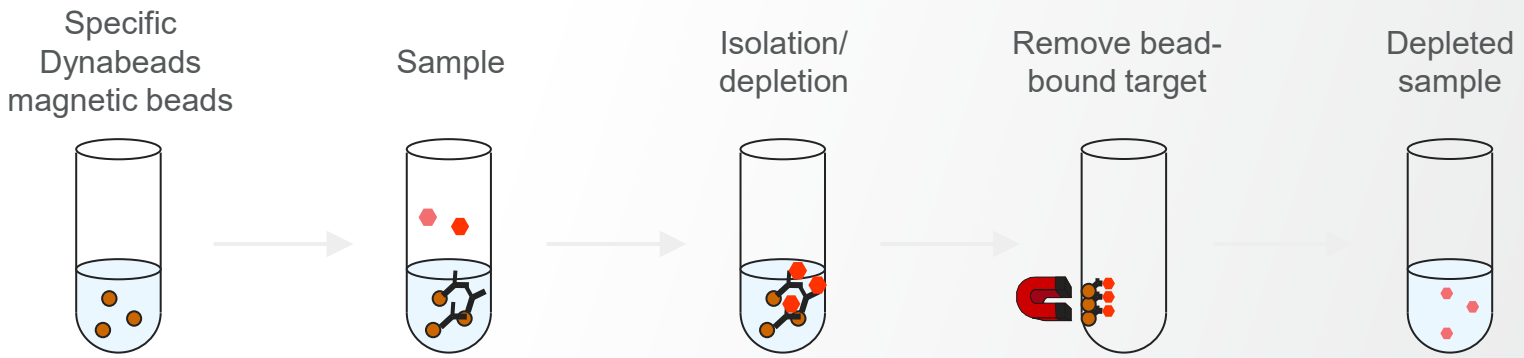
Firmicutes

(Gram-positive)

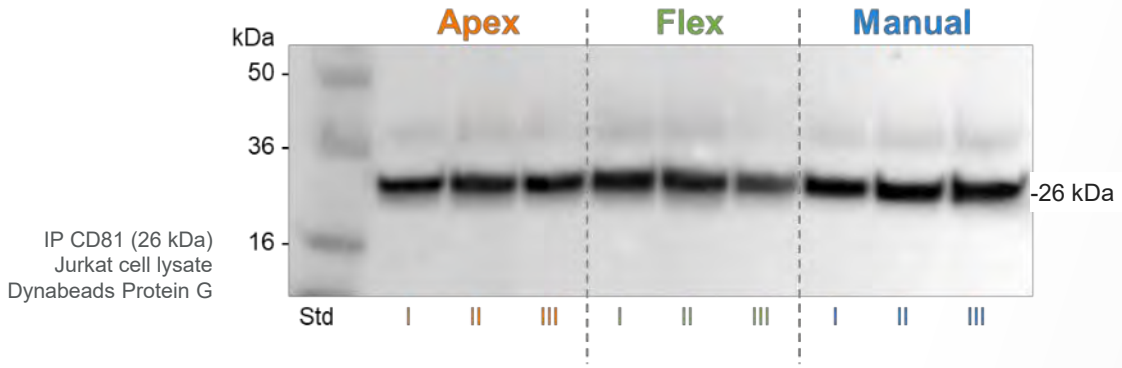


qPCR performed using Applied Biosystems™ TaqMan® Fast Virus 1-Step Master Mix and TaqMan® Assays for Gram-positive and Gram-negative targets. Experiments performed by Madhu Jasti, Staff Scientist, Thermo Fisher Scientific.

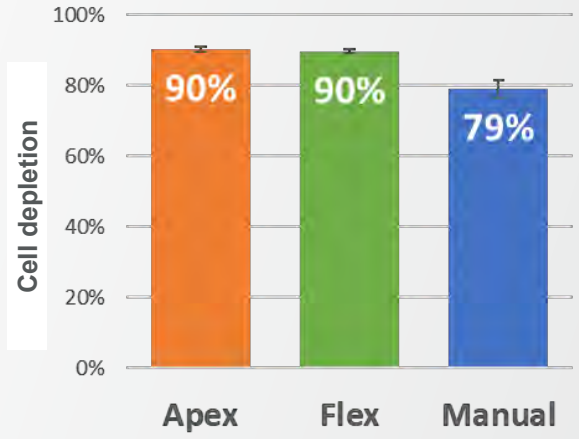
KingFisher Apex Instrument for Proteins and Cells



