Plant Biotechnology

Bayer Russia Plant Biotechnology Conference

July 2023

Molecular Assays

Bayer Russia Biotechnology Conference

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Intro to Molecular Assays for Plant Biotech

Transgene detection and selection in plants

Types of Assays for Transgenes

Gene edit detection and selection in plants

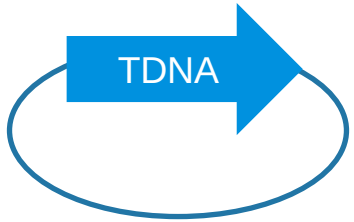
Assays for gene edits and analytics

Considerations for detection workflow automation

About me: Dr. James H. Crowley, PhD

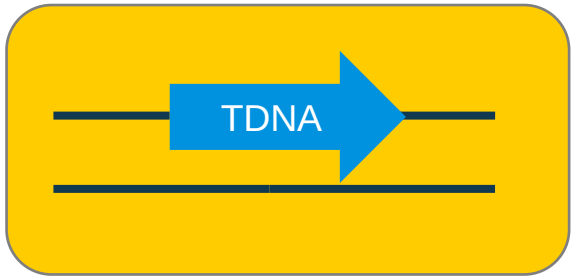
- PhD in Microbiology from North Carolina State University
- Over 25 years experience in Plant Biotechnology at Bayer Crop Science
- Project lead experience leading commercial development of Biotech crops
- Eight years of experience in developing and running molecular screening assays for Biotech crops
- Four years of experience as lead of the STL TaqMan Lab in Chesterfield, MO site

Transformation of plants with Agrobacterium method will produce T0 plants with random insertions in each plant



TDNA in DNA vector

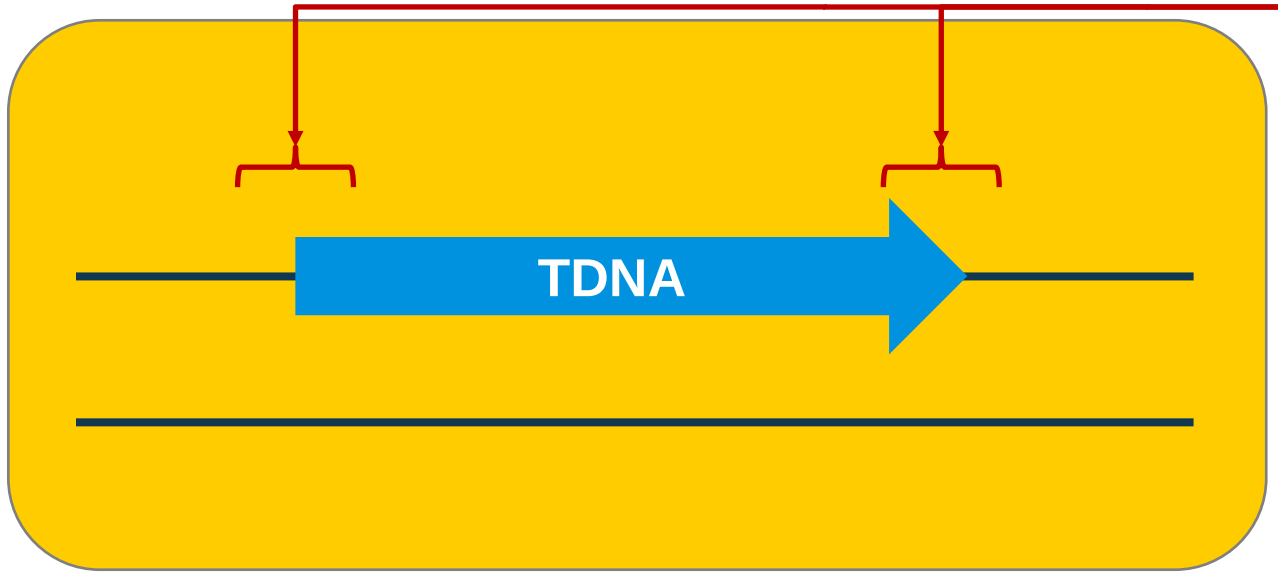
Plant Transformation



TDNA inserted into plant genome (the “event”)

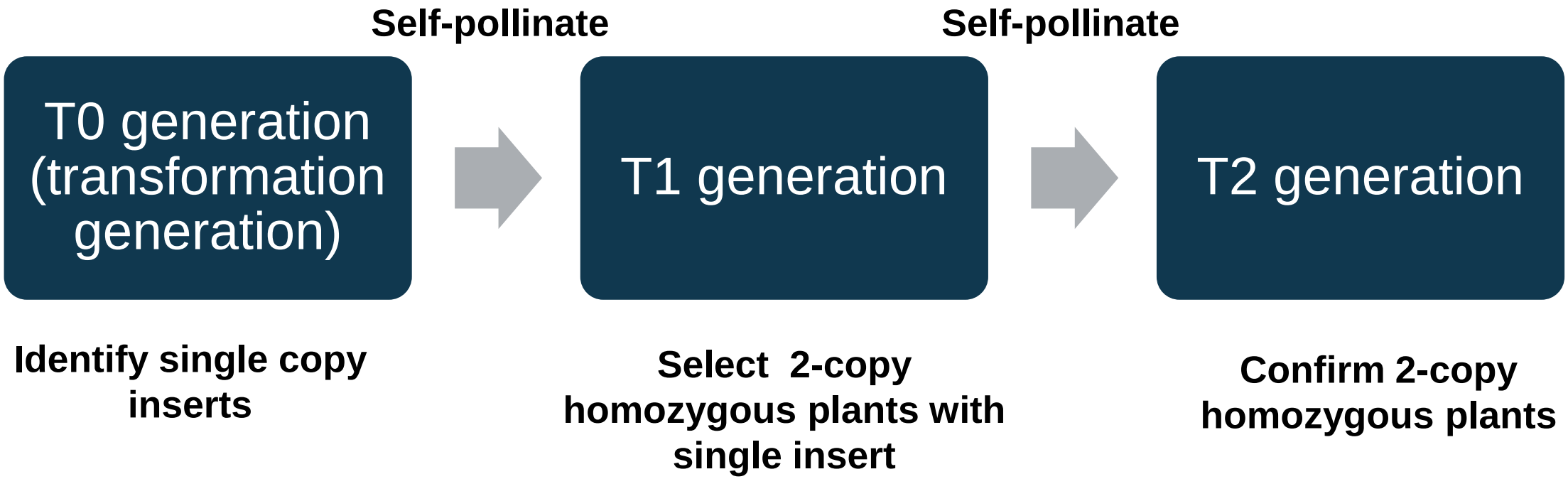
- Each T0 transformed plant will have at least one TDNA insert
- The TDNA insert in each T0 plant will be in a different location in the genome, compared to the T0 other plants (“random insertion”)
- Each TDNA insertion into a unique location in the genome is called an “event”
- Each “event“ has unique junction sequences that can be used to identify the event

