Plant Biotechnology

Bayer Russia Plant Biotechnology Conference

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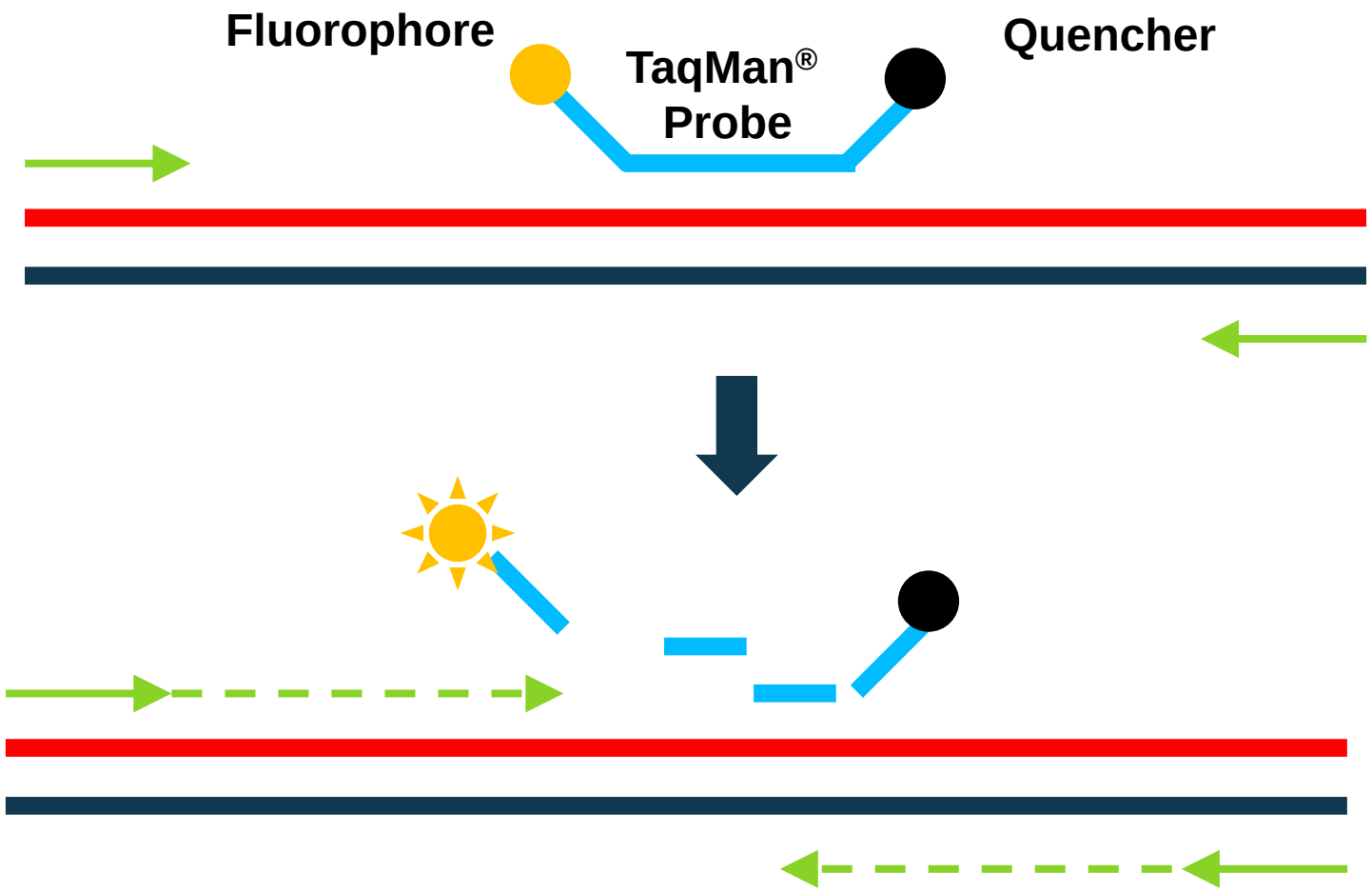
Molecular Assays

