NGT Products – Methods and Matters of Detection

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Corteva Agriscience

What We Do

Dedicated solely to Agriculture, we enrich lives of both the Producer and Consumer through a diverse offering of Seed, Crop Protection, and Digital Technologies

People
~21 000 employees

Products
Seeds (conventional & GMO)
Plant protection products

Facilities
>150 Research Centers
~90 Production Facilities

Global Presence
140 Countries
>10M Customers

Source: www.corteva.com

CRISPR-Cas9 *waxy* Maize

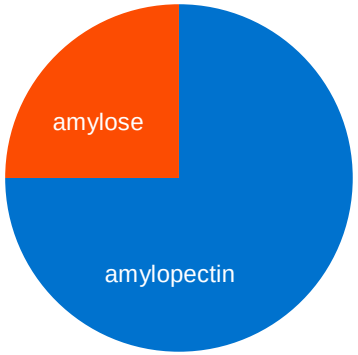
Background

Properties of Waxy Maize Grain

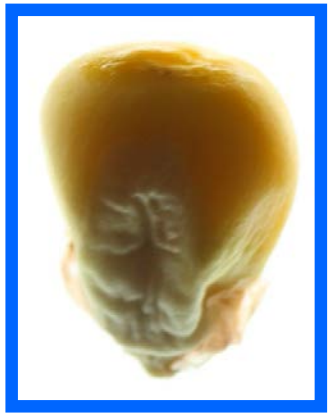
Normal maize kernel

vitreous endosperm
translucent appearance

starch

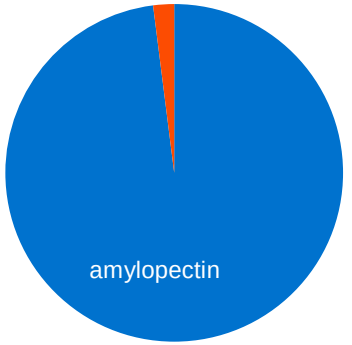


Waxy maize kernel



opaque endosperm
candlewax-like appearance

starch



Source: Maria Fedorova, Corteva Agriscience

Waxy Maize Cultivation and Processing

- Cultivated in the U.S. since the 1940's
- Specialty maize – grown in an identity-preserved system managed by the grower for value capture
- Majority is produced by growers under contracts with U.S. wet-milling industry
- ~0.5% of commercial corn acres in the U.S. annually
- Waxy grain is predominantly processed into starch by wet-milling industry
- Limited amount of U.S. waxy maize grain goes for export or for feed in the livestock, dairy, and poultry industries

Source: Maria Fedorova, Corteva Agriscience

Usage of Corn Starch

- ✓ Essential functions in **food industry** to improve uniformity, stability, and texture in various food products
  - Better freeze-thaw stability of frozen foods
  - Improvement of smoothness and creaminess of canned foods and dairy products
  - More desirable texture and appearance of dry foods
  - Flavor enhancer
  - Emulsifier for salad dressings
- ✓ Binding qualities for the **paper-making** process
  - Remoistening adhesives in the gum tape manufacture
  - Paper strength and printing properties improvements
- ✓ Additional applications in the **textile, corrugating, and adhesive industries**

Diversity of Spontaneous and Induced Mutations in *Wx1* Gene

http://www.maizegdb.org/data_center/stock

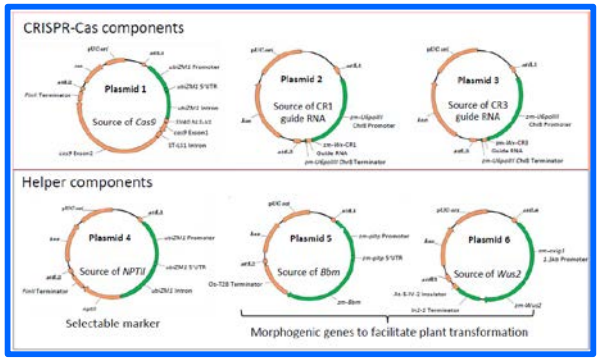
- Loss-of-function *wx1* allele
- > 200 "*wx1*" entries in MaizeGDB
- Extensive literature on *wx1* mutations in maize
- Mutations include insertions, deletions, translocations
- Deletion mutations: variable location and size

