Recombinase Polymerase Amplification (RPA)-Cas12a assay for identification of the tomato leafminer, *Phthorimaea (Tuta) absoluta*

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The tomato leafminer [*Phthorimaea (Tuta) absoluta* (Meyrick) (Lepidoptera: Gelechiidae)], is actively spreading northward towards tomato production regions in the southern US. This insect is a major threat to domestic Solanaceae production, especially tomatoes, because the tomato leafminer can cause 80-100% crop loss (Biondi et al. 2018). This moth has rapidly invaded Europe, Asia, and Africa due to its high reproduction potential, dispersal capacity, and adaptability to newly invaded areas, causing severe physical and economic damage to crops. Large areas of the United States have elevated risk for the tomato leafminer to establish due to their climate and host plant availability, including the Pacific Coast, much of California and Arizona, the Gulf Coast, and the entire state of Florida (Santana et al. 2019). These areas account for more than two-thirds of fresh tomato production and more than 95% of processed tomato production (USDA-NASS 2023).

The South American tomato leafminer is thought to be native to Peru, and in the 1960's and 1980's, spread throughout South America (Vargas 1970, USDA 2019). In 2006, it was accidentally introduced into Spain through tomato transplants from Chile and spread throughout Europe in about 5 years, even with enforcement of stringent regulations to prevent its spread (Desneux et al. 2010, Guillemaud et al. 2015). The moth has continued to spread at a rate of nearly 500 miles (800 km) per year from the Middle East into Africa and Asia. Meanwhile, from Peru it has moved northward into Central America (Panama and Costa Rica), and into Haiti (Giorgini et al. 2019). It is threatening to invade the United States and Mexico.

Identifying the tomato leafminer using morphological methods requires dissection of the genitalia of male moths. Unfortunately, the life stages found in traps or in imported products may not allow identification based on morphology of the male genitalia. Because of this, sequencing of the genome of the tomato leafminer was completed to investigate if molecular methods could be used to identify any life stage of this insect. These sequencing efforts identified single nucleotide polymorphisms (SNPs) that could be used to distinguish the tomato leafminer from two closely related gelechiids, the tomato pinworm (*Keiferia lycopersicella* (Walshingham 1897)) and the potato tuberworm (*Phthorimaea operculella* (Zeller 1873); Tabuloc et al. 2019). Additionally, a real-time PCR assay for tomato leafminer and a multiplexed probe-based quantitative PCR assay were developed (Zink et al. 2020, Lewald et al. 2023). These methods work well but require a thermocycler for the polymerase chain reaction and specialized equipment to visualize the results. There is a need for a method that can provide accurate identification of any life stage of the tomato leafminer, but not require sophisticated equipment.

This manual outlines a molecular method of identifying tomato leafminer without the need for specialized equipment. The method is based on the CRISPR-Cas (Clustered Regularly-Interspaced Short Palindromic Repeats – CRISPR associated proteins) system. In this system, the Cas12a enzyme has indiscriminate single-stranded DNA nuclease activity when it binds to target DNA (Chen et al. 2018). If a single-stranded oligonucleotide probe that has a fluorophore and quencher when the Cas12a enzyme binds to the target DNA, the single-stranded oligonucleotide probe will also be cleaved, releasing the fluorophore and quencher. The fluo-

rescent signal which is proportional to the number of target DNA molecules, can be measured. The Cas12a enzyme reaction is isothermal and occurs between 15°C and 50°C. Coupling this reaction with Recombinase Polymerase Amplification (RPA), an isothermal DNA amplification method, the target DNA from the tomato leafminer can be detected and visualized using a UV illuminator or a green flashlight and cell phone camera fitted with a red transparent filter paper. This method reduces the need for specialized equipment for identifying any life stage of the tomato leafminer. The details of the development of this method can be found in Lewald et al. 2023.

The manual provides the details for one method of DNA extraction from the sample, but other methods of DNA extraction can be used. Once DNA is obtained, the methods of recombinase polymerase amplification (RPA) and the Cas12a assay are given. The flow chart in Figure 1 provides an overview of the process, from insect collection to visualizing the results.

