DNAble®

ENVIROLOGIX

Rapid detection of tNOS in Arctic® Apples

What is DNAble?

DNAble is a patented rapid nucleic acid amplification method that generates results equivalent to end point PCR. However, unlike PCR, DNAble can assay crude plant samples and results are generated in 10 to 15 minutes.

Benefits of DNAble

- **No DNA purification needed**
  Save time & money on consumables
- **Scalable**
  Point-of-need testing
  High-throughput capable
- **3x faster time-to-result vs. PCR**
- **Equivalent result to end-point PCR**
  Accuracy
  Sensitivity
  Specificity

BACKGROUND

In agricultural diagnostics, traditional GMOs express a transgenic protein that can be detected by rapid, inexpensive lateral flow immunoassays. However, as scientific understanding of plant genomes advances, some new traits entering the market do not express a detectable protein. One of the technologies being used for these new crop traits is RNAi, where small specific RNA molecules are produced by the cell through bioengineering, and these RNA molecules interfere with the expression of target genes to create a trait of interest.

For RNAi traits and other, new molecular crop traits, rapid molecular detection methods are required in place of protein-based lateral flow strips. DNAble is an isothermal DNA amplification technology that relies on a nicking enzyme and strand-displacing DNA polymerase, which work with sequence-specific primers that include an engineered tail to amplify target sequence. The amplification reaction is paired with fluorescent molecular probes for real-time detection in under 10 minutes (Fig. 1).