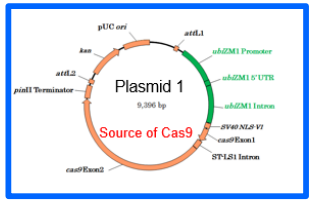
Table 1. Strains used in this study

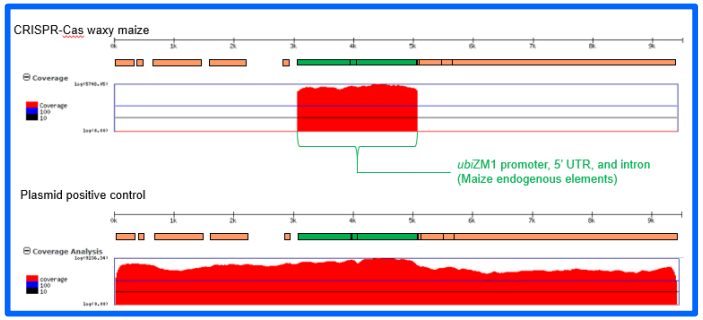
wx allele	Nature of mutation	Lesion*	Molecular progenitor *Wx* allele†	Origin‡
90	Spontaneous	ND	*HY*	Brunson
R	Spontaneous	ND	*HY*	Richardson
B	Spontaneous	Deletion	*HY*	Bear hybrid
G	Spontaneous	Insertion	*W23*	Bear hybrid
I	Spontaneous	Insertion	*HY*	Bear hybrid
K	Spontaneous	Insertion	*HY*	Bear hybrid
M	Spontaneous	Insertion	*HY*	Bear hybrid
C1	Spontaneous	ND	*HY*	Blandy farms
C2	γ rays	ND	*HY*	Blandy farms
C3	γ rays	ND	*HY*	Blandy farms
C4	Spontaneous	Deletion	*HY*	Blandy farms
C31	γ rays	ND	*HY*	Blandy farms
C34	γ rays	Deletion	Unknown	Blandy farms
B1	Spontaneous	Deletion	*W23*	Ashman and Brink
B2	Spontaneous	Insertion	*W23*	Ashman and Brink
B5	Spontaneous	Insertion	*W23*	Ashman and Brink
B6	Spontaneous	Deletion	*HY*	Ashman and Brink
B7	Spontaneous	Deletion	*HY*	Ashman and Brink
B8	Spontaneous	ND	*W23*	Ashman and Brink
c	Spontaneous	ND	*HY*	Collins
Stoner	Spontaneous	Insertion	*HY*	From Assam
BL2	EMS	ND	*HY*	Briggs

Source: Maria Fedorova, Corteva Agriscience

Development and Molecular Analysis of CRISPR-Cas9 Waxy Maize







Example SbS data – Plasmid 1 containing Cas9
No plasmid-genome junctions detected
Endogenous elements (used in plasmids) are detected only in their native genomic context

Characterization of targeted mutation – NGS

Absence of unintentionally inserted plasmid DNA

Southern-by Sequencing (SbS)

- Sequence capture technology + deep sequencing;
- Capture probes covered entire sequences of all 6 plasmids used in transformation;
- Analyses for novel junctions of plasmid within plant genome; none were identified
- Positive controls: plasmid spiked into wild type maize DNA
- Negative controls: wild type maize DNA