Junction DNA sequences can identify TDNA events



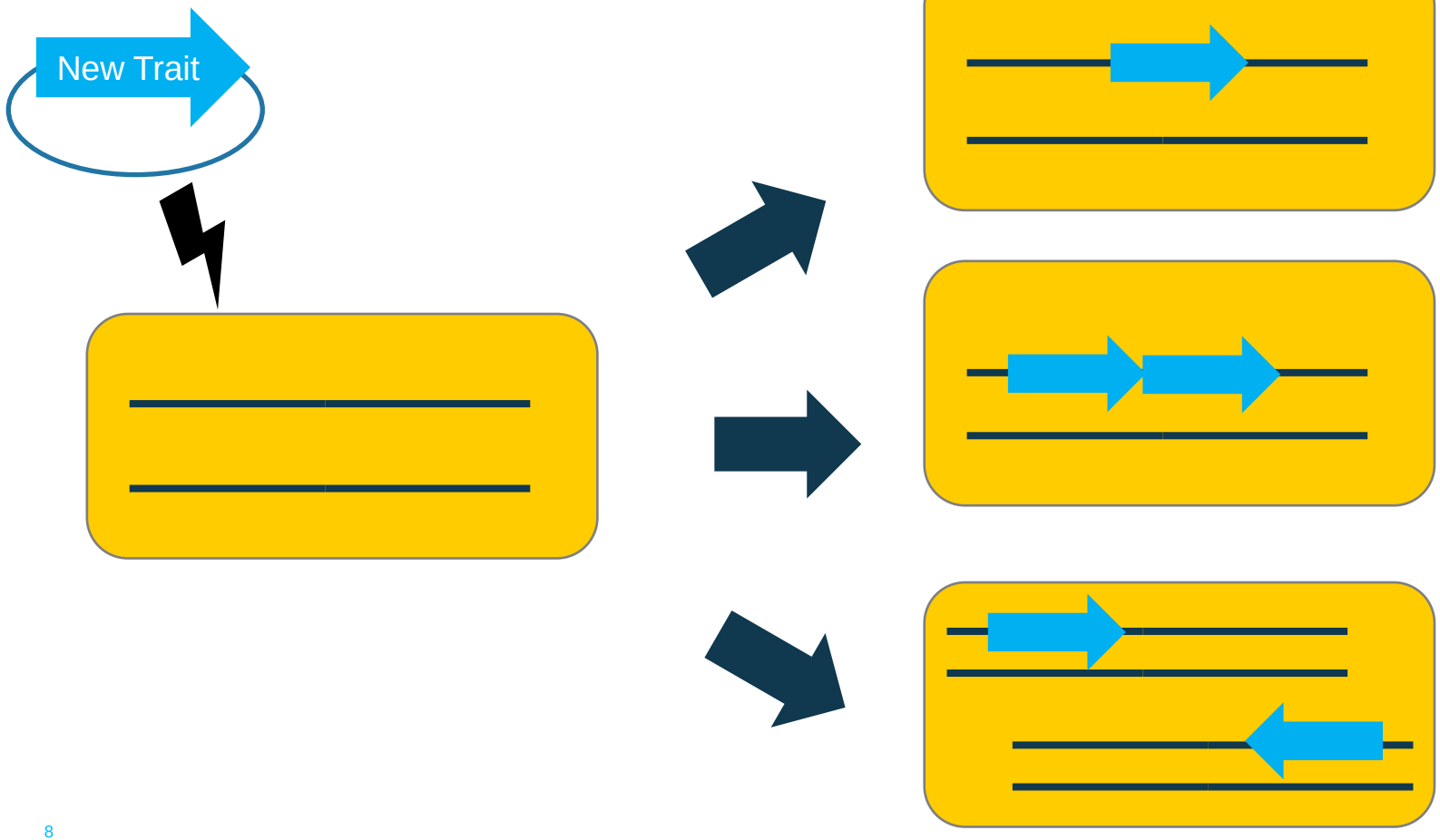
- Each “event“ has two unique junction sequences that can be used to identify each event
- Each left and right junction DNA sequence is a unique combination of sequence from the plant genome and the inserted TDNA
- DNA junctions can be used for unique assays later in development for assays specific to the event

DNA copy number variation (CNV) is used to select single-copy TDNA inserts and identify fixed homozygous lines with transgenic traits



T2 homozygous (“fixed”) lines used as parent donors for further breeding or for use in phenotypic trials to assess trait efficacy

Select for single-copy TDNA plants in first T0 generation



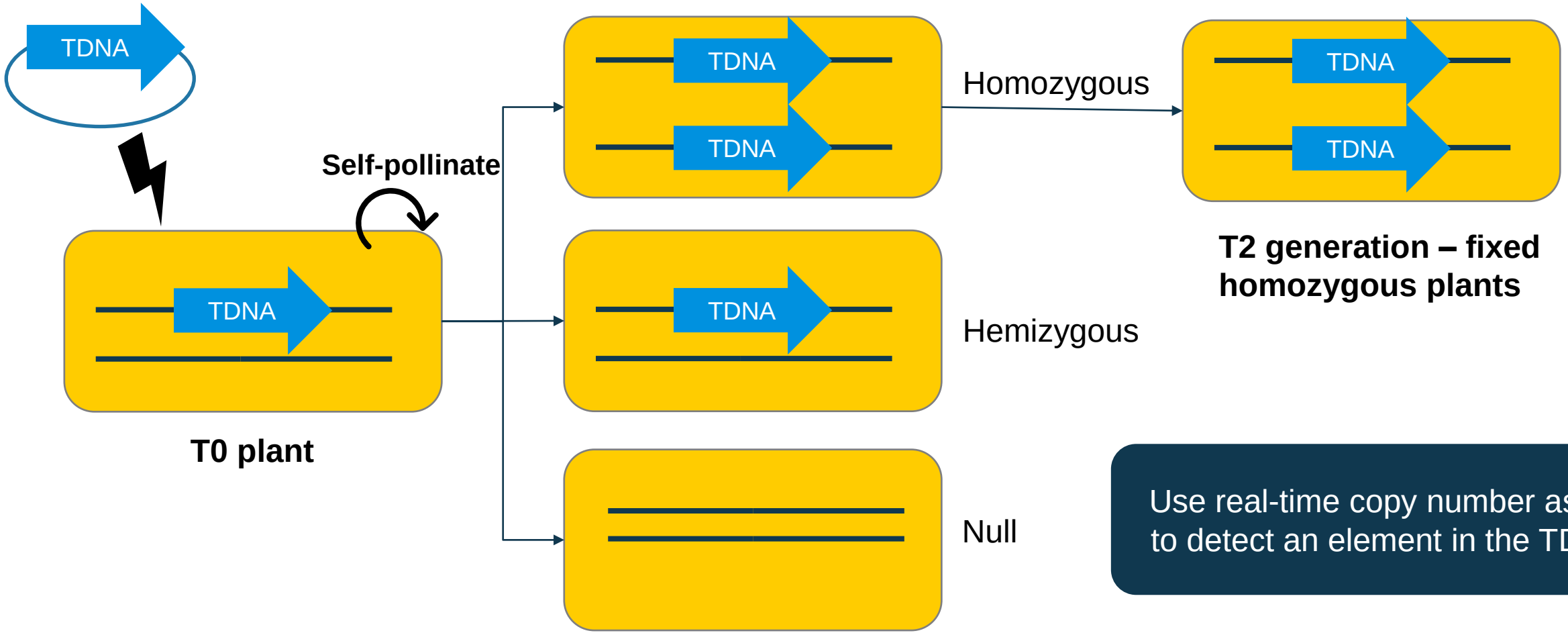
Use real-time copy number assay to detect an element in the TDNA