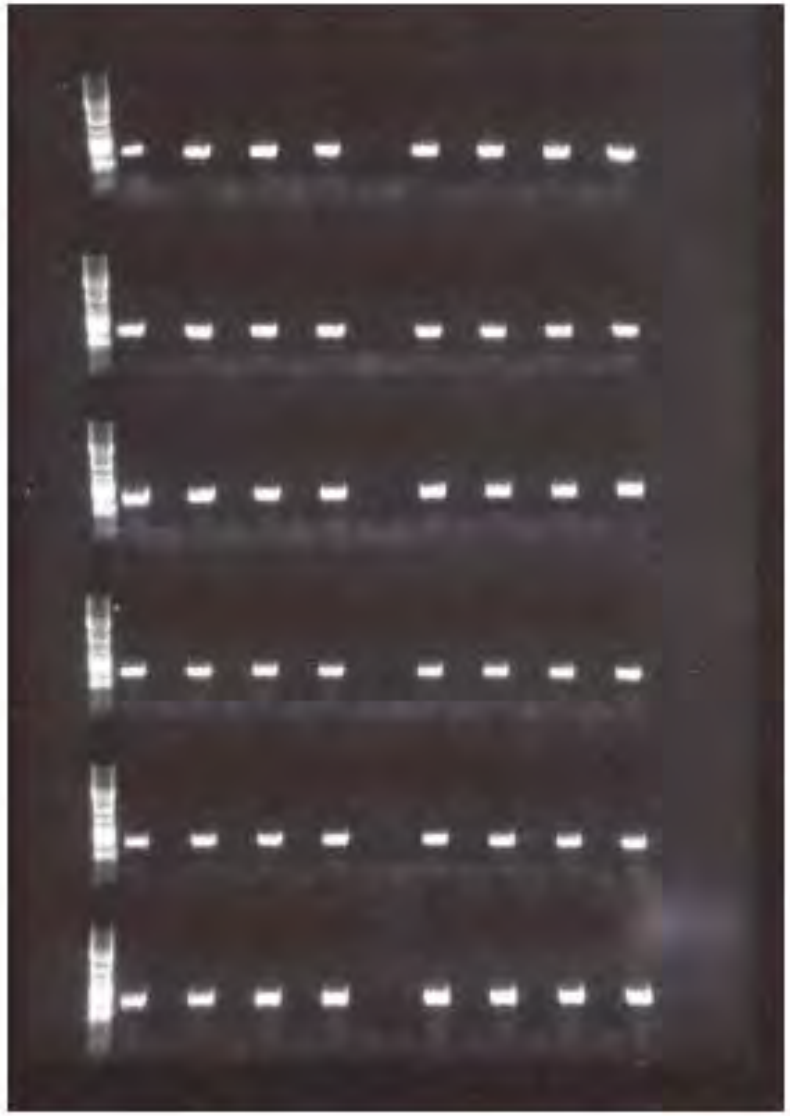
CD81 immunoprecipitation



CD3 cell depletion



No Cross Contamination During Purification

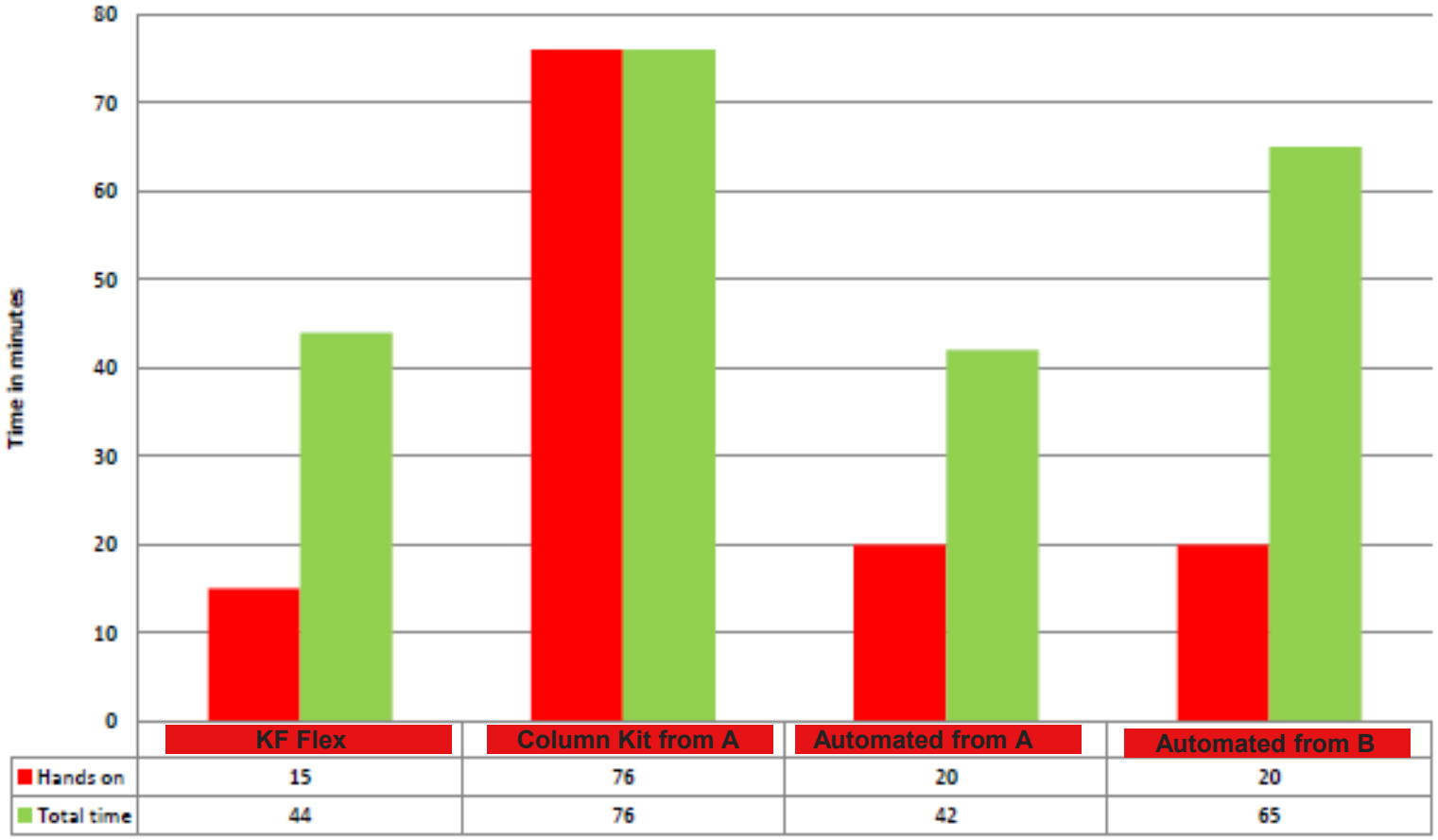


There is no cross contamination during KingFisher 96 processing steps

Procedure:

- Wells filled with blood or water
- gDNA purification
- PCR from all samples
- Run on agarose gel

Hands on and run time comparison



	KF Flex	Column Kit from A	Automated from A	Automated from B
Hands on	15	76	20	20
Total time	44	76	42	65

KingFisher Cell and Tissue DNA Kit with KingFisher Flex was compared with three competitors. The protocols were performed according to manufacturers' instructions. Conclusion: Hands-on time with KingFisher Kit was the shortest.

KingFisher Apex Instrument

Technical specifications

Processing volume by plate format	**96-well plate** • Deep well: 15–1,000 µL • KingFisher standard: 15–200 µL • PCR: 10–80 µL	**24-well plate** • Deep well: 30–5,000 µL	**Storage tube** • 96 tubes: 30–200 µL • 24 tubes: 200–1,000 µL
Customizable protocols	Yes, created with instrument user interface or BindIx Software, which requires Microsoft™ Windows™ 10 Software		
Heating and cooling ranges	Heating up to 100°C Cooling down to 4°C		
Ultraviolet light source	2 UV lamps, maximum 23 hr 59 min		
Dimensions (H x W x D)	45 x 78 x 59 cm (18 x 31 x 23 in.)		
Weight	56 kg (123 lb)		
Plate formats	96 deep-well plate, 96-well KingFisher standard plate, 96-well PCR plate (skirted and semiskirted), 24 deep-well plate, 96 storage tubes, 24 storage tubes		
Instrument memory	~30 GB		
Display interface	Graphical user interface with touchscreen		
Power	100–240 VAC, 50/60 Hz, 280 VA		
Barcode reading	On instrument; 1D barcodes		
Magnetic head options	4 options, customer interchangeable		
Data connectivity	USB device for PC, USB Wi-Fi or LAN adapter for network, RS-232 for automation, cloud-enabled		
Cat. No.	5400910 KingFisher Apex Purification System with 96 PCR Head 5400920 KingFisher Apex Purification System with 96 Combi Head 5400930 KingFisher Apex Purification System with 96 Deep-Well Head 5400940 KingFisher Apex Purification System with 24 Combi Head		

KingFisher Apex Instrument

KingFisher instrument:	Duo Prime	Flex	Apex	Presto
Instrument size	Compact benchtop	Benchtop	Benchtop	Benchtop—integrates with robotic liquid handler
Throughput level	Low to medium	Medium to high	Medium to high	Ultrahigh
Processing volume range	• 50–1,000 µL: 12-pin magnet head • 200–5,000 µL: 6-pin magnet head	• 20–100 µL: PCR plate, skirted • 20–200 µL: 96-well plate • 50–1,000 µL: 96 deep-well plate • 200–5,000 µL: 24 deep-well plate	• 15–1,000 µL: 96 deep-well plate • 15–200 µL: 96-well KingFisher standard plate • 10–80 µL: 96-well PCR plate • 30–5,000 µL: 24 deep-well plate • Storage tubes: 30–200 µL: 96 tubes 200–1,000 µL: 24 tubes	• 50–1,000 µL: 96 deep-well plate • 200–5,000 µL: 24 deep-well plate • 50–150 µL: 96-well KingFisher standard plate
Samples per run	6 or 12	24 or 96	24 or 96	24 or 96
Customizable protocols	Yes, with PC software	Yes, with PC software	Yes, with touchscreen or PC software	Yes, with PC software
Heating/cooling	• 10°C to 75°C (plate row block A) • 4°C to 75°C (elution strip block)	• From 5°C above ambient temperature up to 115°C	• From from 4°C above ambient temperature up to 100°C • Cooling down to 4°C	• From 5°C above ambient temperature up to 115°C
Ultraviolet lamp	8 W (up to 16 hr)	No	2 UV lamps, max. 23 hr 59 min	No

KingFisher Instrument Services and Support

Science can’t wait on instrument downtime

Built on more than 35 years of service expertise, our superior services and support solutions help keep your lab up and running. More than 1,400 trained professionals make up the industry’s largest network, ready to assist you when you need it.