No off-target mutations – PCR amplification + NGS

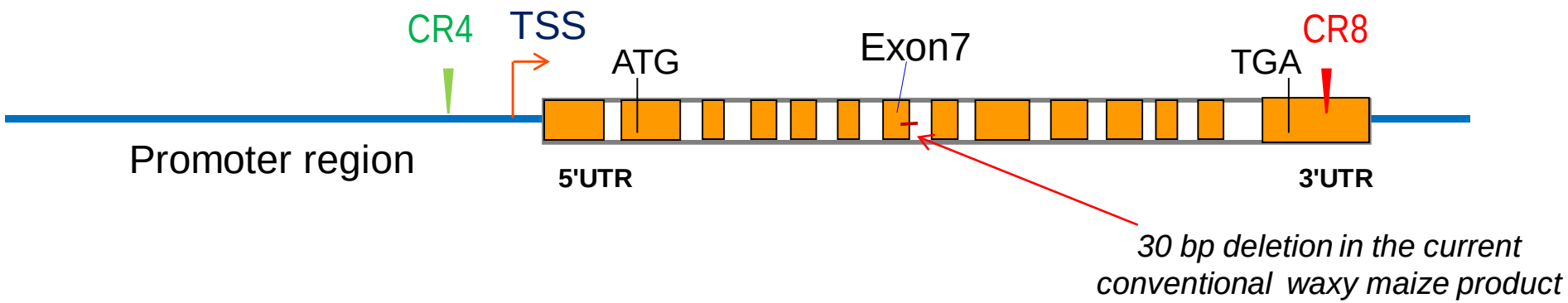
- gRNA were designed to be unique to target sequences
- No mutation *in planta* identified at the closest predicted off-target sites

Source: https://doi.org/10.1038/s41587-020-0444-0

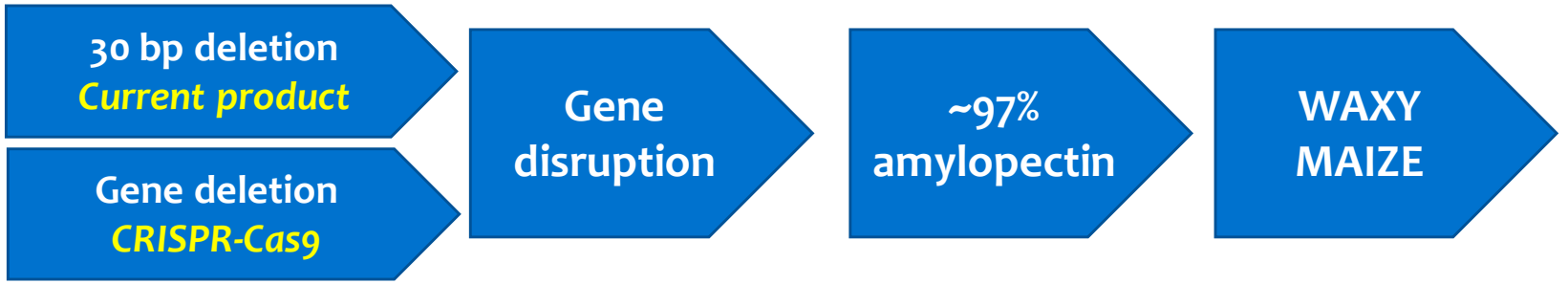
Detection, Identifiability and Traceability

Distinguishing between *Waxy1* Mutations in Food

CRISPR-Cas9 Waxy Maize: *Wx1* Gene Deletion



CR4 and CR8 - two guide RNAs to introduce two DNA double strand breaks



Source: Maria Fedorova, Corteva Agriscience

CRISPR-Cas9 *waxy* Maize and Conventional *waxy* Maize

Common food product from *waxy* maize grain: Corn Starch

Is there adequate and amplifiable DNA recovery?

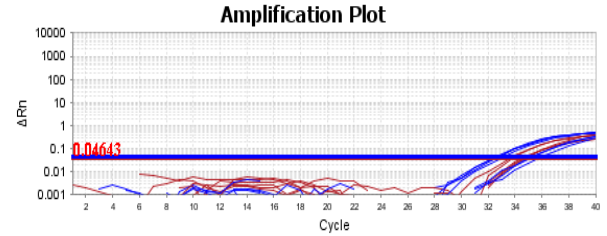
Corn Starch Sample Types Tested	DNA Recovery
Commercial Untreated corn starch	yes
Commercial Conventional waxy corn starch	no
Conventional High Amylose corn starch*	yes
Pioneer CRISPR-Cas9 *waxy* corn starch	yes
Purchased corn starch (grocery)	yes