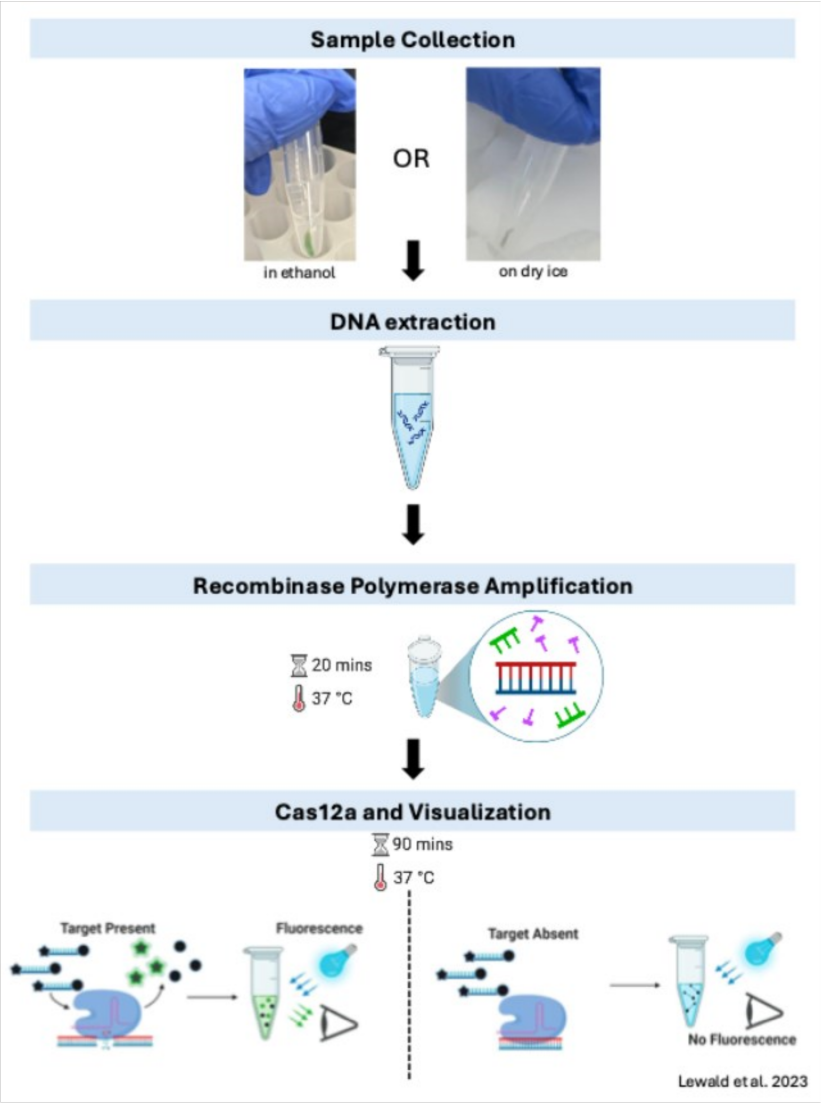

Figure 1. Flowchart of the RPA-Cas12a method

DNA extraction using CTAB
Adapted from Hickner et al. 2016 (*BMC* Genomics)

Composition of the CTAB solution and the purpose of each reagent

Solution	**Purpose**
100 mM Tris-HCl (pH8.0)	Maintain pH of final solution
10 mM EDTA	Prevents DNA degradation
1.4 M NaCl	Aids in DNA precipitation
2% CTAB	Promote cell lysis

Protocol:

For ethanol collected samples:

If the sample was collected in ethanol, the tissue needs to be rehydrated to preserve DNA quality. Remove the ethanol and add enough nuclease free water to submerge the sample. Incubate at room temperature for 20 minutes. Remove water from the sample.

For samples collected on dry ice:

Proceed directly to grinding the sample.