Single copy, Single insert — ADVANCE

Two-copy (Tandem) — DROP

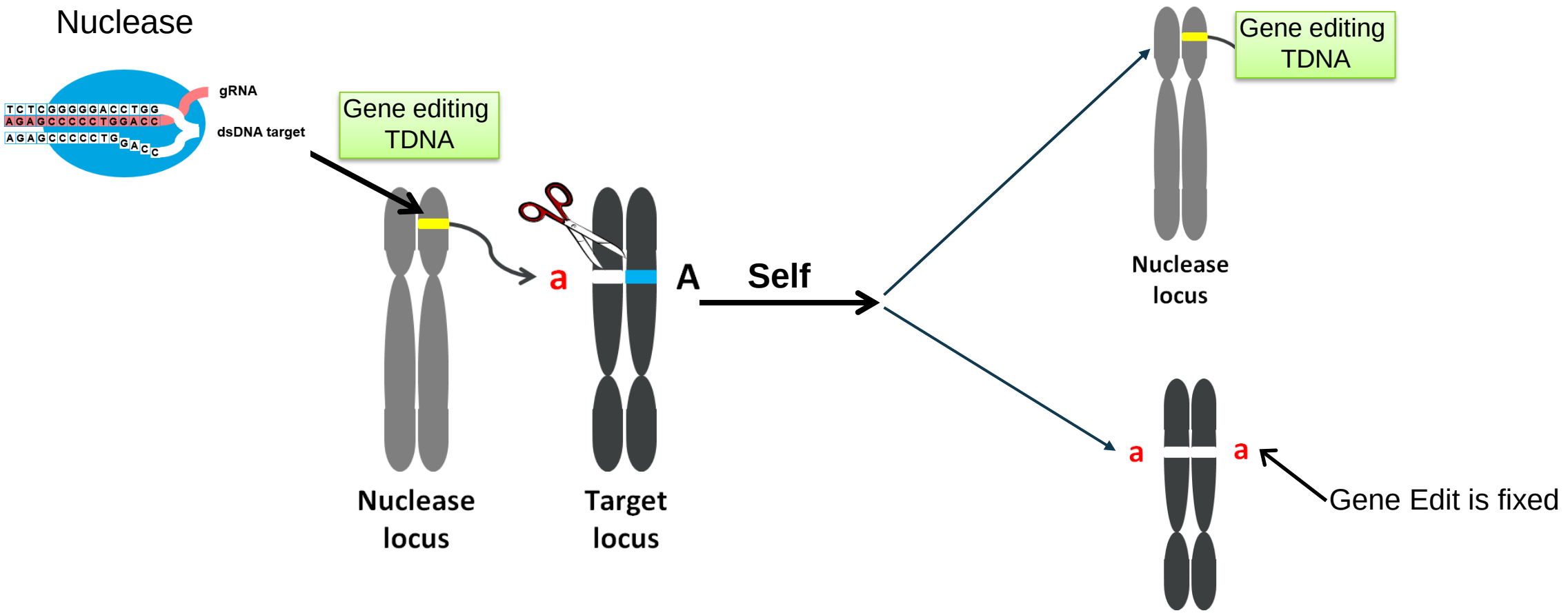
Two-copy (multi-locus) — DROP

Goal in plant advancement for transgenic traits is to select for a plant line that is homozygous for the desired new trait

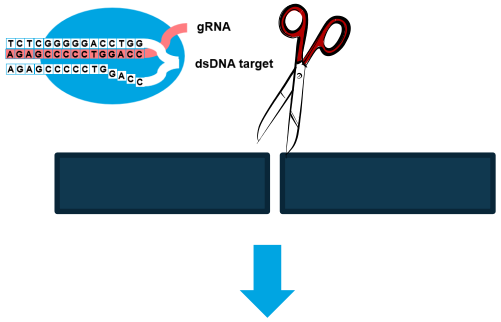


Use real-time copy number assay to detect an element in the TDNA

Gene editing machinery enables making precise edits in the gene of interest followed by segregation of transgenic nuclease from edits



Gene edits can be advanced based on amplicon sequencing



GAGGGC__GCCATCA

GAGGGCATGCCATCA

Select for edit at T0

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

Select for homozygous edit at T1

GAGGGC__GCCATCA

GAGGGC__GCCATCA

Confirm homozygous for edit at T2

Use amplicon sequencing to detect and advance edits

Gene editing involves selection for edits and against TDNA at the same time in plant advancement