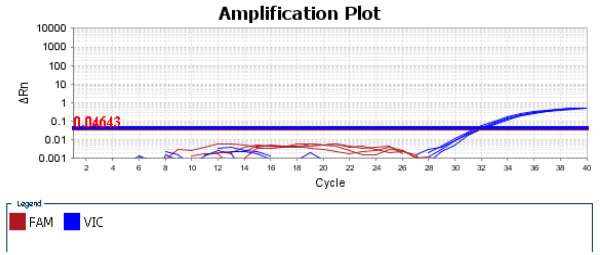
- α-Amylase digestion is critical to prevent gel formation during the DNA extraction process
- DNA extraction method specialized for food products utilized

* High Amylose corn starch is produced from maize varieties that contain ~ 70% Amylose and 30 % Amylopectin

Corn Starch Analysis by Qualitative Real-time PCR – Distinguishing *waxy* mutations?

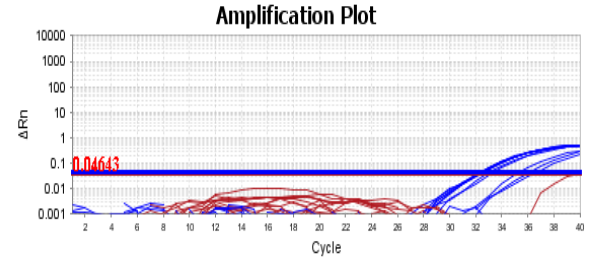
PCR Analysis: Intact *Zm-Wx1* *(no 30-bp deletion)*

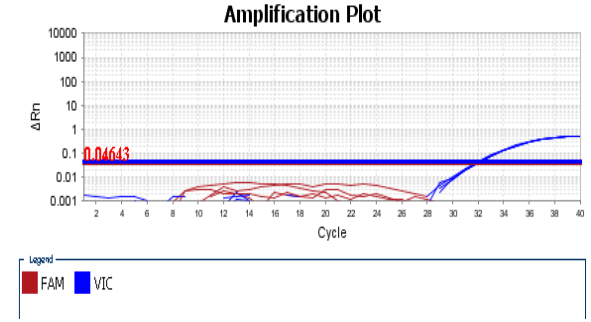




Untreated Corn Starch = Detect
High Amylose Corn Starch = No Detect
CRISPR-Cas9 waxy Corn Starch = No Detect
Grocery store Corn Starch = Detect

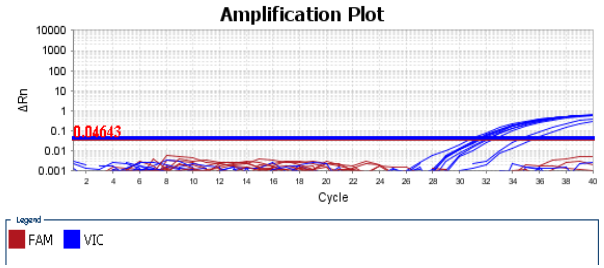
PCR Analysis: *Zm-Wx1* 30-bp deletion

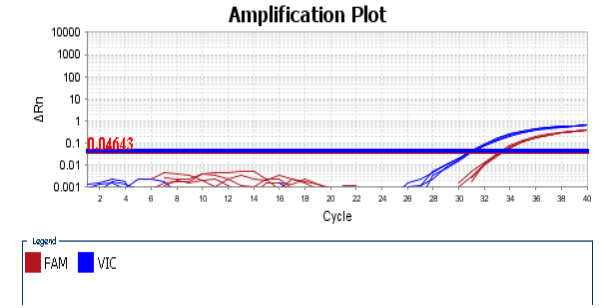




Untreated Corn Starch = No Detect
High Amylose Corn Starch = No Detect
CRISPR-Cas9 waxy Corn Starch = No Detect
Grocery store Corn Starch = No Detect

PCR Analysis *Zm-Wx1* CR4-CR8





Untreated Corn Starch = No Detect
High Amylose Corn Starch = No Detect
CRISPR-Cas9 waxy Corn Starch = Detect
Grocery store Corn Starch = No Detect