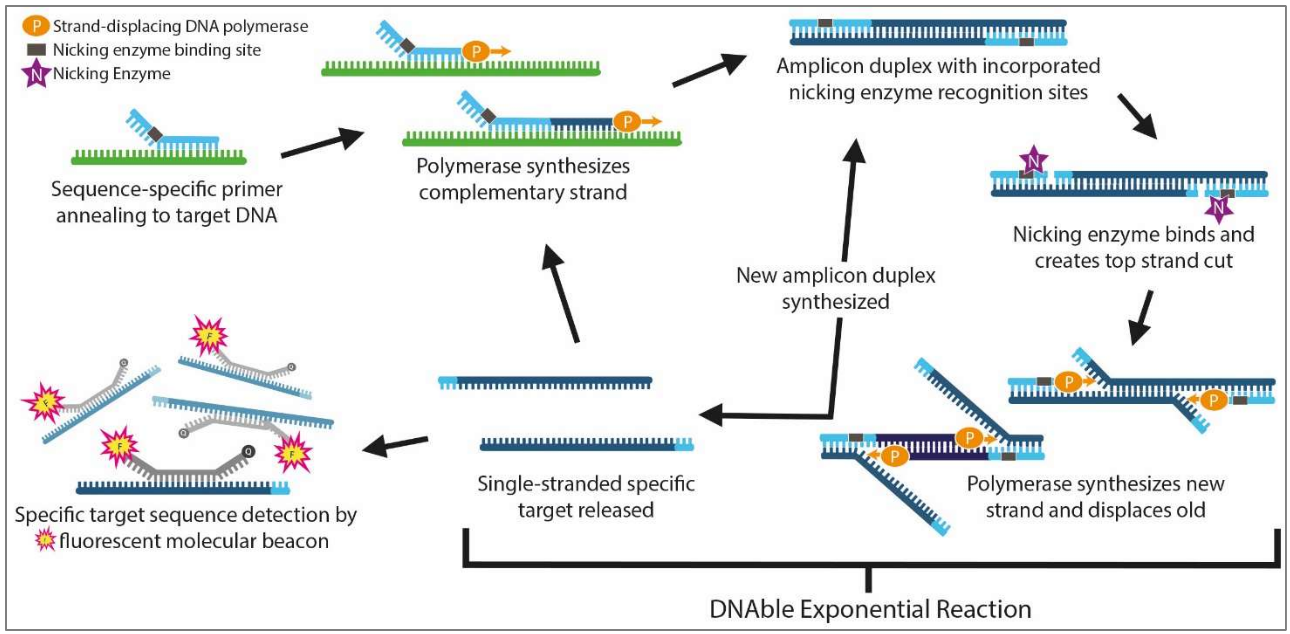

Fig. 1: Molecular mechanics of a DNAble reaction

The Arctic® Apple is a non-browning variety of apple that was developed by Okanagan Specialty Fruits using RNAi technology (Fig. 2). This trait is a hybrid of sequence from other organisms (transgenic) and sequence from apples (cisgenic). The transgenic portions of the Arctic Apple genetic construct include the commonly used CaMV 35s promoter sequence (from cauliflower mosaic virus) and the nopaline synthase gene terminator – tNOS (from *Agrobacterium tumefaciens*). These genetic elements have been used in many other GMOs, including over a dozen commonly planted corn, soy and cotton traits.

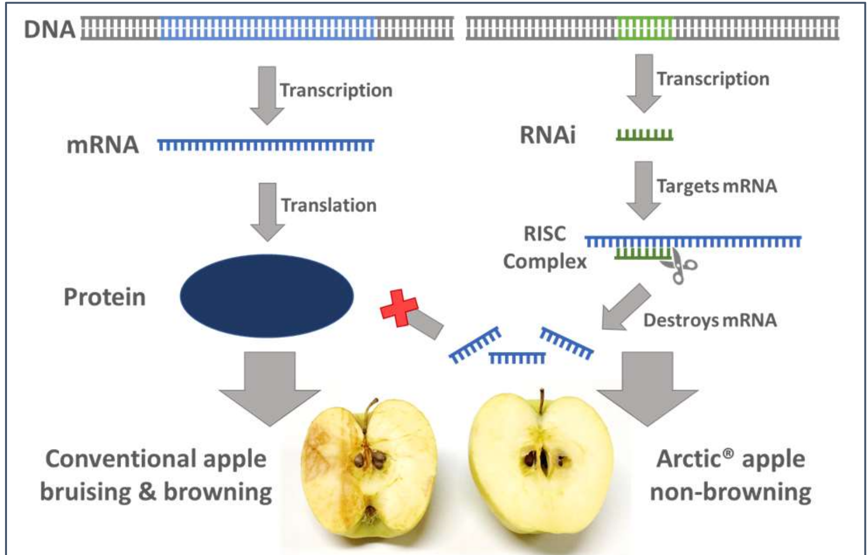

Fig. 2: Molecular mechanism **of** RNAi in Arctic® Apple

Here, EnviroLogix has used DNAble technology in a duplexed reaction to detect tNOS and an apple control sequence simultaneously in under 6 minutes, following a simple 15 minute sample extraction, for a total time to result in less than 25 minutes. The same assay and sample prep may be used in a variety of sample types, including leaf, apple skin, and dried apples.

MATERIALS & METHODS

DNAble Assay Design

The *Agrobacterium* tNOS genetic sequence is frequently used in GMO cassettes, including the non-browning Arctic Apple RNAi trait. A DNAble assay was designed based on published qPCR assay amplicon sequence (Barbau-Piednoir et al, 2014; Lipp et al, 2001; Waiblinger et al 2008), which aligns with the wild type sequence for the *Agrobacterium* Nopaline Synthase gene (Fig. 3a).

To design a control assay for Malus domestica (apple), a sequence alignment for all available ribosomal RNA genes in the NCBI nucleotide database was generated using the alignment program T-coffee (Notredame et al, 2000). The DNAble control assay was designed against a conserved region of the internal transcribed spacer 2 (Fig. 3b), which does not share sequence homology with rDNA sequence from common crops with commercialized tNOS-containing GMO varieties (corn, cotton, canola, and soy).