Self-pollinate **Self-pollinate**

TDNA
EDIT

- **Select single copy inserts**
- **Select edits**

- **Select null for TDNA**
- **Select homozygous for edits**

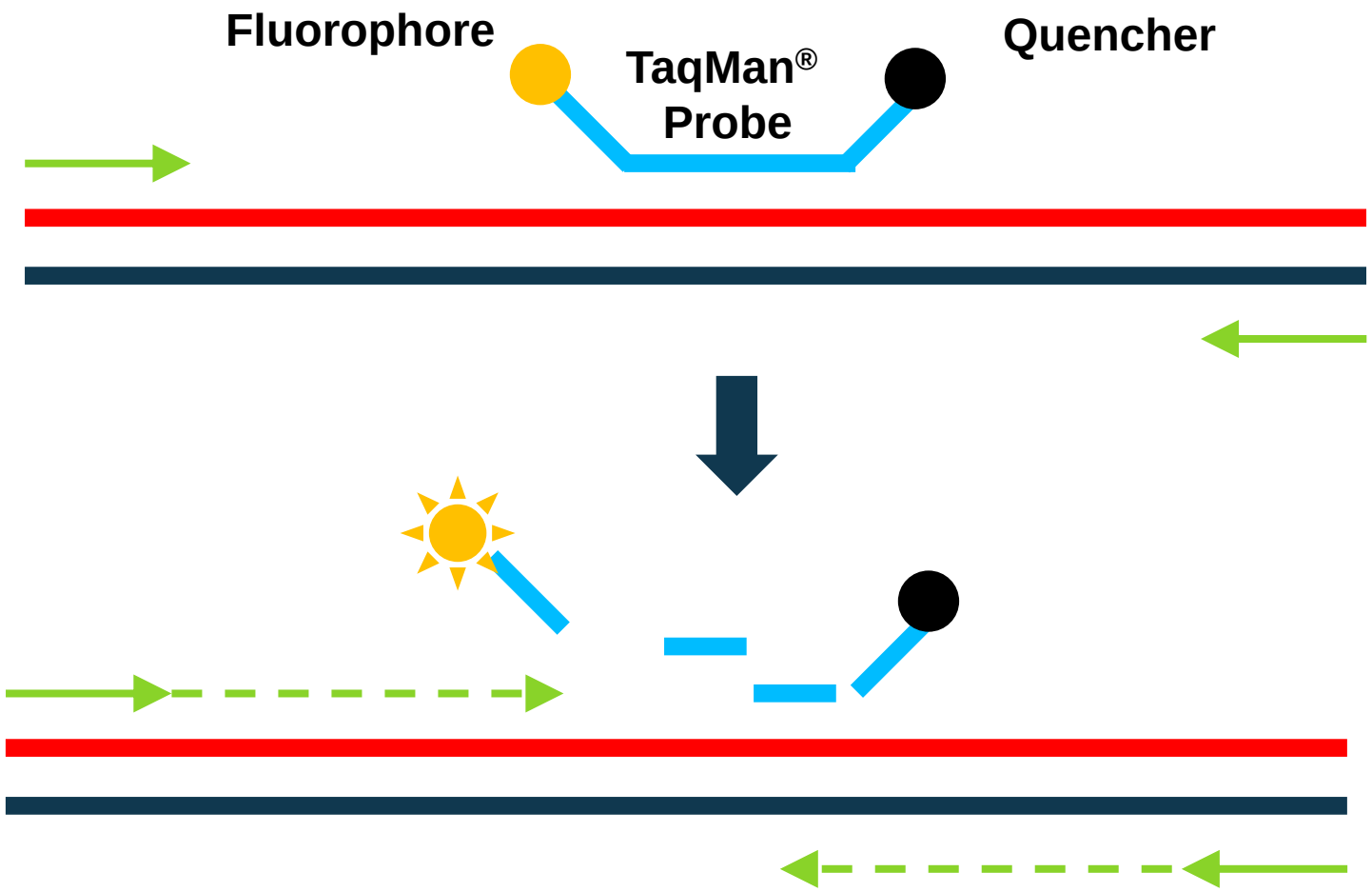
- **Confirm null for TDNA**
- **Confirm homozygous for edits**

T2 homozygous (“fixed”) lines used as parent donors for further breeding or for use in phenotypic trials to assess trait efficacy

Molecular assays for transgene and gene edit detection

- TaqMan® for Transgenes
  - Real-Time
    - Copy number variation
      - Insert number
      - Zygosity
    - Gene Expression
  - Endpoint
    - Presence/absence
- Amplicon sequencing for gene edits
- TaqMan® assays for gene edits

TaqMan® uses PCR and fluorophore-labeled probes to detect DNA sequences

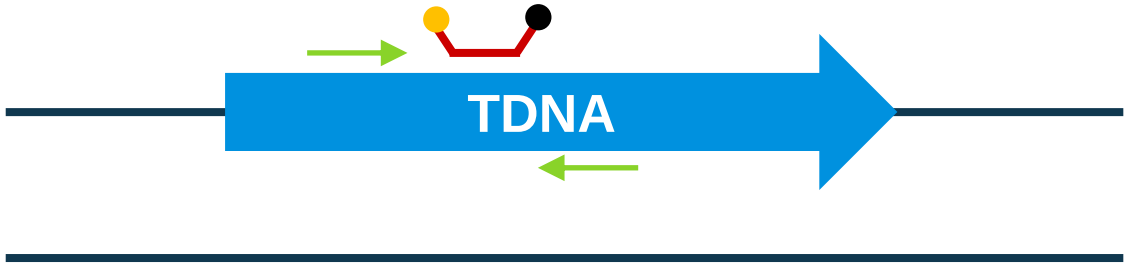


Fluor is released following exonuclease activity of polymerase

TaqMan® is a registered trademark of Roche Molecular Systems, Inc.
For more detail on TaqMan assays see ThermoFisher Scientific website (www.thermofisher.com)

Genetic element-based assays and event junction assays can both be used to assay for transgenic events