Explore our instrument services at
thermofisher.com/instrumentservices

Warranty and AB Assurance service plan

Every KingFisher system comes with a one-year warranty. Extended coverage service plans are also available at the time of instrument purchase. We can also customize a service solution based on your lab’s needs.

Find out more at **thermofisher.com/kingfisher-services**

SmartStart training*

The KingFisher system includes SmartStart orientation to get you up and running quickly in your lab. The orientation includes basic familiarization and setup with online instrument management and includes on-site training.

Qualification services

Add an Installation or Operational Qualification (IQ/OQ) to your instrument purchase and receive timely, audit-ready qualification documentation to help ensure your instrument is installed and operating to manufacturer’s specifications.

See what our compliance services can do for you at
thermofisher.com/iqoqpq

KingFisher Apex Ordering Information

KingFisher instruments	Cat. No.
KingFisher Apex instrument with 96 PCR Head	5400910
KingFisher Apex instrument with 96 Combi Head	5400920
KingFisher Apex instrument with 96 Deep-Well Head	5400930
KingFisher Apex instrument with 24 Combi Head	5400940
Plastics for 96 deep-well format	
KingFisher Apex 96 Combi Tip Comb	97002570
KingFisher 96 deep-well plate, barcoded	95040450B
KingFisher 96 deep-well plate, sterile, barcoded	95040460B
KingFisher 96 tip comb for deep-well magnets, barcoded	97002534B
KingFisher 96 deep-well tip comb and plate, sterile, barcoded	97002820B
96 storage tube (Nunc)	374066

KingFisher plastics	Cat. No.
Plastics for 96 standard and PCR formats	
KingFisher Apex 96 PCR Tip Comb	97002560
KingFisher 96 microplate (200µL), barcoded	97002540B
PCR plate, skirted, clear	AB2396
PCR plate, semi-skirted, low profile	AB2496
KingFisher 96 tip comb for KingFisher magnets, barcoded	97002524B
Tip comb for PCR magnets	97002514
Plastics for 24 deep-well format	
KingFisher Apex 24 Combi Tip Comb	97002580
KingFisher 24 deep-well plate, barcoded	95040470B
KingFisher 24 deep-well plate, sterile, barcoded	95040480B
KingFisher 24 deep-well tip comb and plate, barcoded	97002610B
KingFisher 24 deep-well, tip comb and plate, sterile, barcoded	97002620B
24 storage tube (Nunc)	374323

KingFisher Apex Replacement Ordering Information

KingFisher accessories	Cat. No.
Replacement Magnet Heads	
KingFisher Apex 96 PCR Head	24079910
KingFisher Apex 96 Deep-Well Head	24079930
KingFisher Apex 96 Combi Head	24079920
KingFisher Apex 24 Combi Head	24079940
Replacement Heating Blocks	
KingFisher Apex PCR heating block	24075910
KingFisher Apex 96 KF heating block	24075920
KingFisher Apex 96 DW heating block	24075930
KingFisher Apex 24 DW heating block	24075940
KingFisher Apex 96 Storage tube heating block	24075950
KingFisher Apex 24 Storage tube heating block	24075960

KingFisher accessories	Cat. No.
Accessories	
KF Apex 24 storage tube adapter	N21445
KF Apex 96 PCR semi skirted plate adapter	N21446
KF Apex UV lamp	N21447

KingFisher Apex BindIx™ Software

BindIx software for your Desktop

- BindIx software allows you to create or edit protocols for KingFisher Apex directly from your PC
- All the functionality of the Apex instrument on your PC
- Conveniently create and modify protocols
- Easily transfer new or edited protocols to KingFisher Apex
- Access protocol library
- Available for PC laptops or desktop computers
- Compatible with KingFisher Apex instruments only

How to Install BindIx software*

1. **Download BindIx Here:**
   http://downloads.thermofisher.com/BindIx/FlexPlusUI_1.0.44.0_x86_ReleaseSideload.msixbundle
   - Double-click the downloaded package to start the install process

2. **Download BindIx from Microsoft Store** here:
   https://www.microsoft.com/store/apps/9NDGKHMNSTLJ
   - BindIx from Microsoft Store can be installed by selecting “Install” button on MS Store.

3. **USB stick (delivered from manufacturer)**
   - BindIx from USB stick can be installed by double-clicking .msix bundle file and after that selecting “Install” button

**Note: System requirements for BindIx software is Windows 10 version 1809 (17763.0) or higher*

WELCOME TO THE APEX OF PURIFICATION.

Thank you

ThermoFisher SCIENTIFIC

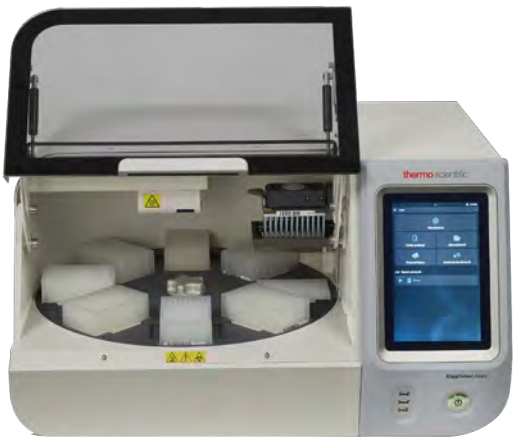
KingFisher Instruments

KingFisher Instruments	
Magnetic Beads	
Apex	
Magnetic heads/volumes	
KingFisher Consumables	

Service/Support /Software	
Miscellaneous	
Questions	
Contact Information	**Christian Kis**: Field Application Scientist Email: christian.kis@thermofisher.com Phone: (615) 681-7970 **Alex Durkin**: Sample Prep Technical Sales Specialist (Midwest) Email: alex.durkin@thermofisher.com Phone: (312) 340-9703 **Lennie Bane**: Genetic Analysis, Sr. Sales - DNA/RNA Sequencing, qPCR Solutions, dPCR Solutions Email: lennie.bane@thermofisher.com Phone: (760) 814-0003 Please reach out with any question. If you fall outside of the territory we cover, we will get you connected with your local rep.

KingFisher Apex

Link to KingFisher landing page
https://www.thermofisher.com/us/en/home/life-science/dna-rna-purification-analysis/automated-purification-extraction/kingfisher-systems.html



Agdia is seeking to collaborate with state certification agencies and researchers to develop and validate test kits for direct tuber testing. The goal of this effort is to provide an IC testing method and / or test kit that has a defined procedure, results in a high degree of accuracy, and can be offered as an IC kit to states or laboratories that wish to harmonize their methods. To accomplish this, we intend to conduct the following research:

1. Procure and investigate the most appropriate antiserum source for IC-PCR / IC-qPCR testing. Currently various sources are being used that may or may not have similar capture properties. We are hoping to compare all currently used sources, including some new polyclonal and monoclonal antibodies Agdia is producing. Our goal is to select the best performing antibody(s) and produce an optimized IC coating procedure and / or pre-coated plates.
2. Compare all existing PCR / qPCR methods being used for direct tuber testing applications using the antibody selected in objective 1. The best method will be selected for downstream validation purposes. Selection criteria will be based on overall analytical sensitivity, diagnostic specificity, and inclusivity of strains.
3. Validate the method or methods selected in collaboration with state certification agencies who are willing to participate. Agdia would need to receive tuber cores from the same tubers being sent to HI or FL for the WGO test. Agdia would test these cores using the optimized IC method and later compare the data to the WGO results.
4. Agdia will share all data produced from this research with collaborators as well as presented at the subsequent WERA-89 meeting, or other appropriate venues. Data shall be shared in a manner that provides anonymity to the sample providers, unless permitted otherwise.
5. Should the data demonstrate that the method provides a sufficient level of accuracy Agdia would offer the method as a service within our high-throughput laboratory as well as in the form of validated and quality-controlled IC test kit.