Fig. 3a: tNOS sequence and assay recognition sites

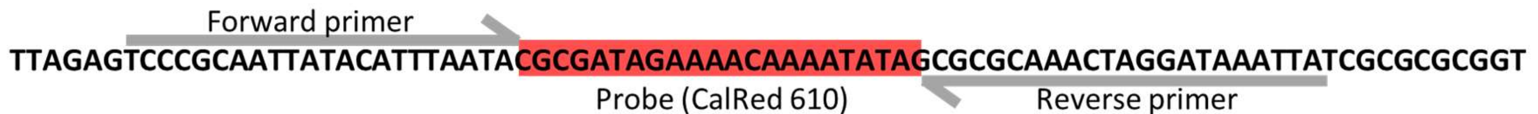

Fig. 3b: Apple ribosomal RNA internal transcribed spacer 2 consensus sequence and assay recognition sites

Plant Material & Sample Preparation (Fig. 4)

Commercially available Arctic® ApBitz (dried Arctic® Golden Apples) and Bare® Baked Apple Chips (Fuji & Red apple varieties) were ground for 60 seconds in a NutriBullet blender (model # NB-101B). A 10% GMO corn sample (Herculex XTRA™ RoundupReady™ 2 maize, a 6-event stack with two copies of tNOS) was prepared weight-to-weight (90% conventional mixed with 10% GMO corn) and ground for 2x 60 seconds in a NutriBullet® blender. For all ground samples, 2 scoops (LaMotte 0.05 g scoop) of sample were added to a 2 mL microcentrifuge tube. Apple leaf and conventional golden delicious apple samples were obtained locally; leaf and skin punches were made using a 2 mL microcentrifuge tube cap.

Samples were extracted in 1.6 mL of a 1:1 mix of previously developed EnviroLogix extraction buffers MB11 and MB14. Samples were vortexed briefly, then heated on a dry heat block at 85°C for 10 minutes to facilitate extraction. Samples were vortexed again, then centrifuged at 2000 xg (RCF) for 1 minute. Samples were diluted 1/10 in previously developed EnviroLogix reaction buffer 1x RB1.

Complete reaction lyophilized mastermix includes all enzymes, dNTPs, and assay-specific primers and probes for dual detection of the tNOS and apple rDNA targets. Per sample, six tubes of mastermix were resuspended with 50 µL of diluted sample each, then distributed to five 10 µL technical replicates on a 384-well plate, for a total of 60 technical replicates. The 384 well plate was sealed and the assay run on a 56°C 15 min. isothermal protocol with FAM/CalRed610 fluorescent detection on a Roche LC480.