Genetic element-based assay

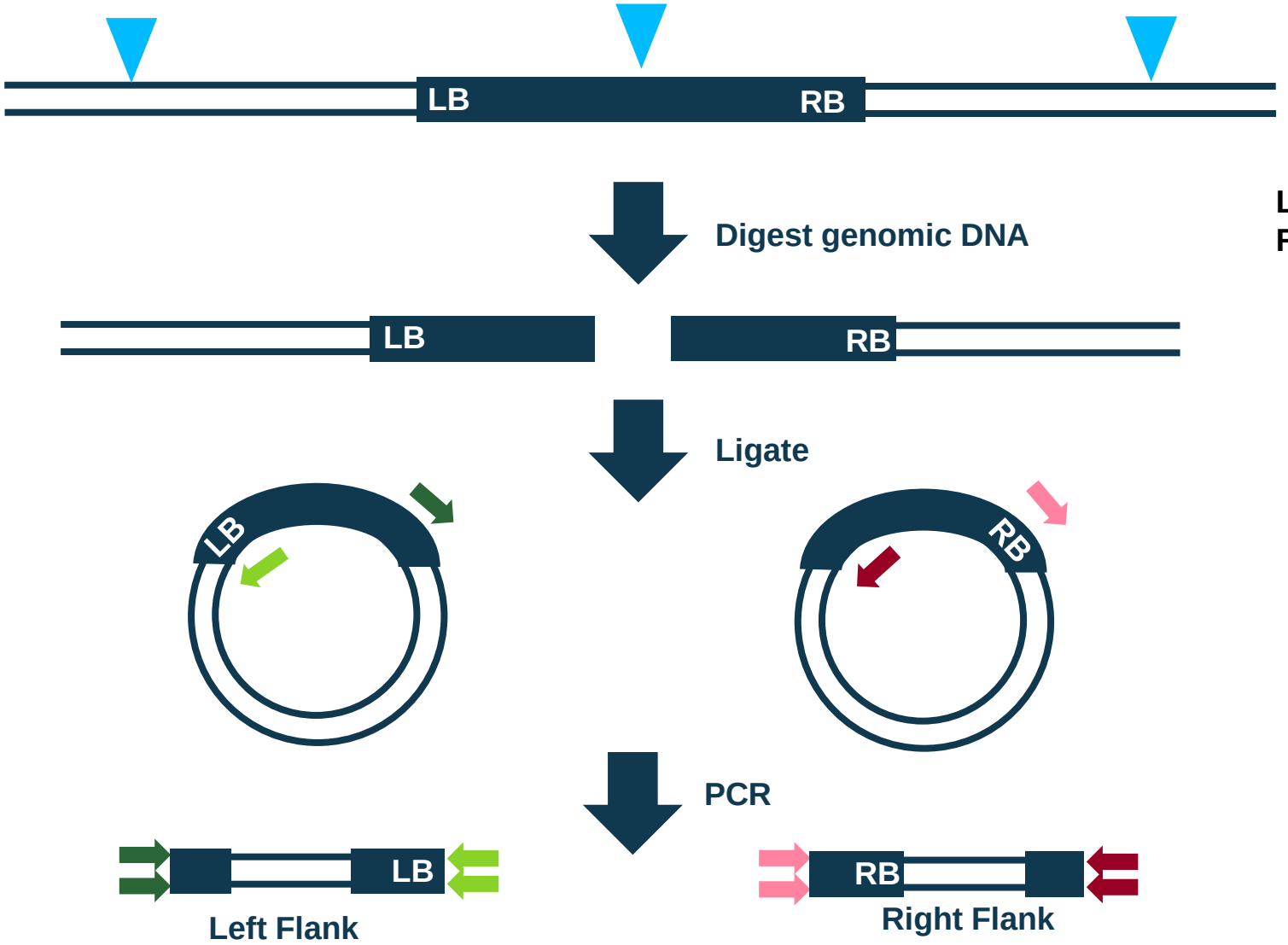


- Detects all events from construct with the element sequence
- Can detect events from different constructs if constructs contains the same element sequence
- Used to determine copy number of TDNA
- Does not require genomic flank DNA sequence
- For example: NPTII assay in this training is an element-based assay

Event-specific junction assay

- Typically designed to detect one TDNA insertion
- Often used when the same event will be used or grown many times (as in commercial traits)
- Will not detect multiple events or events from a different construct
- Can be designed as RT-PCR assay or endpoint TaqMan® assay
- Requires isolation of genomic flank DNA for assay development

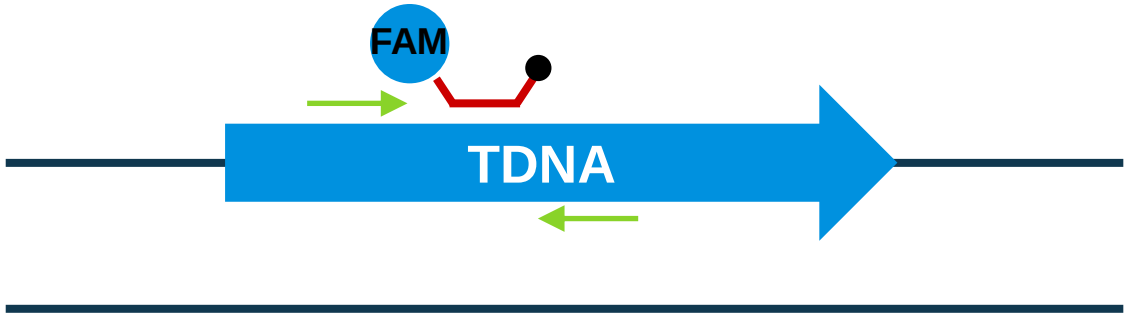
Inverse PCR can be used to obtain DNA sequence of genomic flank of transgene inserts to develop event-specific assays



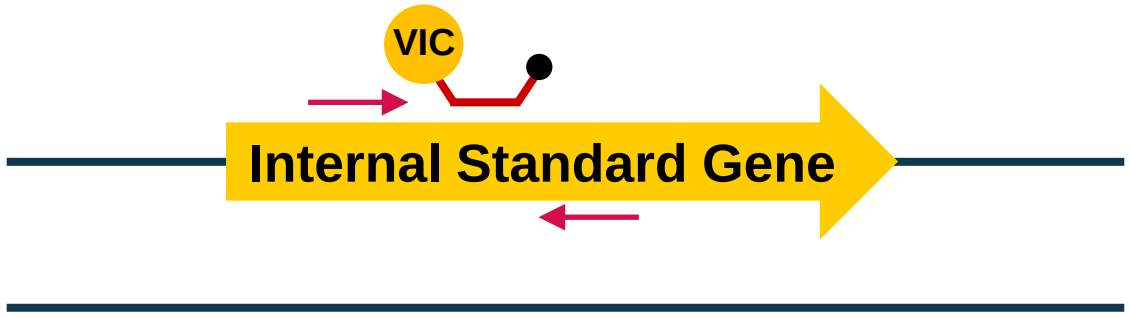
LB = Left border TDNA element
RB = Right border TDNA element