If you are presently conducting research on direct tuber testing, and are interested in collaborating, please email Keith Schuetz (kschuetz@agdia.com) or Tara Gauthier (tara.gauthier@agdia.com). We are seeking collaborators willing to provide samples, current testing methods, or unique antibodies.

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The simultaneous differentiation of *Potato virus Y* strains including the newly described strain PVY^{NTN-NW} by multiplex PCR assay

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A B S T R A C T

New recombinant strain and genotype of PVY, designated as PVY^{NTN-NW} and SYR-III, respectively, shared properties with PVY^{NTN} and PVY^{N}W has been reported recently. PVY^{NTN-NW} predominated in potato fields in Syria and was able to induce potato tuber necrotic ringspot disease (PTNRD). Due to the rapid spread of the recombinant strains of PVY which might be the case of PVY^{NTN-NW}, a specific and reliable detection method is an essential step to control this strain and minimize its spread. The shared properties of PVY^{NTN-NW} and SYR-III with PVY^{NTN} and PVY^{N}W, however, complicate their identification involving multiple detection methods. Therefore, a multiplex polymerase chain reaction (PCR), that relies on a combination of previously published and newly designed primers was developed for the detection and identification of PVY^{NTN-NW} and SYR-III in single or mixed infections with the main PVY strains, PVY^{O}, PVY^{N}, PVY^{NTN} and PVY^{N}W. In addition, the present PCR assay was able to detect the recombination points in the P1 region enabling the differentiation of the variable genotypes of the recombinant strains PVY^{NTN-NW}, PVY^{NTN} and PVY^{N}W. The reliability of this PCR assay was confirmed using a significant number of well characterized PVY isolates collected from Syria and Japan including those of PVY^{NTN-NW}, SYR-III, PVY^{O}, NA-PVY^{N}, PVY^{N}W and PVY^{NTN}.

1. Introduction

Potato virus Y (PVY) is the most common and destructive virus found in most potato production area (Valkonen, 2007). It is the type species of the genus *Potyvirus*, family *Potyviridae* with a single-stranded positive-sense genomic RNA of approximately 9.7 kb (Berger et al., 2005). According to the reaction of potato cultivars carrying different resistance genes and *Nicotiana tabacum*, potato isolates of PVY are classified into five strain groups PVY^{O}, PVY^{C}, PVY^{N}, PVY^{Z} and PVY^{E} (Singh et al., 2008). According to their sequences, isolates of PVY^{N} strain group fell into two genetic subgroups which are the European (PVY^{N}) and North American (NA-PVY^{N}) (Ogawa et al., 2008). Among these strain groups and subgroups, PVY^{O} and PVY^{N} are the most frequent strains in potato whereas PVY^{C} is not common in potato fields and has less economic importance (Blanco-Urgoiti et al., 1998a; Kerlan et al., 1999). Isolate of PVY^{Z} and PVY^{E} have been reported only once in the original reports and have not been noted elsewhere (Jones, 1990; Blanco-Urgoiti et al., 1998b; Kerlan et al., 1999; Singh et al., 2008) which decrease their significance with regard to potato production.

Genomic recombination plays a significant role in PVY evolution and has led to the emergence of new genotypes/strains including the recombinant PVY^{NTN}, PVY^{N}W and PVY^{NTN-NW} (Glais et al., 2002; Chikh Ali et al., 2007, 2010). PVY^{NTN} and PVY^{N}W were first found in Hungary and Poland, respectively but within a comparatively short time frame they have become common in potato fields in most potato production area (Kerlan et al., 1999; Piche et al., 2004; Glais et al., 2005; Lorenzen et al., 2006; Crosslin et al., 2006; Schubert et al., 2007; Chikh Ali et al., 2007). Many factors might help these new strains to increase their fitness. PVY^{N}W is more infectious in potato, able to infect a larger potato cultivar range and to cause milder symptoms than PVY^{N} or even symptomless infection (Chrzanowska, 1991; Glais et al., 2005) allowing such isolates to escape from the field inspection. In the same way the recombinant PVY^{NTN}, the causal of potato tuber necrotic ringspot disease (PTNRD) was reported to be able to overcome the field resistance of potato cultivars (Van den Heuvel et al., 1994). In addition, the lack of sufficient identification methods is another important factor helped these strains to build up their population.

PVY^{N}W has two main recombination structures, “A” with a recombination point at the HC-Pro/P3 and “B” with an additional

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Detection of *Potato virus Y* in industrial quantities of seed potatoes by TaqMan Real Time PCR

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Abstract Potato (*Solanum tuberosum*) is the largest crop in Israel. Production is based on the import of seed tubers from Europe for the spring crop. Imported tubers are generally free from virus infection. The most important virus infecting potato is *Potato virus Y* (PVY), which may cause severe damage to marketable yields. In Israel, tubers from the spring harvest are stored over the summer for planting in the autumn. It is important to be able to determine the infection rate of seed tuber lots from the spring harvest prior to storage. Commonly, infection is measured by sprouting tubers and measuring virus titre in the leaves using ELISA (the "Growing-On test"), which takes at least 6 weeks to give results. There is a need for a faster method to produce results, such as Taqman Real Time PCR (qPCR), for direct analysis of viral infection in tubers at harvest. To use qPCR as a diagnostic tool, it is necessary to demonstrate that both techniques give comparable results on batches of field-grown tubers. Such a comparison was performed on potential seed tuber lots of 14 different cultivars over three Israeli spring harvests (2013–2015). The agreement between the results of the two techniques was not of high statistical significance. However, the qPCR technique can distinguish well, by binary classification, between tuber lots with a low PVY infection rate (<5% by Growing On test; suitable for seed) and those unsuitable for seed (≥5% by Growing On test). Therefore, qPCR is an appropriate technique for determination of the PVY infection rate of seed tuber lots in Israel.

Keywords *Potato virus Y* · PVY · Seed potatoes · Real Time PCR · Growing-On test · Virus detection

Liat Avrahami-Moyal and Yehudit Tam equally contributed to this work.

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Introduction

Potato (*Solanum tuberosum* L.), the world's fourth largest food crop (Karasev and Gray 2013), is vegetatively propagated and is therefore disposed to accumulate devastating virus diseases. *Potato virus Y* (PVY) is currently the most important potato viral pathogen

Chapter 19

Direct Detection of Plant Viruses in Potato Tubers Using Real-Time PCR

Neil Boonham, Lynn Laurenson, Rebecca Weekes and Rick Mumford

Summary

Virus indexing of seed potatoes can be carried out by growing eye plugs to produce small plants and then testing them by ELISA, but this method is time consuming. Direct testing of the eye plugs by ELISA is not reliable, and so a method has been developed for the routine testing of seed potatoes for virus by PCR.

Key words: Virus, ELISA, RT, PCR, Potatoes.