Commercial Corn Starches

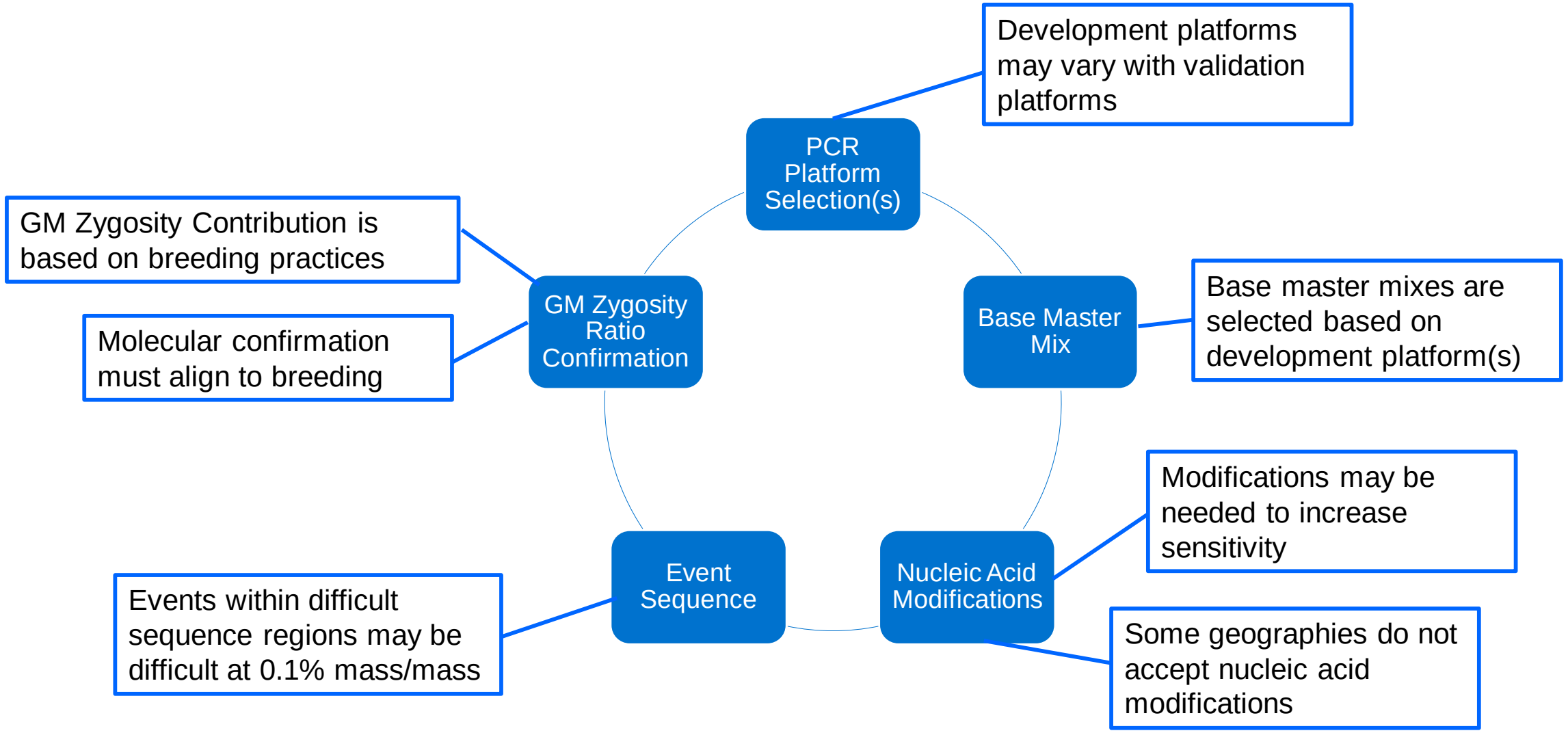
CRISPR-Cas9 *waxy* Corn Starch

GM Detection Methods

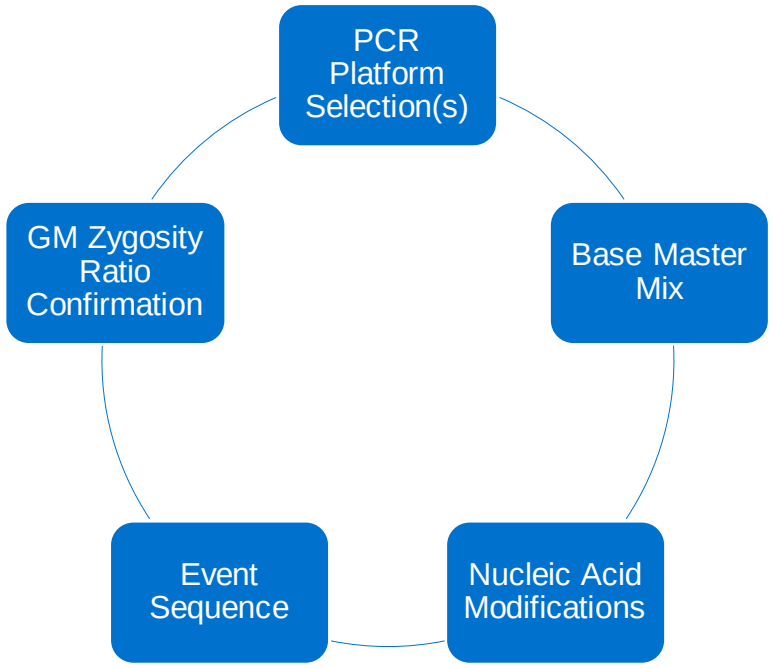
Challenges and Hurdles with GM Detection Methods – Examples

GM Detection Method

Hurdles of Influencing and Interrelated Parameters: Challenges to Achievement of Trueness and Precision