TaqMan® assays can be run as duplex assays

Gene of interest Target



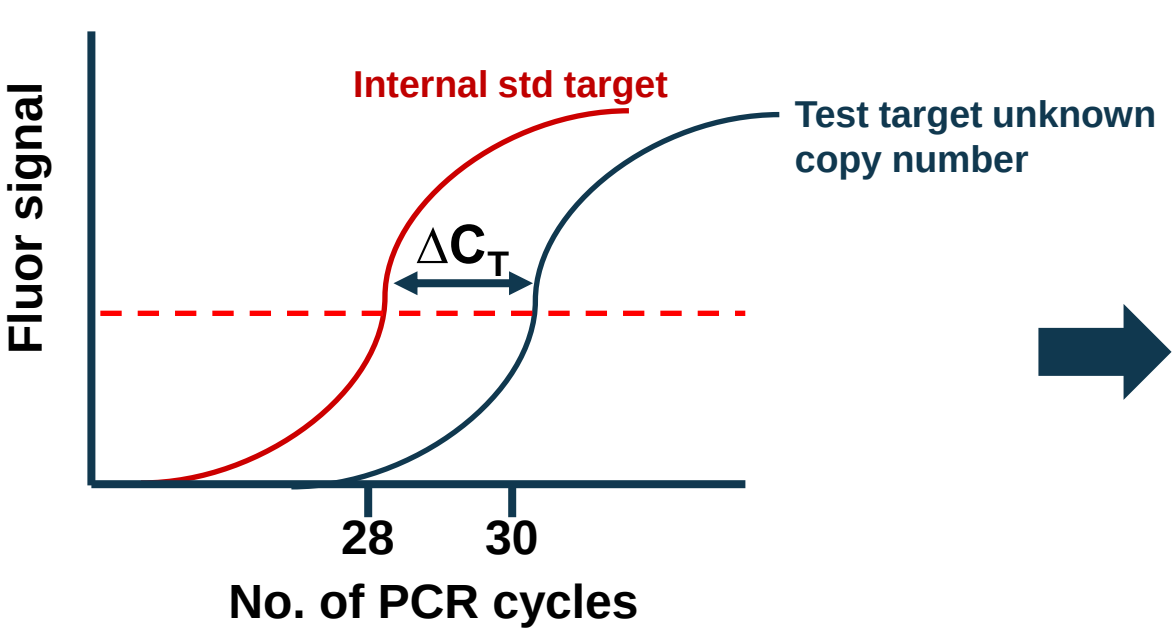
Internal Standard Target

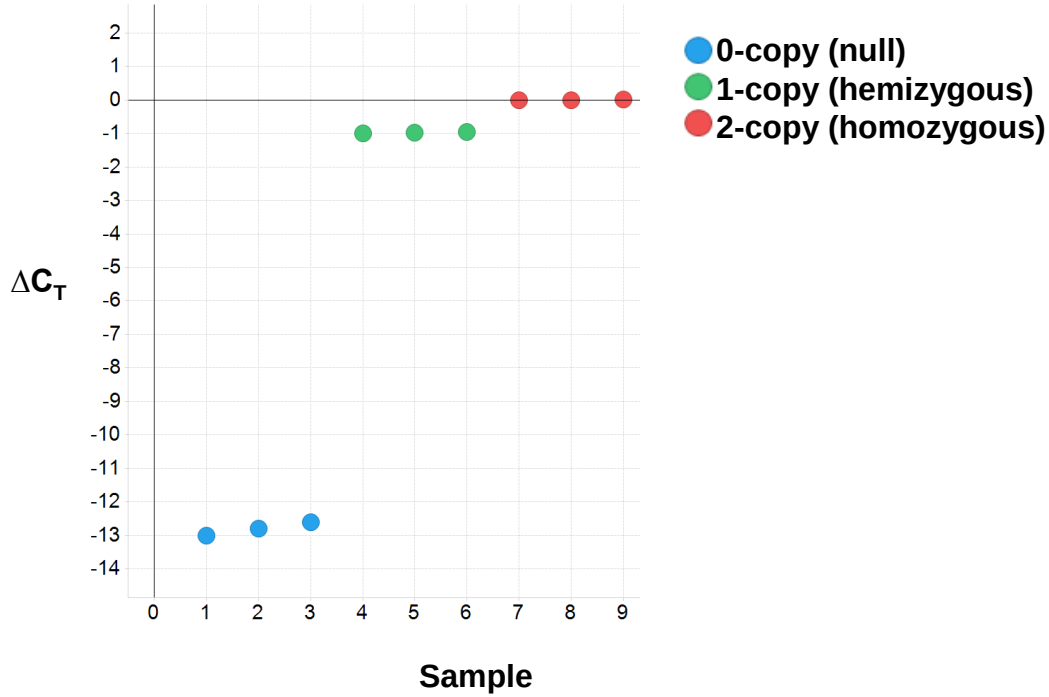


- Typically run TaqMan® assays as a duplex

Step	Reagent	Stock Conc. (µM)	Vol. (ml)	Final Conc. (µM)
--	Reaction Volume		5	
1.	2X Master Mix		2.50	
2.	Primer 1 - GOI	100	0.02	0.4
3.	Primer 2 – GOI	100	0.02	0.4
4.	FAM-Probe -GOI	100	0.01	0.2
5.	Primer 3 – Internal Std	100	0.02	0.4
6.	Primer 4 – Internal Std	100	0.02	0.4
7.	VIC-Probe – Internal Std	100	0.01	0.2
8.	Extracted DNA (template): • Leaf Samples to be analyzed • Negative control (non-transgenic DNA) • Negative water control (No template control) • Positive DNA control		2.4	

Real-time TaqMan® can be used to determine gene copy number by measuring ΔC_T of transgene target relative to internal standard target





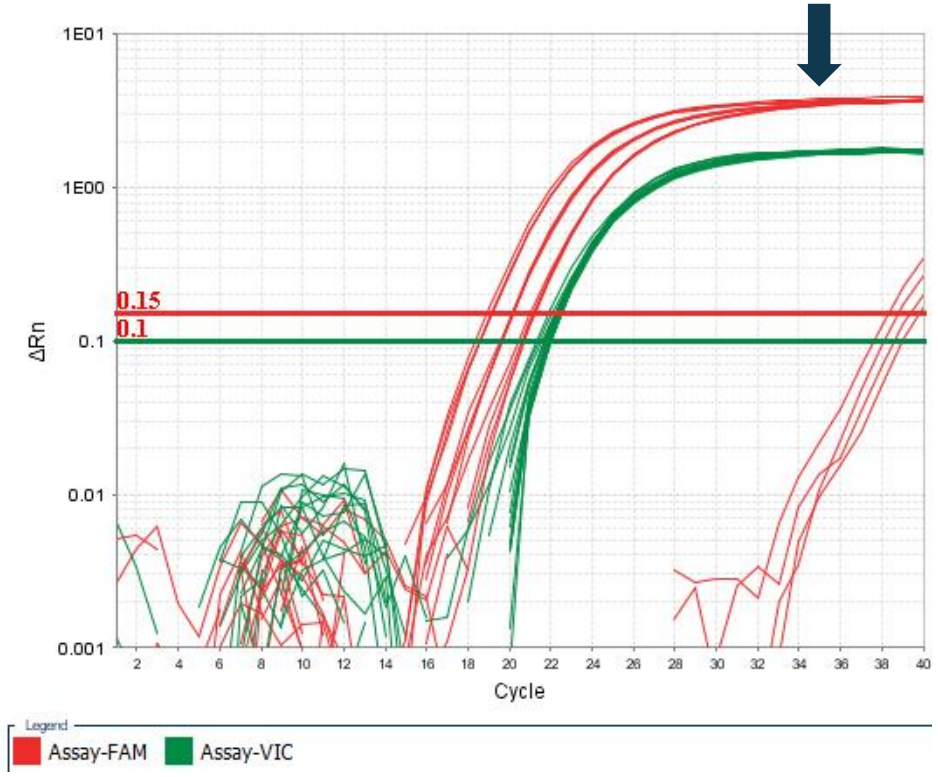
C_T = cycle threshold, the PCR cycle number at which the fluorescent signal exceeds background

