Development of a new molecular diagnostics tool for *Agrobacterium tumefaciens*

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Introduction

The plant pathogen *Agrobacterium tumefaciens* is the cause of an economically harmful plant abnormality known as a root or crown gall (**Figure 1**). These galls are the result of abnormal growth that manifests itself on either the root or the stem and can be devastating for the ornamental plant industry. Introduction of *A. tumefaciens* to a nursery can be highly destructive and thus early detection of the pathogen is crucial in order to prevent widespread disease throughout a nursery.

Genes necessary for virulence are found on a Ti plasmid (**Figure 1**). These include the *vir* regulon, auxin and cytokinin genes necessary to provoke gall formation, and opine synthesis genes. The gene, *virD2*, is required for virulence and is found in all pathogenic strains, making it a good candidate for a diagnostic assay.

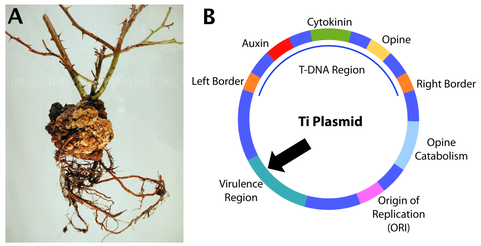


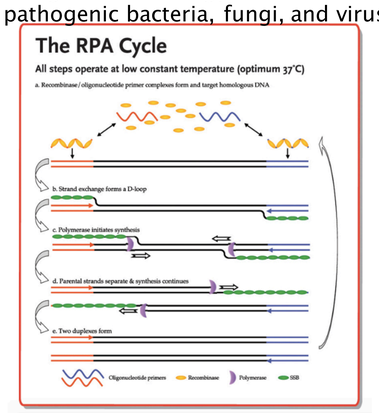
Figure 1. A. Crown gall symptoms caused by *A. tumefaciens*. Image Source: OSU Plant Clinic. **B. Schematic of the Ti plasmid.** Arrow indicates location of the *virD2* gene.

Project Objectives

- Identify and validate gene targets for TwistPCR assays that are specific to multiple strains of phytopathogenic *A. tumefaciens*.
- Optimize the TwistPCR assays for specificity and selectivity.
- Extend assay to work with TwistAmp nfo and lateral flow analysis.
- Provide growers with a fast, inexpensive, and accurate method of detecting phytopathogenic *A. tumefaciens*.

The RPA Cycle and TwistPCR

Recombinase Polymerase Amplification (RPA) is a method of isothermal amplification of DNA (**Figure 2**). TwistPCR assays such as TwistAmp® Basic and TwistAmp® nfo are based on this method of DNA amplification. Assays using this technology have been developed as diagnostic tools for pathogenic bacteria, fungi, and viruses.



Benefits of TwistPCR Assays:

- Low, constant temperature
- Sample does not require purification
- Speed: capable of diagnosis in less than 30 minutes
- Sensitivity: capable of single copy detection
- Specificity: capable of identification among background noise
- Multiple detection: combine multiple primers and probes in one tube
- Stabilized format: easy transport and capable of storing without refrigeration

Figure 2. Steps of the RPA (Recombinase Polymerase Amplification) Cycle. A. The two oligonucleotide primers form a complex with the recombinase proteins. **B.** This complex is able invade the target DNA and directs the primer to homologous sequences. **C.** A continuous amplification is catalyzed by a DNA polymerase while single-strand binding proteins (SSB) stabilize the displaced strand. **D.** Amplification continues. **E.** Two double-stranded fragments are formed. Figure Source: www.twistdx.co.uk

TwistAmp ® specifically detects a wide range of phytopathogenic *A. tumefaciens* isolates

Phytopathogenic *A. tumefaciens* vary in their host range, opine type, and Ti plasmids (**Table 1**). However, the virD2 protein is conserved. Sequence variation exists within the *virD2* gene sequence, therefore it was necessary to design primers for TwistAmp® Basic and a probe for TwistAmp® nfo that would work for a wide range of *A. tumefaciens* isolates (**Figure 3**).

Strain ID	Host	Octopine
AtuC58	Cherry	Nopaline
AS1C6	Peach/ Almond Rootstock	Nopaline
AS1E5	Yarrow	Nopaline/ Chrysopine
AS1F6	Raspberry	Nopaline
AS1G6	Peach/ Almond Rootstock	Octopine/ Agropine
JL5166	Clematis	Agrocinopine
JL5177	Raspberry	Agrocinopine
JL5268	Crabapple	Succinamopine
JL5274	Yarrow	Succinamopine
JL5182	Rose	Succinamopine

Table 1. Strains of *A. tumefaciens* used for the development of the TwistPCR assay.