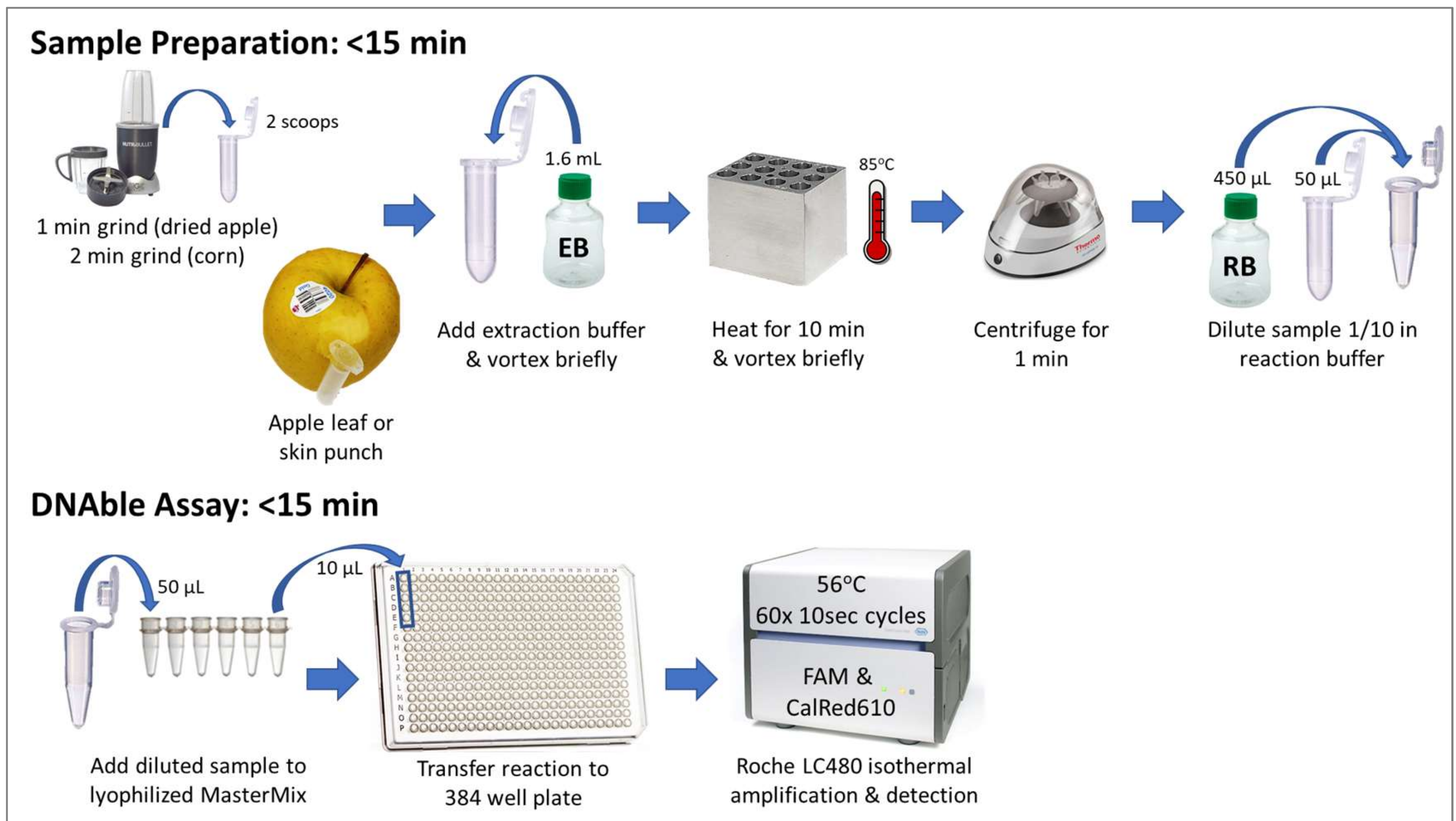

Fig. 4: DNAble sample preparation and assay conditions provide a result in less than 30 minutes

RESULTS & DISCUSSION

To compare assay performance with duplexed test and control targets, the DNAble tNOS-Apple assay was evaluated with ground Arctic® ApBitz (dried Arctic® Golden Apples), ground Bare® Baked Apple Chips (Fuji & Red apple varieties), a skin punch from a conventional golden delicious apple, a leaf punch from a conventional apple tree, and a ground corn grain sample with 10% tNOS-containing GMO present.

The duplexed assay demonstrated 100% accuracy in all samples tested, with a time to result in 6 minutes or less (Figure 5 and Table 1). Reaction amplification is called positive when/if the normalized fluorescence passes 0.3 RFU (crossing point – Cp). This assay demonstrates that DNAble can amplify and detect more than one genetic target simultaneously. These results also show that rapid, simple detection of new crop traits that do not express a transgenic protein is possible for a wide variety of sample types.

Where DNA purification and lengthy thermocycling required for PCR are not feasible, DNAble is a rapid, simple alternative for detection of molecular traits in a variety of crop samples. Detection is reliable and highly specific to the intended genetic sequence. As agricultural traits diversify, EnviroLogix will continue to focus on innovation in detection methods to make science practical for the industry.