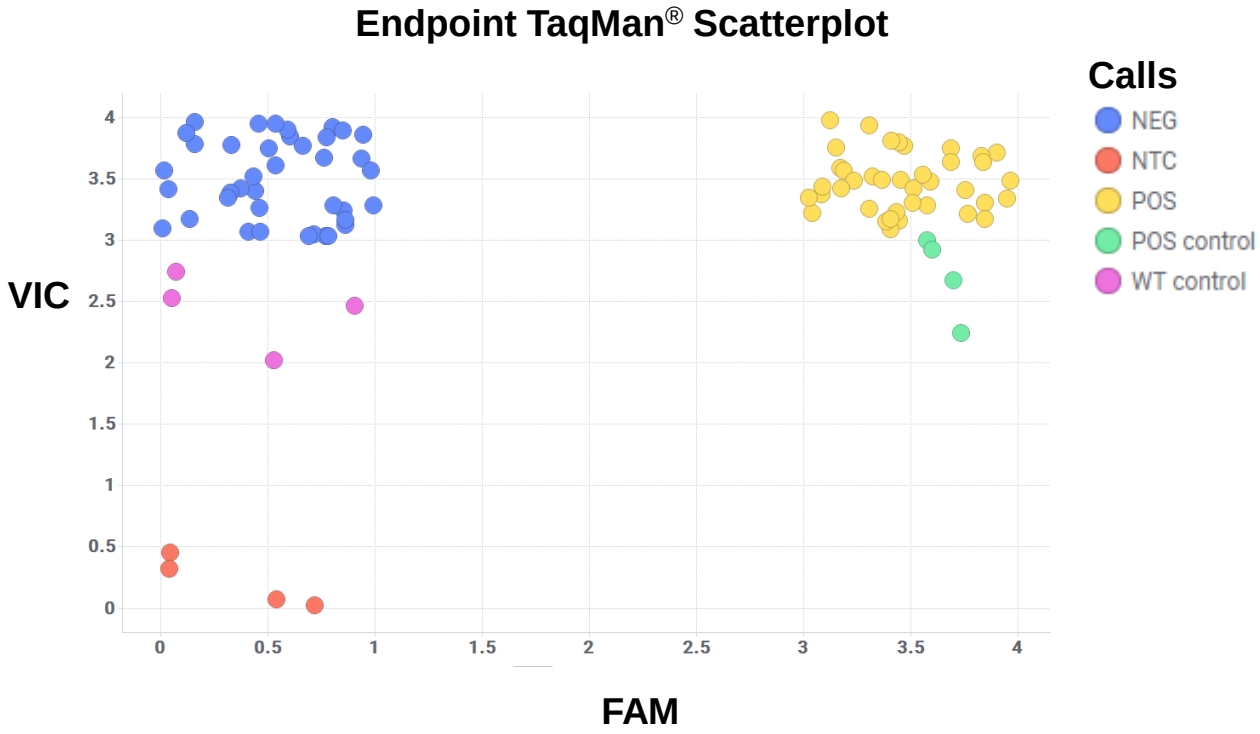
ΔC_T is the difference between C_T of test target of unknown copy number and internal standard target of known copy number

Used for both insert number determination and zygosity

Endpoint TaqMan® assays can be used as qualitative test for presence or absence of a transgene

“Endpoint” TaqMan® uses one fluorescence reading after a specified number of cycles

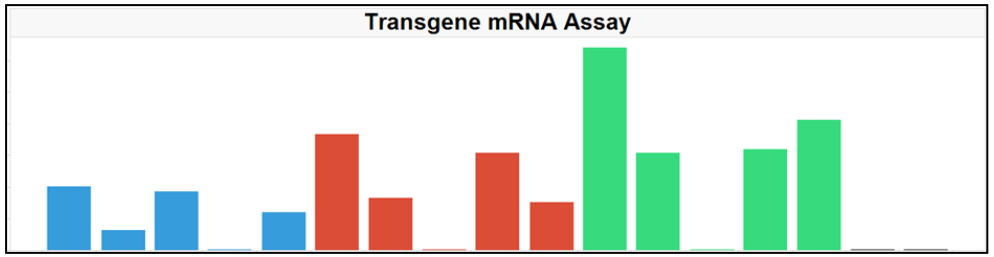




Gene expression level can be measured by reverse transcription Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) method

qRT-PCR

Transcription

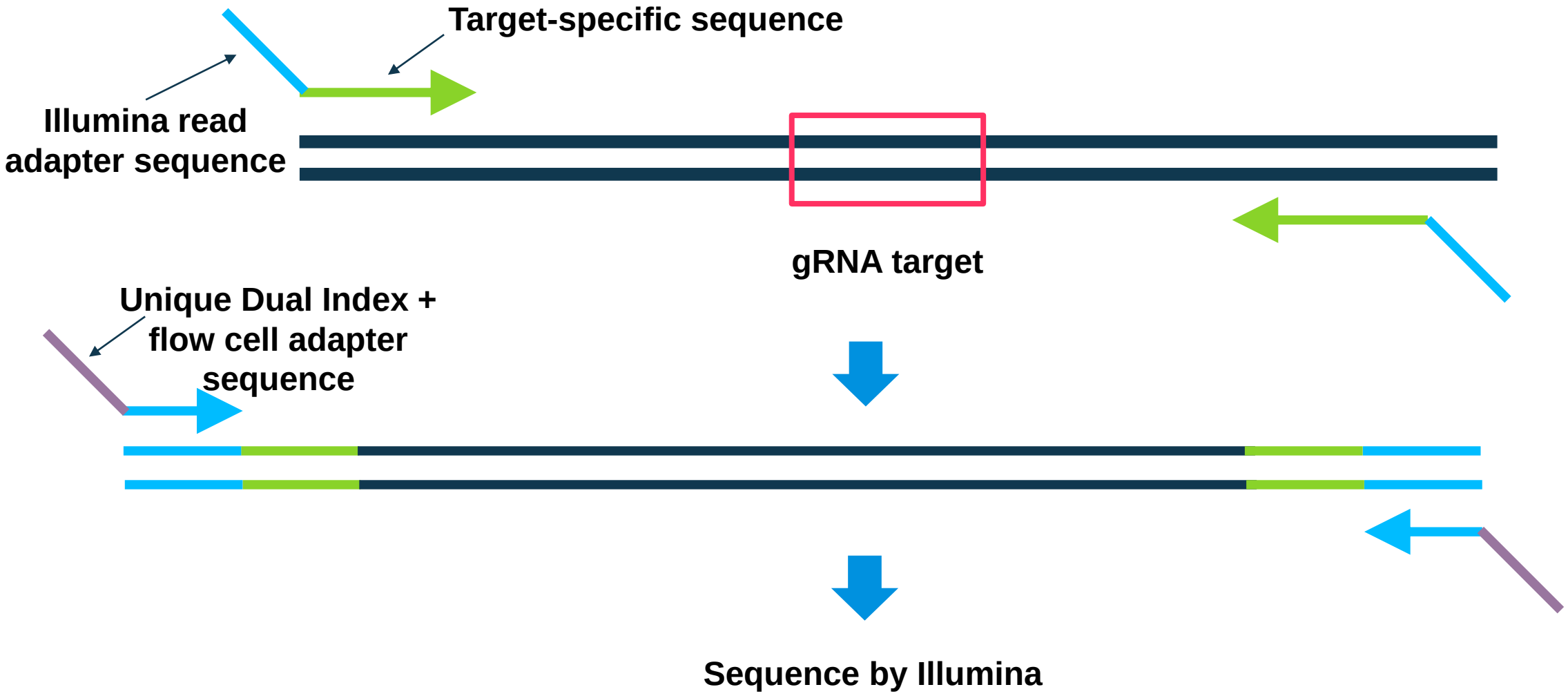


Reverse transcription

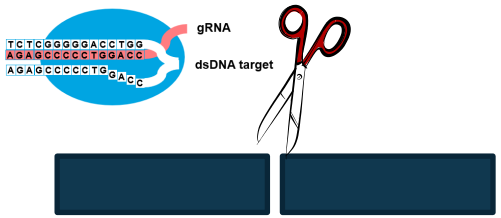
cDNA

Quantitative PCR

Amplicon sequencing for gene edits involves a two-step PCR method



Gene edits can be identified by amplicon sequencing



GAGGGC__GCCATCA

GAGGGCATGCCATCA

Select for edit at T0 with amplicon sequencing

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGC__GCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