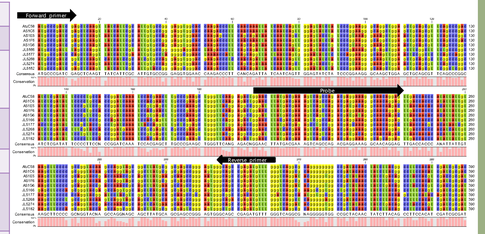


Figure 3. Sequence alignment of *virD2* sequences from *A. tumefaciens* isolates with location of oligonucleotides used for TwistPCR. The first 390 bp of *virD2* was aligned using CLC Sequence Viewer. Arrows indicate locations of forward and reverse primers and probe.

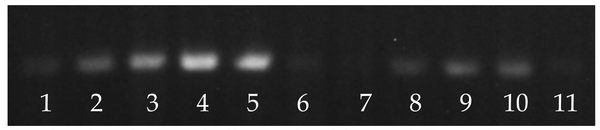


Figure 4. PCR showing designed oligonucleotides work for various strains of *A. tumefaciens*. 1) AtuC58 2) AS1C6 3) AS1E5 4) AS1F6 5) AS1G6 6) JL5166 7) JL5177 8) JL5268 19) JL5274 10) JL5182 11) Col-0

TwistAmp® nfo and Lateral Flow Analysis

Primers designed for the TwistAmp ® Basic assay can be extended for use with TwistAmp® nfo and Lateral flow analysis. For the TwistAmp® nfo assay, an additional probe is designed (**Figure 3, Figure 5**) and incorporated into the RPA product (**Figure 5**). Finally, this product which has a 5' FAM tag and a 3' Biotin label is detected using lateral flow strips (**Figure 6**).

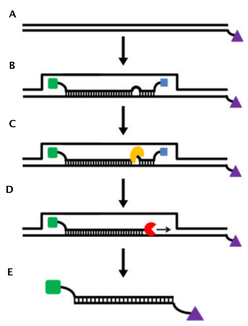


Figure 5. Schematic for TwistAmp® nfo. A. The initial product contains a 3' biotin label (purple triangle). **B.** A probe internal to this product is added in the reaction. The probe is labelled with a FAM tag at the 5' end (green square) and has a 3' polymerase blocking group (blue square). The probe also contains an abasic site (black loop). **C.** When the probe fully binds to a homologous sequence the nuclease (yellow shape) is able to cut through the DNA double strand and release the blocking group. **D.** Additional polymerase extension substrate occurs. **E.** The continuation of strand synthesis results in a dual-labelled amplicon. Figure Source:

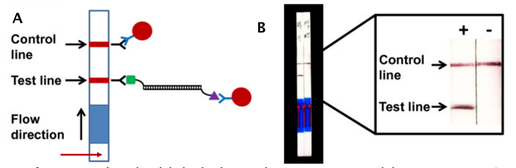


Figure 6. Lateral Flow Strip Detection. A. The dual labeled amplicon generated by TwistAmp® nfo is applied to a test strip (red arrow). Antibody-labeled gold particles (red circles) bind to the 3' biotin label and are carried by capillary action through the strip. Anti-FAM antibodies at the test line bind to the probe, giving a positive result. B. Example of Lateral flow detection.

Twist PCR can selectively amplify *A. tumefaciens vird2* at low concentrations

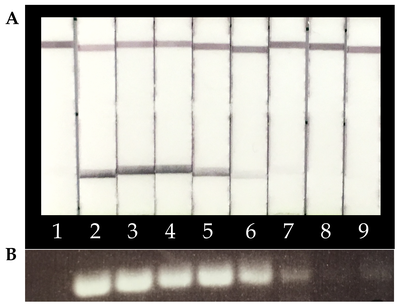


Figure 7. Sensitivity of TwistAmp® Basic and TwistAmp® nfo. A serial dilution of genomic DNA from the *A. tumefaciens* isolate C58 was used to test the analytical sensitivity of the assay. A. TwistAmp® nfo visualized on lateral flow strip. B. TwistAmp® Basic reaction products (200bp) visualized on an agarose gel (2%)
1) Water 2) AtuC58 (35 ng) 3) 1:10 4) $1:10^2$ 5) $1:10^3$ 6) $1:10^4$ 7) $1:10^5$ 8) $1:10^6$ 9) $1:10^7$

Project Outcomes

- Designed and validated primers for Twist PCR that work on a range of *A. tumefaciens* isolates varying in host range, Ti plasmid type, and opine type.
- Designed a probe for TwistAmp® nfo and tested with Lateral Flow strips
- Determined that TwistAmp® Basic and TwistAmp® nfo specifically detect a wide range of isolates
- Determined sensitivity of both TwistAmp® Basic and TwistAmp® nfo

Future Directions

- Test TwistAmp® nfo and Basic on DNA from *A. tumefaciens* galls.
- Optimize method to be useful in a field work setting.
- Quantify success of TwistAmp® nfo on Lateral Flow strips.

Acknowledgements

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