Misalignment of one parameter during validation process



All parameters are interrelated, especially for low copy samples

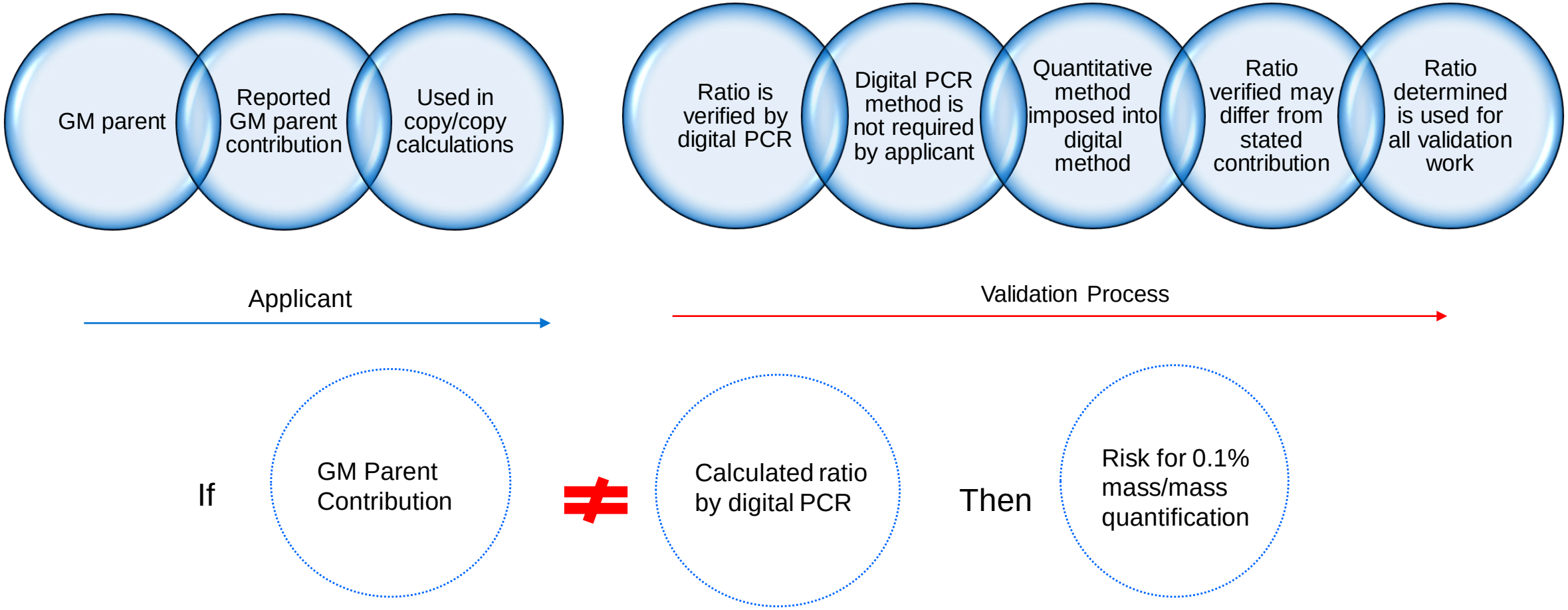
A single parameter or a combination of parameters can negatively influence trueness and precision at 0.1% mass/mass