GAGGGCATGCCATCA

Select for homozygous edit at T1 with amplicon sequencing

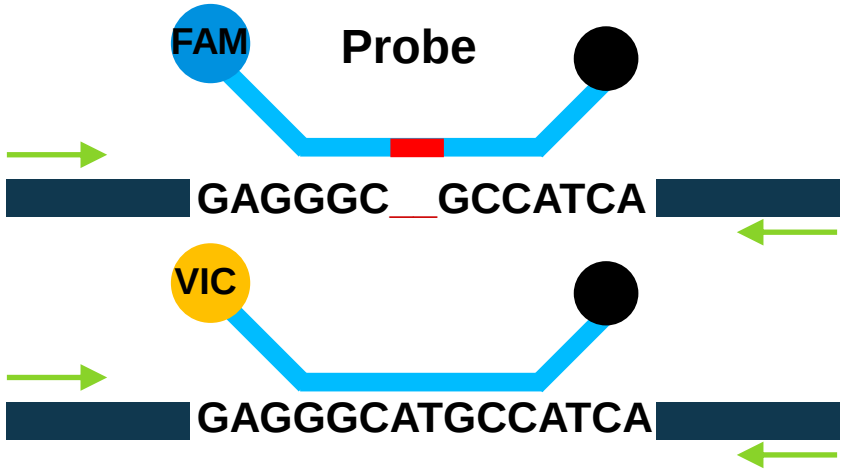
GAGGGC__GCCATCA

GAGGGC__GCCATCA

Confirm homozygous for edit at T2 with amplicon sequencing

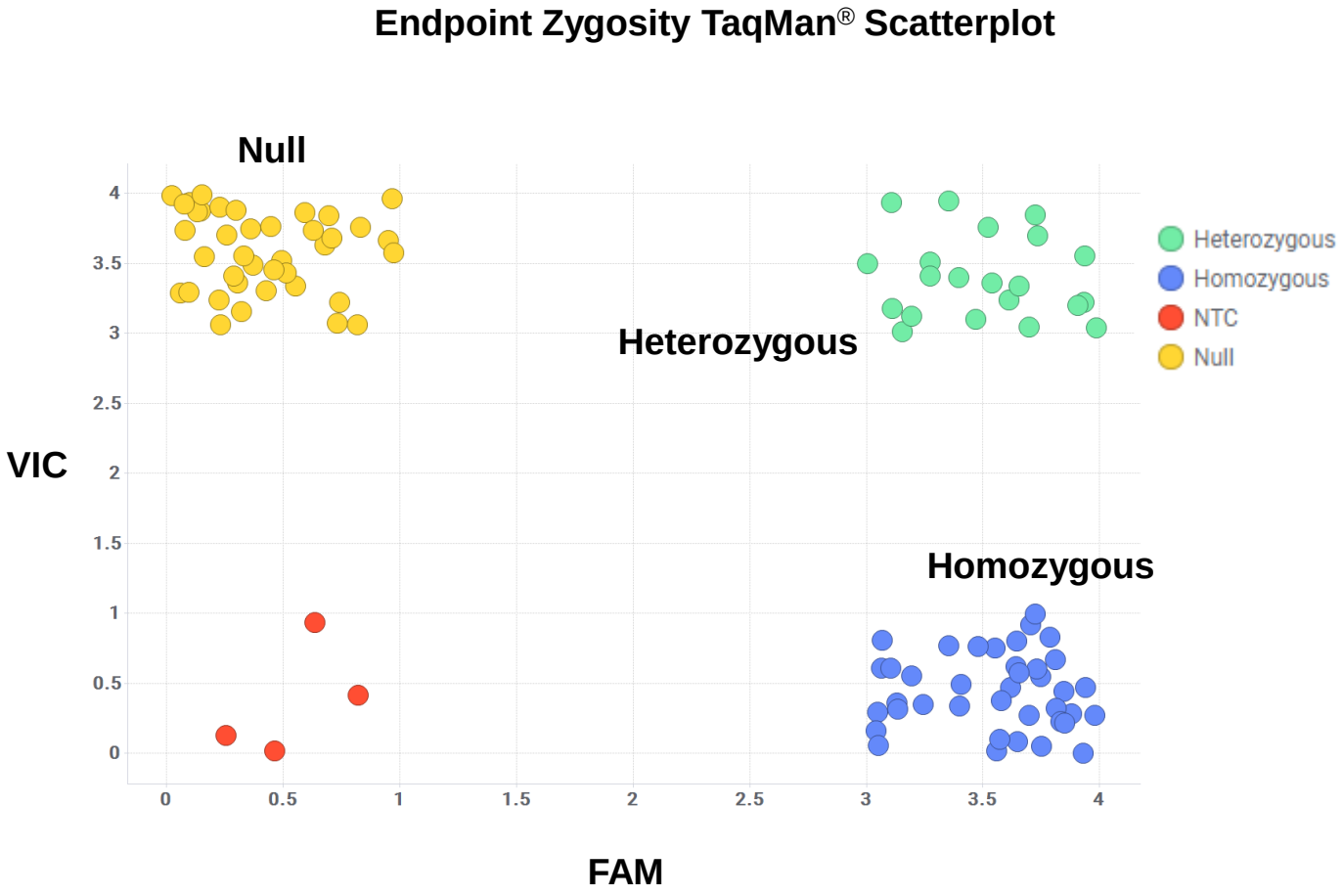
Develop edit-specific TaqMan® assay

Edit-specific TaqMan® assay can be used for breeding after a gene edit is fixed as homozygous



Edit-specific assay is like a typical SNP (single nucleotide polymorphism) assay

(2 primers, 2 probes)



Considerations for detection workflow and automation

- Using 384-well format increases throughput, but also increases sample tracking complexity
  - Consolidating tissue sampling in 96-well format to 384-well assay format increases complexity
- Laboratory information management systems improve sample tracking
- Automated liquid handlers that can used to automate liquid transfer steps for all assays
  - Example manufactures: Agilent, Hamilton, Tecan, Formulatrix, SPT Labtech
- RT-PCR instruments can be used for multiple assays:
  - Copy number variation for DNA targets (copy number assays)
  - Copy number for zygosity
  - Qualitative Endpoint TaqMan® assays for presence-absence
  - Gene expression determination
- Endpoint TaqMan® can also be determined using combinations of thermocylers and fluorescence readers
  - Having separate thermocylers and fluorescence plate readers gives flexibility for qualitative TaqMan® assay throughput

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Thank you!

Any questions?