19.1. Introduction

The virus indexing of seed potatoes is one of the most widespread testing procedures performed by virologists. In general, a common approach is used, based on taking eye cores from dormant tubers and growing these on in a greenhouse for several weeks, before testing the sprouts produced from these cores by ELISA. By testing at least 100 tubers, individually or in small batches, the test can be used to estimate the percentage of virus-infected tubers found in a particular seed stock, hence indicating its suitability for planting or the grade at which it should be classified. Over the last 30 years or more, this method has become almost universally adopted because of the advantages it offers: not only can it be used for most common potato viruses, it is also robust, simple to perform, and is well suited to high-throughput testing. As a result, testing laboratories can routinely test hundreds or thousands of seed stocks in a season.

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Virology

Distribution of *Potato virus Y* in Potato Plant Organs, Tissues, and Cells

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ABSTRACT

Kogovšek, P., Kladnik, A., Mlakar, J., Tušek Žnidarič, M., Dermastia, M., Ravnikar, M., and Pompe-Novak, M. 2011. Distribution of *Potato virus Y* in potato plant organs, tissues, and cells. Phytopathology 101:1292-1300.

The distribution of *Potato virus Y* (PVY) in the systemically infected potato (*Solanum tuberosum*) plants of the highly susceptible cultivar Igor was investigated. Virus presence and accumulation was analyzed in different plant organs and tissues using real-time polymerase chain reaction and transmission electron microscopy (TEM) negative staining methods. To get a complete insight into the location of viral RNA within the tissue, in situ hybridization was developed and optimized for the detection of PVY RNA at the cellular level. PVY was shown to accumulate in all studied leaf and stem tissues, in shoot tips, roots, and tubers; however, the level of virus accumulation was specific for each organ or tissue. The highest amounts of viral RNA and viral particles were found in symptomatic leaves and stem. By observing cell ultrastructure with TEM, viral cytoplasmic inclusion bodies were localized in close vicinity to the epidermis and in trichomes. Our results show that viral RNA, viral particles, and cytoplasmic inclusion bodies colocalize within the same type of cells or in close vicinity.

The initial entry of viruses into plant cells occurs via physical penetration of the cell wall, for example by insects that feed on the plant or following mechanical wounding of leaf trichomes or epidermal cells (44). From the entry site the virus spreads by cell-to-cell movement and then systemically through the phloem which is connected with photo assimilate transport in a source-to-sink manner (37). At a later stage, when leaves become older and mature, the import of the virus and the photo assimilates stops and in the process of senescence the photo assimilates and other components are transported from old leaves to actively growing ones. In parallel with virus movement, symptom development was also shown to be dependent on the developmental stage of the leaf at the time of infection (29,30). In addition, viruses can spread through the xylem, unrelated to photo assimilate transport (12,22,39). During the infection process, the virus accumulates to high levels in different organs and tissues. Identification of the sites of virus accumulation would contribute to better understanding of the virus pathogenicity.

There is only limited information on the distribution of potyviruses within the whole systemically infected plant. In systemically infected apricot, *Plum pox virus* (PPV) was localized within the stem pith and cortex and in close vicinity to leaf veinal tissue, where an accumulation of viral RNA and viral particles was detected (22). Accumulations of various *Potato virus A* (PVA) isolates were analyzed in plants of wild potato and it was shown that different virus isolates accumulate at different levels in roots and systemic leaves (37). When analyzing localization of PVA proteins within the infected leaves, coat protein was immunolocalized in the vascular parenchyma cells but not in the companion cells (37). Additionally, PVA cytoplasmic inclusion protein, helper component-proteinase, proteinase, genome-linked protein, and viral RNA were found to colocalize in parenchyma and mesophyll cells (38). The spread of *Tobacco etch virus* construct that encodes β-glucuronidase (TEV-GUS) was followed in potato leaf and trichoma cells (20).

Potato virus Y (PVY), a member of the family *Potyviridae*, is a filamentous virus possessing single-stranded (+)RNA (16,24). It is one of the most common viruses that infect a wide range of plant species, especially from the family *Solanaceae*, and is naturally transmitted by aphids in the nonpersistent manner (41). It can also be mechanically transmitted (24). PVY-infected plants express diverse symptoms, depending on the cultivar and virus isolate (5,9,47), and symptoms also greatly differ according to primary or secondary infection. The PVY^{NTN} isolates present one of the biggest problems for potato production out of all PVY isolates, as they have been shown to cause potato tuber necrosis ring spot disease (PTNRD) (6), apart from some other PVY isolates, e.g., $PVY^{N\text{-}Wi}$ (35,45). PTNRD was first reported in Hungary in the 1980s (6) and by the end of the century, PTNRD was reported globally (7,8,14,17,19,23,28,31,34,42,49). Besides necrosis on tubers, naturally or artificially infected potato plants of highly sensitive cultivars normally develop chlorotic and necrotic ringspots on leaves, which appear a few days following mechanical inoculation. On systemically infected leaves, wrinkles and chlorosis, leading to senescent phenotype, develop. However, plants of various cultivars, being highly susceptible, moderately susceptible, or tolerant, express different symptoms after infection with PVY^{NTN} (32) where the severity of symptoms was shown not to correlate with the virus titer in infected potato plants following mechanical inoculation. Nevertheless, 3 weeks after inoculation the amount of virus was shown to be similar in the majority of potato plant organs of differently sensitive susceptible cultivars (32).

The accumulation and spread of PVY through the plants in potato cultivars with different sensitivity to infection was previously analyzed at the level of organs using the enzyme-linked immunosorbent assay (ELISA) method (32,43). In the potato and tobacco stem tissues, PVY^{O} coat protein (CP) was localized by a tissue-print in the epidermal and phloem tissue, and in tobacco

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Direct tuber testing for *Potato Y potyvirus* by real-time RT-PCR and ELISA: reliable options for post-harvest testing?*

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The method currently used for testing potato tubers for viruses following harvest involves a growing-on test. This takes up to 6 weeks to complete, and there is therefore a demand for more rapid test results. The sensitivity and reliability of direct tuber testing by DAS-ELISA and real-time RT-PCR (TaqMan) were compared with the growing-on test. In addition, the reliability of all three methods for the detection of *Potato Y potyvirus* (PVY) in tubers was compared over post-harvest intervals of 6, 10, 14 and 18 weeks. The test material came from plots of tubers (cv. 'Maris Piper') containing a primary infection of strains PVY^N and PVY^O, following aphid transmission from marked infector plants grown during the 2003 season. Sample material was homogenized and divided, to provide comparative test material for detection of PVY by ELISA and real-time RT-PCR. Tuber eye-plugs were then taken and subjected to the growing-on test. The remainder of the tuber was also grown on and tested, to ensure infection was not missed as a consequence of an uneven distribution of virus throughout the tuber material. The results obtained using the two methods for direct testing of the tubers, and those results obtained from the traditional growing-on test, are compared. The advantages and disadvantages of each method are discussed.

Introduction

An accurate assessment of virus transmission during the growing season is essential in the prediction of virus levels in subsequent growing potato crops. This has important consequences for both plant health and consumer satisfaction. The detection of primary infection by the *Potyvirus Potato virus Y* (PVY) in foliar material has been found to be unreliable (Kurppa & Tiilikkala, 1987) resulting in the use of tuber testing to predict virus levels in subsequent crops in seed certification schemes. A post-harvest growing-on test has accordingly been widely adopted to give a reliable measure of the virus content of potato stocks. This involves treating rose-end eye-plugs with gibberellic acid and growing in a glasshouse for 5–6 weeks. The resulting plantlets are tested for virus using ELISA. However, the time taken for this test method means that seed producers can risk missing markets for their stocks and there is therefore a demand for a reliable and more rapid test that can be carried out directly on tubers. PCR methods have been shown to be highly sensitive (Boonham *et al*., 2000) and have broad specificity for field isolates of PVY (Singh & Singh, 1996). Real-time RT-PCR also has the advantage of being used for multiplex assays, i.e. the detection and diagnosis of multiple viruses in a single closed tube assay (Boonham *et al*., 2000; Klerks *et al*., 2001). The main advantage of ELISA has been the ease with which the system can deal with a high throughput of samples, but when applied to tubers, there have been mixed results (Gugerli, 1979; Hill & Jackson, 1984; Jones & Barker, 1998; Browning *et al*., 2004).