If parameter(s) cannot be rectified, the detection method is at risk

For GM events, validation is not a straightforward process

Misalignment of one parameter during validation process

Example: GM Zygosity Ratio and Digital PCR



Quantified Detection for NGT Products

Detectability, Identifiability, Practicability and Applicability

NGT Product Detection Method – Quantification Potential at 0.1% mass/mass?

Detectability –
Ability to state something is present or absent

Possibilities
- Detectability by qualitative PCR
- Applicable to known edits only

Challenges
- Applicability of assay to food matrices
- DNA Extraction
- Competition of endogenous DNA
- Assay efficiency
- Specificity
- Sensitivity
- Transferability and Robustness

Identifiability –
Ability to distinguish something from another

Possibilities
- Applicable to known edits or known sequence

Challenges
- Distinguishing NGT from mutations may exist with many variants not characterized
- Germplasm variances may have other SNPs
- Common genetic elements for screening may not exist
- High throughput whole genome screening methods do not yet exist

NGT Product Detection Method – Quantification Potential at 0.1% mass/mass?

Practicability – how feasible is a quantification detection method for an NGT product?

Some Considerations:

Specialized PCR

- Ease of repeatability and transferability
- Nucleic Acid modifications
- Acceptance by other geographies

Sensitivity

- Detection of small change in bulk grain may be unpredictable

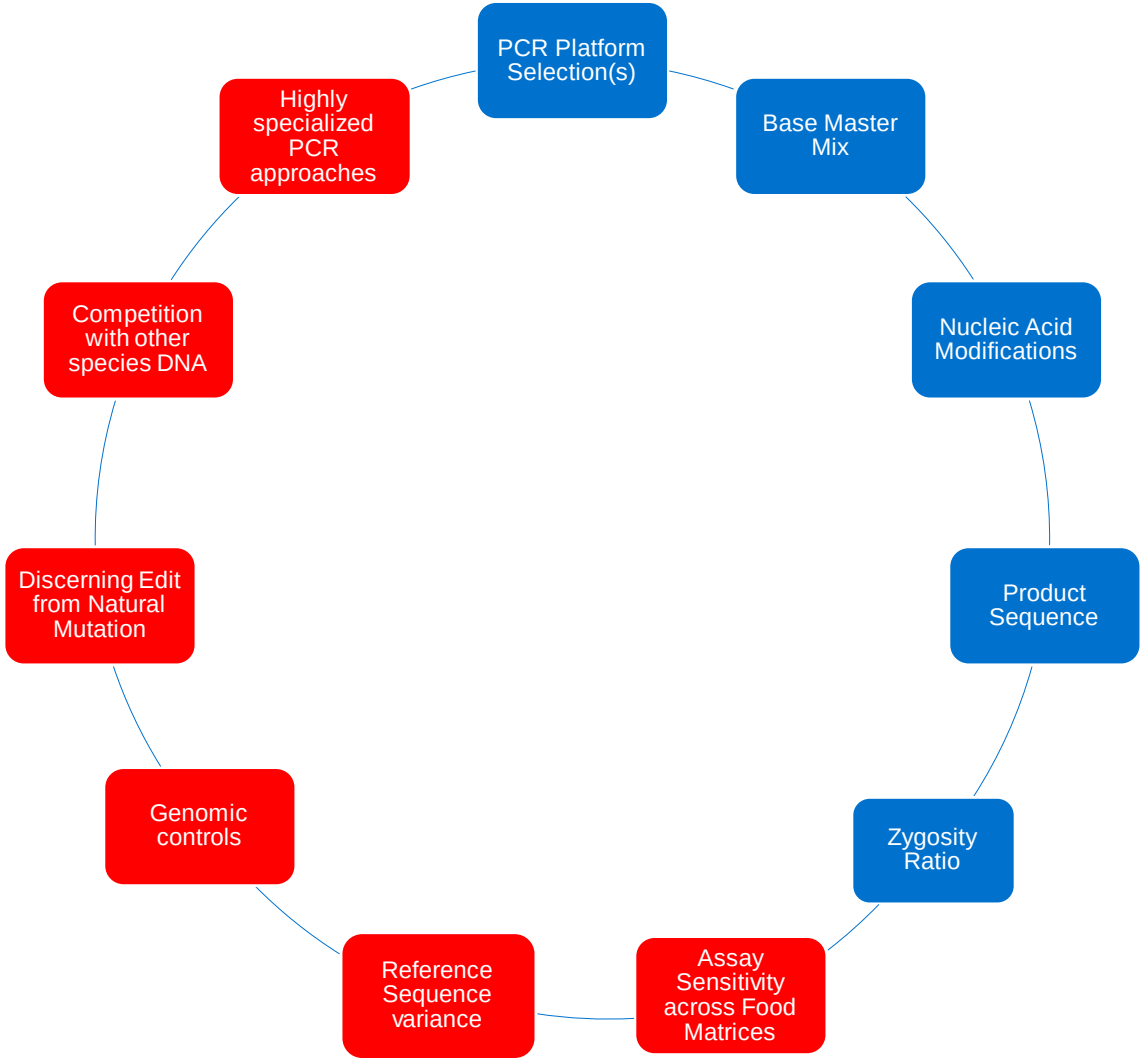
Discernment

- Ability to distinguish edit from other natural mutations in current products
- Sequence variance across germplasm
- Competition with other species DNA in food
- Certified Reference Material required

Reference Sequence

- Ploidy influence on method performance across genomes
- Sequence region of interest may be difficult
- Knowledge of sequence change