Real Time-PCR

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

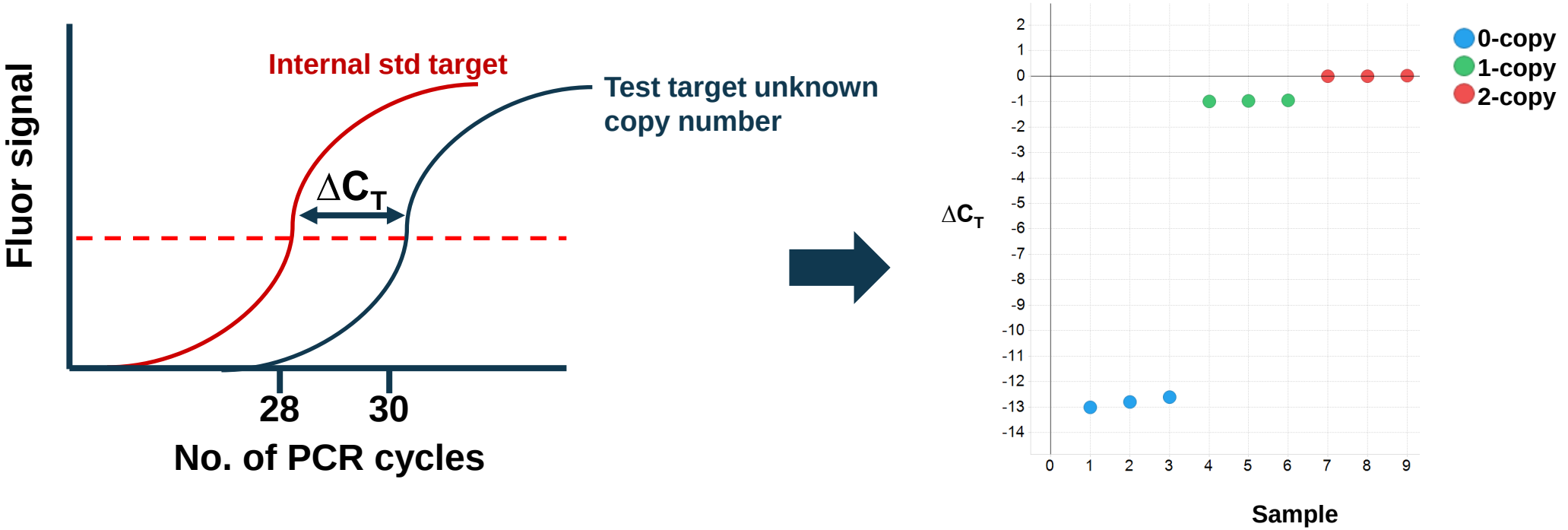
TaqMan® uses PCR and fluorophore-labeled probes to detect DNA sequences



Fluorophore 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)

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

General guidelines for new assay primer design

- Use primer design software, such as Primer Express™ (ThermoFisher)
- Test multiple primer and probe combinations
- Overall for the amplicon
  - Keeping the probe close to the forward primer, without overlap, often contributes to a strong assay
  - 40-60% GC content is best
  - Avoid runs of an identical nucleotide, particularly for guanine, keep it under 4 G's
  - Keep the amplicon length between 50-150 bp
  - Confirm amplicon sequence to target sequence to ensure specificity
- For the probe
  - Select a probe with more Cs than Gs.
  - Melting temperature (Tm) should be 9°C -11°C higher than the primers' Tm when using Primer Express software (or other)
  - Do not put a G on the 5´ end. The cleaved G will quench the FAM or VIC signal. (It is a checkbox within Primer Express)

General guidelines for new assay primer design (continued)