It has been noted that PVY detection by ELISA, in both primarily and secondarily infected dormant tubers is more reliable soon after lifting than following storage (Gugerli & Gehriger, 1980; de Bokx, 1981; Vetten *et al*., 1983). The reliability of detection over time may vary between cultivars (de Bokx & Cuperus, 1987). In contrast, the increased sensitivity of real-time RT-PCR that has been demonstrated for other potato viruses (Boonham *et al*., 2000) could be of use in detecting low levels of PVY resulting from inconsistent distribution of virus within tubers. Previous attempts to improve titre and virus distribution have involved breaking tuber dormancy, either by exposure to oxygen and carbon dioxide (Reust & Gugerli, 1984) or by treating tubers with Rindite[1] (e.g. Denny, 1945). Rindite breaks dormancy and results in an increased concentration and more even distribution of virus throughout the tuber (Gugerli & Gehriger, 1980; Vetten *et al*., 1983). However, tubers require 2–6 weeks storage after treatment, providing little advantage in time taken compared with the growing-on test. Most samples that are drawn for testing as a consequence of contraventions of the Scottish Seed Potato Classification Scheme (SSPCS) arrive soon after harvest. However, it is not uncommon to receive tuber samples from seed producers shortly before the following growing season. As part of an ongoing programme to improve service delivery for post-harvest testing at the Scottish Agricultural Science Agency (SASA), direct tuber

*Paper presented at the EPPO Conference on Quality of Diagnosis and New Diagnostic Methods for Plant Pests (Noordwijkerhout, NL, 2004-04-19/22).

[1]A preparation composed of a mixture of 2-chloroethanol, 1,2-dichloroethane and tetrachloromethane.

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Single-step RT real-time PCR for sensitive detection and discrimination of Potato virus Y isolates

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Abstract

Potato virus Y (PVY) has a worldwide distribution and infects several economically important crops from the *Solanaceae* family. The emergence and spread of the PVY^{NTN} strain, which is the causative agent of potato tuber necrotic ringspot disease (PTNRD), has lead to large economic losses and highlighted the need for accurate discrimination of the different PVY strains. Detection and differentiation of PVY isolates is mainly based on a combination of ELISA, RT-PCR and bioassays; however, PVY^{NTN} isolates are particularly difficult to differentiate from standard PVY^{N} without the use of time-consuming bioassays.

A strong correlation has been identified previously between the ability to induce PTNRD and the presence of a recombination point in the virus coat protein. An RT real-time PCR assay has been developed to enable detection of isolates with the recombination point, therefore, enabling rapid differentiation between potentially tuber necrotic PVY^{NTN} isolates and standard PVY^{N} isolates. The assay is also able to detect the presence of PVY^{O} isolates. To aid with routine testing, immuno-capture and post-ELISA virus release were introduced; when coupled with RT real-time PCR the sensitivity of the assays were up to seven orders of magnitude higher than ELISA. The assay was shown to be a suitable method for rapid large-scale diagnostic testing of PVY in different types of plant material including tubers, and specific screening for potentially tuber necrotic recombinant isolates.

Keywords: Potato virus Y; PTNRD; RT real-time PCR; Specificity; Sensitivity; Recombination

1. Introduction

Potato virus Y (PVY) is one of the most important viruses infecting potato and has a worldwide distribution. There are three commonly described PVY strains: the ordinary or common strain (PVY^{O}), the stipple streak strain (PVY^{C}) and the veinal necrosis strain (PVY^{N}). PVY^{O} and PVY^{C} are described on the basis of hypersensitive reactions in cultivars of *Solanum tuberosum* bearing resistance genes N_{tbr} and *Nc*, respectively, (DeBokx and Huttinga, 1981; Jones, 1990). PVY^{N} does not elicit a hypersensitive resistance response in *S. tuberosum,* but causes veinal necrosis in *Nicotiana tabacum*, which is distinct from the mild mosaic and rugosity induced by isolates of PVY^{O} and PVY^{C} (DeBokx and Huttinga, 1981). These three main strains are commonly differentiated through bioassays for the biological characteristics described above in combination with serological testing.

Isolates classified serologically as PVY^{O} but those which are able to overcome N_{tbr} and *Nc* genes have been found and the name PVY^{Z} was proposed (Jones, 1990). Members of the PVY^{N}–Wilga ($PVY^{N–Wi}$) group have the biological properties of PVY^{N} isolates but are serologically classified as PVY^{O}; they have been shown to have a recombinant genome consisting of both PVY^{N} and PVY^{O} sequences (Glais et al., 2002). Isolates of the $PVY^{N–Wi}$ group are thought to be more infectious than PVY^{O} and cause less severe symptoms in potato than standard PVY^{N} isolates (Blanco-Urgoiti et al., 1998). Isolates from this group (also referred to as $PVY^{N:O}$) have been described after

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Special Report

The MIQE Guidelines: *M*inimum *I*nformation for Publication of *Q*uantitative Real-Time PCR *E*xperiments

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BACKGROUND: Currently, a lack of consensus exists on how best to perform and interpret quantitative real-time PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

CONTENT: The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher. By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement.

SUMMARY: Following these guidelines will encourage better experimental practice, allowing more reliable and unequivocal interpretation of qPCR results.

The fluorescence-based quantitative real-time PCR (qPCR)[15] *(1–3)*, with its capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources, is the enabling technology par excellence of molecular diagnostics, life sciences, agriculture, and medicine *(4, 5)*. Its conceptual and practical simplicity, together with its combination of speed, sensitivity, and specificity in a homogeneous assay, have made it the touchstone for nucleic acid quantification. In addition to its use as a research tool, many diagnostic applications have been developed, including microbial quantification, gene dosage determination, identification of transgenes in genetically modified foods, risk assessment of cancer recurrence, and applications for forensic use *(6–11)*.

This popularity is reflected in the prodigious number of publications reporting qPCR data, which invariably use diverse reagents, protocols, analysis methods, and reporting formats. This remarkable lack of consensus on how best to perform qPCR experiments has the adverse consequence of perpetuating a string of serious shortcomings that encumber its status as an independent yardstick *(12)*. Technical deficiencies that affect assay performance include the following: (*a*) inadequate sample storage, preparation, and nucleic acid quality, yielding highly variable results; (*b*) poor choice of reverse-transcription primers and primers and probes for the PCR, leading to inefficient and less-than-robust assay performance;

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[15] Nonstandard abbreviations: qPCR, quantitative real-time PCR; MIQE, Minimum Information for Publication of Quantitative Real-Time PCR Experiments; RT-qPCR, reverse transcription–qPCR; FRET, fluorescence resonance energy transfer; C_q, quantification cycle, previously known as the threshold cycle (C_t), crossing point (C_p), or take-off point (TOP); RDML, Real-Time PCR Data Markup Language; LOD, limit of detection; NTC, no-template control.