Grind sample on ice in 200µl of 2% CTAB solution with a motorized pestle, and then heat the sample at 65°C for 5 minutes.

Add 200 µl of chloroform and invert the sample 10 times to mix.
The chloroform separates the nucleic acids from the proteins

Centrifuge at 13,000 rpm for 15 minutes at 4°C.

Transfer the upper layer containing the DNA to a clean tube and add an equal volume of 100% isopropanol to the sample to precipitate the DNA in the sample.

Place the sample at -20°C overnight to complete the precipitation step.

The next day, centrifuge the sample at 13,000 rpm for 15 minutes at 4°C to pellet the DNA.

Discard the supernatant and add 200µl of 70% ethanol to the tube to wash the pellet.

Centrifuge the tube at 13,000 rpm for 5 minutes at 4°C.

Discard the ethanol, and with the cap open, lay the tube on its side on a Kimwipe to allow the pellet to air dry. The pellet is dry when it changes color from white or opaque to translucent.

Resuspend the pellet in 50µl of nuclease-free water.

The resuspended DNA can be stored at –20°C.

Recombinase Polymerase Amplification (RPA)
Using the TwistAmp Liquid Basic Kit
Adapted from Lewald et al. 2023

Materials:

TwistAmp™ Liquid Basic Kit (TwistDx; Ref: TALQBAS01)
10mM dNTP (ThermoScientific; Cat: R0191)
Forward Primer (5'- GCTTTAACTGTTTAATCTGTTCATCCATTG -3')

Protocol:

Assemble the reaction below. If doing more than one sample, create a master mix for all the samples.

Master Mix	**1 reaction**
2X Buffer	12.5µl
Nuclease-free Water	3.4µl
10mM dNTPs	1.2µl
10X E-mix	2.5µl
10uM Forward Primer	1.2µl
10uM Reverse Primer	1.2µl

Mix by flicking or inverting.

Add 3.75µl per reaction of 20X Core Reaction solution.

Invert to mix and aliquot 23.25µl of master mix to a separate tube for each sample.

Dilute DNA with nuclease-free water to a final concentration of 1ng/µl. Add 1µl of the diluted DNA to the master mix.

Add 1.25µl magnesium acetate to the lid of the tube. This is so all reactions can start at the same time.

Gently mix and spin down.

Incubate at 37°C for 20 minutes.

The RPA products can be stored at 4°C for up to 1 week. For longer term storage, the products should be stored at –20°C.

Cas12a Assay and Visualization
Adapted from Lewald et al. 2023

Materials:

2X Binding Buffer (made fresh)

2X Binding Buffer	**1ml**
1M Tris-HCl pH7.5	40µl
1M KCl	200µl
1M $MgCl_2$	10µl
50% Glycerol	200µl
1M DTT	2µl
Nuclease-free water	548µl

3µM Cas12a Enzyme
10µM crRNA (5'-UAAUUUCUACUAAGUGUAGAUUAUAGUUCACAGAGUCUUGA-3')
10µM reporter probe (CALRed610-ACGTTTTCAG[C]AACACTTT-BHQ2Plus)
Nuclease-free water
96-well plate and seal (compatible with the qPCR machine you will be using)
qPCR machine (alternatively, a 37°C heat block or water bath can be used)
302 nm UV transilluminator (alternatively, a green LED flashlight and red correction lighting gel filter sheet can be used)

Procedure:

Assemble the reaction below. If doing more than one sample, create a master mix for all the samples.

Master Mix	**1 reaction**
2X Buffer	10µl
Nuclease-free Water	5µl
Cas12a Enzyme	2µl
crRNA	0.6µl
Reporter probe	0.4µl

Mix by flicking or inverting.

Aliquot 18µl of master mix to a 96-well plate. Add 2µl of RPA product.

Seal the plate and spin down in a plate spinner.

Incubate at 37°C for 90 minutes with a heat block, water bath or qPCR machine. If using a qPCR machine, a fluorescence reading can be taken every minute.