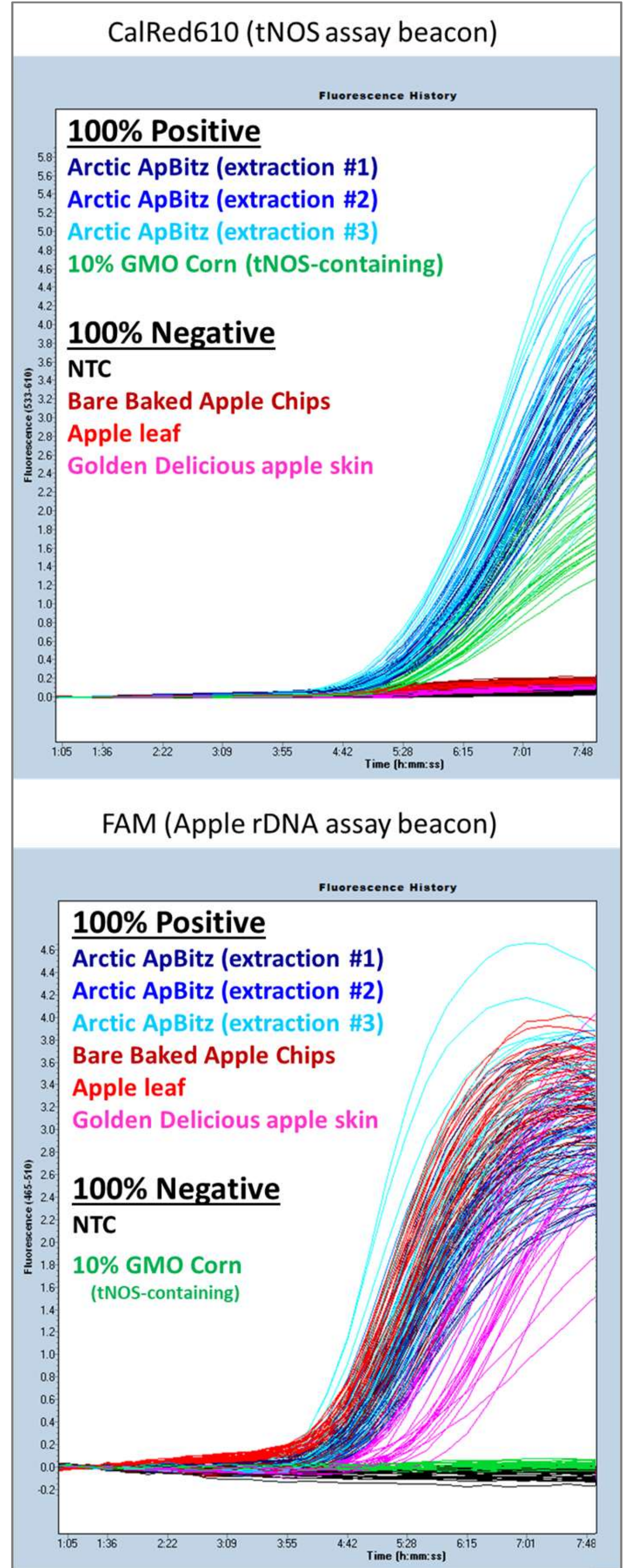

Fig. 5: DNAble results for duplexed reactions demonstrating 100% assay accuracy for both tNOS and Apple rDNA control assay targets

Table 1 Sample	Extraction Replicates	Assay Replicates per Extraction (10µL reactions)	tNOS Assay Results		Apple rDNA Assay Results	
			# Reps. Positive	Mean Cp (minutes)	# Reps. Positive	Mean Cp (minutes)
NTC	N/A	30	0 / 30 (0%)	–	0 / 30 (0%)	–
Arctic® ApBitz	3	30	90 / 90 (100%)	5.4	90 / 90 (100%)	4.5
Bare® Baked Apple Chips	1	30	0 / 30 (0%)	–	30 / 30 (100%)	4.5
Apple leaf	1	30	0 / 30 (0%)	–	30 / 30 (100%)	4.2
Golden Delicious apple skin	1	30	0 / 30 (0%)	–	30 / 30 (100%)	5.3
10% GMO Corn (tNOS-containing)	1	30	30 / 30 (100%)	5.8	0 / 30 (0%)	–