A Multiplex PCR Assay to Characterize *Potato virus Y* Isolates and Identify Strain Mixtures

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ABSTRACT

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Potato virus Y (PVY) has become a serious problem for the seed potato industry, with increased incidence and rejection of seed lots submitted for certification. New PVY strains and strain variants have emerged in recent decades in Europe and North America, including the PVY^N strain that causes veinal necrosis in tobacco, and strain variants that represent one or three recombination events between the common strain (PVY^O) and PVY^N. Several reverse transcription–polymerase chain reaction (RT-PCR) assays have been described that characterize PVY isolates as to strain type, but they are limited in their ability to detect some combinations of mixed strain infections. We report here the development of a single multiplex RT-PCR assay that can assign PVY strain type and detect mixed infections with respect to the major strain types. Validation of this assay was achieved using 119 archived PVY isolates, which had been previously characterized by serology and bioassay and/or previously published RT-PCR assays. Results for single-strain isolates were comparable to previous results in most cases. Interestingly, 16 mixed infections were distinguished that had previously gone undetected. The new multiplex RT-PCR assay will be useful for researchers and seed production specialists interested in determining PVY infection type using a single assay.

Potato virus Y (PVY) is the type member of the *Potyvirus* family and infects several important crops of the Solanaceae family, including potato, tomato, pepper, and tobacco (4,15). There are several widely recognized strains of PVY, including the common strain (PVY^O), the tobacco necrosis strain (PVY^N), and PVY^C causing stipple streak (4). The common strain of PVY that produces foliar mosaic symptoms has been the predominant strain in PVY-infected seed potatoes in North America (5). In recent years, other PVY strain variants have been reported from within North America (10,13). The PVY^N strain causes veinal necrosis on tobacco leaves (4). The origins of PVY^N are difficult to pinpoint, although early reports suggest it was first detected in South America more than 70 years ago (16,17). More recently, variants and recombinants of PVY have been detected. Tuber necrosis strains of PVY (PVY^{NTN}) were first detected in Europe (Eu-PVY^{NTN}) and are characterized by a PVY^N serotype and by having three recombination junctions (6,8). Tuber necrosis strains in North America are also characterized by a PVY^N serotype but have either no or a single recombination junction (10,13). Other PVY recombinants such as $PVY^{N:O}$ also have a single recombinant junction but are unique in that they have a PVY^O serotype (2,11). Some of the latter types of $PVY^{N:O}$ can cause an atypical tuber necrosis (13). We have recently completed the confirmation of these recombination events by whole-genome sequencing of the PVY^N and PVY^{NTN} strains in North America (9). Based on the sequence analysis of the PVY genomes and on symptomatology (13), it is evident that PVY^N and PVY^{NTN} isolates from Europe are different than those recovered from North America.

Our research programs have worked with three widely accepted reverse transcription–polymerase chain reaction (RT-PCR) assays used to identify PVY strain types (3,11–13). A multiplex RT-PCR assay developed by Nie and Singh (11) queries the P1 cistron and differentiates PVY into two main groups: those that produce leaf necrosis on tobacco indicator plants (European PVY^N, European PVY^{NTN}, $PVY^{N:O}$ (or PVY^N-Wi), North American PVY^N and North American PVY^{NTN}) and those that do not produce leaf necrosis on tobacco (PVY^O) (11,12). For simplicity, North American PVY tobacco necrotic isolates will be designated as NA-PVY^N and NA-PVY^{NTN}, while European PVY^N or European PVY^{NTN} will be referred to as PVY^N and PVY^{NTN}, respectively. The Nie and Singh (11) assay can detect mixtures of PVY^O plus PVY^N (including PVY^{NTN} and $PVY^{N:O}$). A second multiplex assay, developed by the same research group (12), was used in conjunction with the P1 assay to identify isolates capable of producing necrotic lesions on tobacco leaves, and it also identifies recombinants of the PVY^{NTN} and $PVY^{N:O}$ types based on amplification of three amplicons or one amplicon, around the respective recombination junctions. A third assay is based on *Hin*cII restriction digests of P1 amplicons and can differentiate PVY^O from the necrotic strains of PVY^N, PVY^{NTN}, $PVY^{N:O}$, PVY^{NA-N}, and PVY^{NA-NTN} (3). This assay will also detect strain mixtures of PVY^O and PVY^N, PVY^{NTN}, NA-PVY^N, or NA-PVY^{NTN}. The above assays are limited in providing unambiguous identification of strain mixtures from within the necrotic groups of PVY^N, PVY^{NTN}, $PVY^{N:O}$, NA-PVY^N, or NA-PVY^{NTN}. Two other assays have been described to detect mixtures of PVY^O and PVY^N serotypes using specific fluor-labeled primers to the coat protein region (18), or separate PCR assays for PVY^O, PVY^N, or PVY^C (1).

A recent report indicated the presence of new variants of some of the above strains (13). These "strain variants" included individual isolates that reacted with monoclonal antibodies specific for both PVY^N and PVY^O. Although the authors used available RT-PCR tests to characterize strain types and identify strain mixtures (11,12), reaction with both antibody types indicated either a changed epitope profile that would react to both specific antibodies or a mixture of strains that was not detectable using the current nucleic acid-based assays. During the course of characterizing these "novel" isolates before sequencing to try to predict the amino acid change(s) responsible for the epitope change, it became apparent that two of these isolates were mixed infections of PVY^{NTN} and

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Large-Scale RT-qPCR Diagnostics for Seed Potato Certification

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Abstract

Every year, Agroscope examines nearly 300,000 tubers for the presence of viruses, as regulated for the certification of seed potatoes intended for Swiss growers. Since 2016, this examination has been performed via RT-qPCR on dormant tubers directly after harvest. This method offers fast results and eliminates the need for the use of Rindite, which is a toxic and polluting gaseous compound previously used in Switzerland to break the dormancy of seed tubers. The implementation of this molecular analytical method for the routine diagnosis of regulated viruses makes it possible to conduct additional analyses via Illumina sequencing to assess the conformity of the primers and probes used with the sequences of the different viral isolates. This form of quality control in routine diagnosis is a source of information that can answer more fundamental scientific questions related to the epidemiology of viral strains related to certification. The datasets produced in this framework can also be used to explore the diversity of rare or unknown virus species in potato crops.

Keywords Certification · Deep sequencing · Molecular diagnostics · Quality control · Real-time RT-PCR · Viral disease

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Optimization of a Real-Time RT-PCR Assay and its Comparison with ELISA, Conventional RT-PCR and the Grow-out Test for Large Scale Diagnosis of *Potato virus Y* in Dormant Potato Tubers

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Abstract A TaqMan real-time reverse transcription-PCR (real-time RT-PCR) procedure was developed, optimized, and compared with other routine methods to detect *Potato virus Y* (PVY) in dormant potato tubers. Three pairs of primers and probes were designed and evaluated for their suitability to facilitate the real-time RT-PCR detection of PVY for all strain groups including PVY^{O}, PVY^{N}, $PVY^{N:O}$ (= $PVY^{N\text{-}Wi}$) and PVY^{NTN}. Among the primer and probe combinations tested, the combination PVY-1 produced the lowest threshold cycle (Ct) value of 25.75. The procedure was further optimized by adjusting various parameters including primer/probe concentration, reaction volume, amplification cycles, and master mixes from different sources. The real-time RT-PCR was then employed to detect PVY from dormant tubers of different cultivars and potato fields, and the results were compared with those obtained from conventional RT-PCR, enzyme-linked immunosorbent assay (ELISA) on sprouts and grow-out testing. Out of 1,069 single-virus infected (PVY only) tubers tested, both formats of RT-PCR detected PVY in 52 samples, ELISA on sprouts in 45, ELISA on leaves in 54 and visual observations in 53. However, in 61 multiple-virus infected tubers tested, both formats of RT-PCR, and ELISA on both sprouts and leaves detected a similar number of positives, thus, making all the methods equally sensitive. Considering that ELISA requires sprouting of dormant potato tubers for PVY testing, grow-out testing takes approximately 6–8 weeks to obtain results, and conventional RT-PCR needs post-PCR processing, real-time RT-PCR offers a speedy alternative for large scale detection of PVY from dormant tubers. The method is therefore recommended for testing of PVY in potato tubers on a commercial scale in a diagnostic laboratory.