- Variability of DNA replication may cause sequence change(s) over time

NGT Product Detection Method – Quantification Potential at 0.1% mass/mass?



Applicability of a single detection method for single edit

Parameters that impact GM events in quantification of 0.1% mass/mass will still exist

For NGT products, additional combination(s) of parameters may also negatively affect the practicability to achieve trueness and precision

And

Even if detected and quantified, it may be impossible to identify or distinguish the exact source of detection for some gene edits

Quantified Detection for NGT Products

- To inform and give consumers a choice in their food purchase requires:
  - Unique and known sequence recognition
  - A validated detection method to detect and quantify

Example: CRISPR-Cas9 *waxy* maize as a fit for purpose detection method?

Practicability: May require specialized PCR and special handling of DNA for food matrices

Applicability: May be impossible to confidently assert

Detectability: Potential for this NGT product, but this cannot be expected for all

Identifiability: Uncertainty exists

Conclusions

- Certain NGT products may be able to have robust validated detection methods
  - However - this does not mean that there is ability to create methods for every NGT product

- A fit for purpose detection method can only be developed if:
  - There is an awareness of the gene edit(s) completed
  - There is unique sequence recognition

- How can these be carefully tested?
  - To date, there are no high-throughput whole genome screening methods available nor common markers
  - This impacts the ability to test across high numbers of potential edits

- Overall uncertainty in specificity and unique sequence recognition supporting NGT products
  - Negatively impacts the possibilities of enforcement of the current legislation




Thank you for your attention