View the reactions with a 302 nm UV transilluminator. Fluorescent samples are positive for *P. absoluta*. Figure 2 has examples of the amplification curves and fluorescence in tubes.

Alternatively, the results of the assay can be visualized using a green LED flashlight and red transparent filter paper. In a dark room, shine a green LED flashlight on the underside of the plate. Tape a piece of red correction lighting gel filter sheet to your camera lens and then take an image of the sample.

The Cas12a products can be stored at 4°C for up to 1 week. For longer term storage, the products should be stored at –20°C.

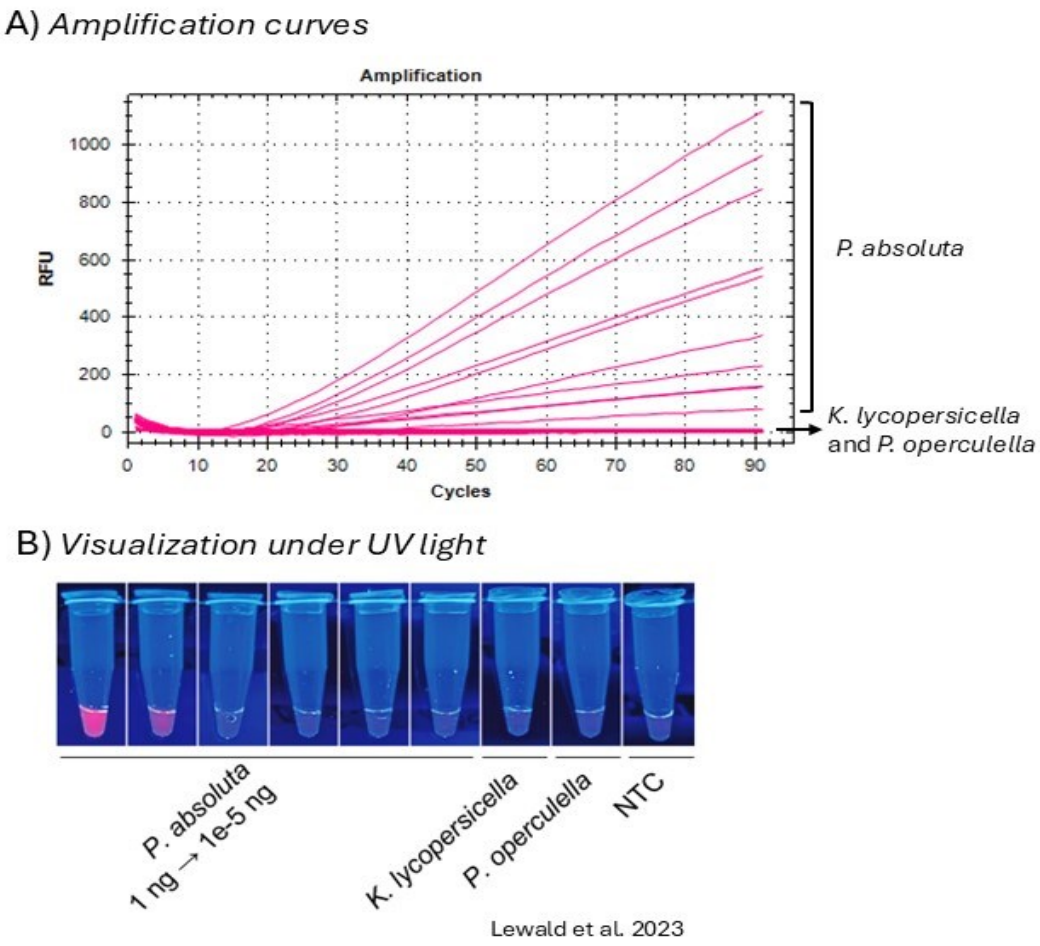

Figure 2: Example of assay results. A) Amplification curves from UV transilluminator; B) visualization using a green LED flash light with red correction lighting gel filter paper. From Lewald et al. 2023 .

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