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Resumen Se desarrolló y optimizó un procedimiento de transcripción reversa de tiempo real de TaqMan (RT-PCR de tiempo real), y se comparó con otros métodos rutinarios para detectar al virus Y de la papa (PVY) en tubérculos de papa en dormancia. Se designaron tres pares de iniciadores y sondas y se evaluaron para su conveniencia para facilitar la detección de PVY por RT-PCR de tiempo real para todos los grupos de variantes, incluyendo PVY^{O}, PVY^{N}, $PVY^{N:O}$ (=$PVY^{N\text{-}Wi}$) y PVY^{NTN}. Entre las combinaciones probadas de iniciadores y sondas, la combinación de PVY-1 produjo el valor del ciclo del umbral más bajo (Ct) con 25.75. El procedimiento se optimizó mas adelante mediante el ajuste de varios parámetros, incluyendo la concentración iniciador/sonda, volumen de reacción, ciclos de amplificación, y muestras maestras de diferentes fuentes. Se empleó posteriormente el RT-PCR de tiempo real para detectar PVY de

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Suggested guidelines for validation of real-time PCR assays in veterinary diagnostic laboratories

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Abstract. This consensus document presents the suggested guidelines developed by the Laboratory Technology Committee (LTC) of the American Association of Veterinary Laboratory Diagnosticians (AAVLD) for development, validation, and modification (methods comparability) of real-time PCR (rtPCR) assays. These suggested guidelines are presented with reference to the World Organisation for Animal Health (OIE) guidelines for validation of nucleic acid detection assays used in veterinary diagnostic laboratories. Additionally, our proposed practices are compared to the guidelines from the Foods Program Regulatory Subdivision of the U.S. Food and Drug Administration (FDA) and from the American Society for Veterinary Clinical Pathology (ASVCP). The LTC suggestions are closely aligned with those from the OIE and comply with version 2021-01 of the AAVLD Requirements for an Accredited Veterinary Medical Diagnostic Laboratory, although some LTC recommendations are more stringent and extend beyond the AAVLD requirements. LTC suggested guidelines are substantially different than the guidelines recently published by the U.S. FDA for validation and modification of regulated tests used for detection of pathogens in pet food and animal-derived products, such as dairy. Veterinary diagnostic laboratories that perform assays from the FDA Bacteriological Analytical Method (BAM) manual must be aware of the different standard.

Key words: accreditation; methods comparability; real-time PCR; validation; verification.

Introduction

Real-time PCR (rtPCR) assays have become the workhorse molecular assays in veterinary diagnostic laboratories (VDLs) since ~2000. PCR assays targeting commonly encountered veterinary pathogens are commercially available (standard test methods) but often laboratories choose to develop their own nonstandard test method (laboratory-developed tests, LDTs). Nonstandard rtPCR assays targeted for use in the laboratory may be completely novel, conversions of conventional PCR assays to a rtPCR format, or extensions of a standard test method outside its intended scope (e.g., new specimen matrix). Whatever the scenario, all nonstandard test methods must be validated for use prior to implementation in an accredited laboratory. In addition, a standard test method, or a previously validated nonstandard test method, may need to be modified in order to accommodate sequence changes within the pathogen, incorporation of a new specimen type or species, or to take advantage of new reagents or instrumentation. Regardless of whether the assay is new or modified, it must be demonstrated to be fit-for-purpose through a predefined process.

Consensus guidelines exist for laboratory assays utilized in human medicine and are regulated by the FDA, which oversees commercial assays[18,45] and has proposed guidelines for LDTs.[18] Validation pathways for human medicine exist

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Simultaneous detection of potato viruses, PLRV, PVA, PVX and PVY from dormant potato tubers by TaqMan® real-time RT-PCR

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Abstract

The requirements of sprouting dormant potato tubers for biological or serological assays or RNA extraction for nucleic acid and PCR assays add to the cost of virus screening. Recently, cheaper, reliable and more rapid methods for the screening of potato tuber-seed pieces for viruses have been developed that do not require sprouted tubers for indexing, including TaqMan® real-time RT-PCR. Although the assays are often designed for minimal time and reagent use, they still require a time-consuming and laborious RNA extraction step. This paper describes an assay where four common potato-infecting viruses, *Potato leafroll virus*, *Potato virus A*, *Potato virus X* and *Potato virus Y*, were detected simultaneously from total RNA and saps of dormant potato tubers in a quadruplex real-time RT-PCR. Factors critical for the detection of these viruses in saps of dormant potato tubers included: optimum dilution and inhibition of RNAses, and the optimization of the reverse transcription and PCR steps. Potato virus detection directly from tuber saps was comparable to that from purified total plant RNA, and this represents significant savings of time and expense. The TaqMan® system developed in this study detected between 200 and 400 copies of potato virus RNA.

Keywords: Multiple virus detection; Potato tubers; TaqMan®; Real-time RT-PCR; Multiplex detection

1. Introduction

In the field, potato (*Solanum tuberosum* L.) is infected frequently with several viruses during a growing season (McDonald, 1984), which leads to reduced yield and quality tubers. Among the most common viruses affecting potato crops are: *Potato leafroll virus* (PLRV, genus *Polerovirus*), *Potato virus X* (PVX, genus *Potexvirus*), *Potato virus A* and *Potato virus Y* (PVA, PVY, both genus *Potyviruses*), and *Potato virus S* (PVS, genus *Carlavirus*) (Singh, 1999). These viruses can occur in single or as mixed infections within the potato crop.

Planting seeds that are free or/and resistant to viruses is a way of controlling viral diseases. Reliable and sensitive indexing techniques are indispensable tools for determining the virus status of potato seeds. There are several systems extant for seed indexing and certification, but most utilize a combination of field-based inspections for visual symptoms and serological testing using enzyme-linked immunosorbent assay (ELISA) (Singh and Singh, 1996). However, these methods are time consuming, expensive and generally cannot be carried out on dormant tubers (Huttinga, 1996). Consequently, several reverse transcription polymerase chain reaction (RT-PCR) protocols have been developed for detection of individual viruses from dormant tuber-derived RNA (Singh and Singh, 1997, 1998).

Detection of several individual viruses separately by RT-PCR reactions is also expensive and time-consuming. Multiplex reverse transcriptase polymerase chain reaction, which accommodates several pairs of primers in one reaction, has been investigated as a means to reduce cost and increase efficiency (Singh and Nie, 2003). For example, duplex RT-PCR detection of PLRV and PVY has been reported (Singh et al., 2000), as has multiplex detection of PVY strains (Nie and Singh, 2002; Singh and Nie, 2003). Multiplex RT-PCR detection of five potato viruses (PVA, PVS, PVX, PVY and PLRV) has also been reported (Nie and Singh, 2000). Most recently, multiplex RT-PCR was used to differentiate strains of PVY (Lorenzen et

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