- Primer guidelines
  - Secondary Structure (Primer Dimer): Keep oligo interactions at no more than four.
  - The Tm of each primer should be 59 to 61 °C. If two designs cannot be made in this range, then 55-57°C may be tried for copy number.
  - The five nucleotides at the 3´ end of each primer should have no more than two G and/or C bases (it is a checkbox within Primer Express™)
- It is recommended to follow additional instructions from the manufacturer of the Real-time PCR instrument that is used

Example duplex TaqMan® Assay Protocol

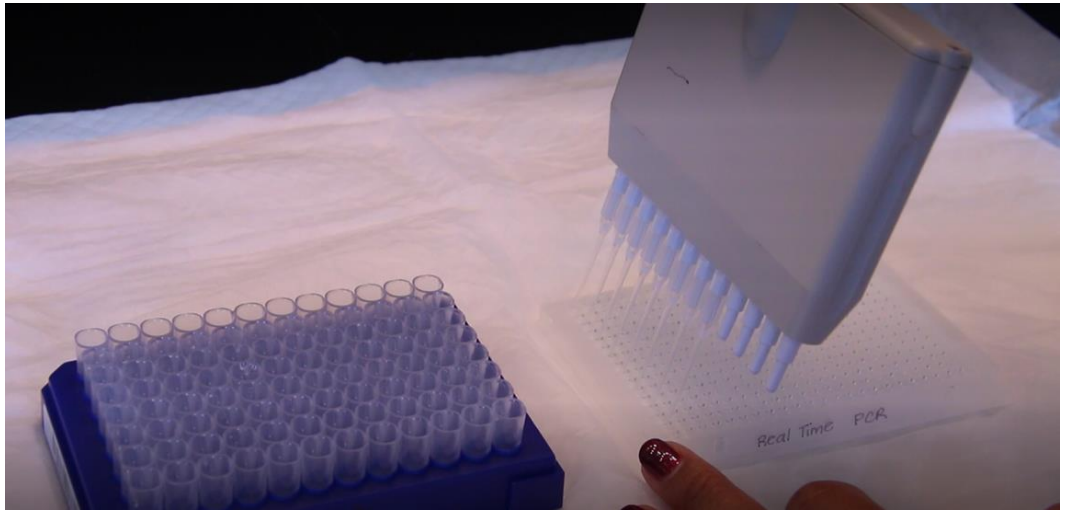
PCR mix

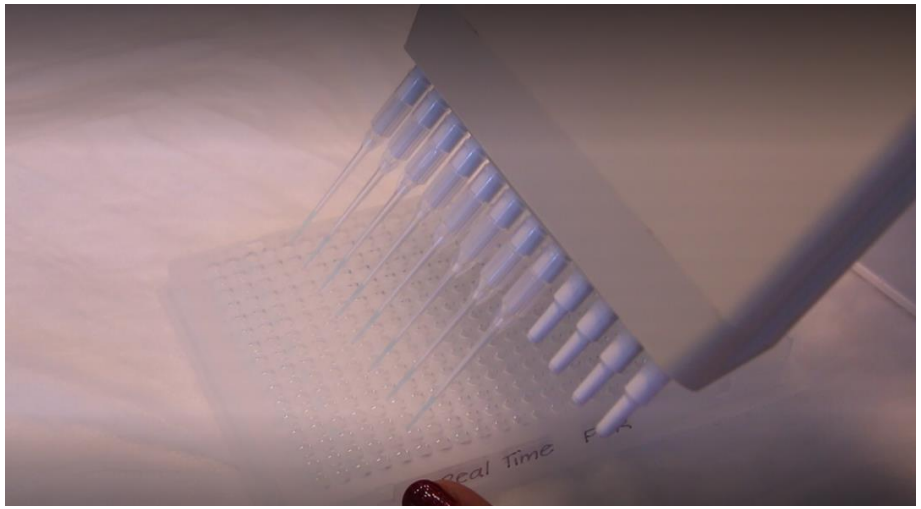
Step	Reagent	Stock Conc. (µM)	Vol. (ml)	Final Conc. (µM)
--	Reaction Volume		5	
1.	2X Master Mix		2.50	
2.	Primer 1 - GOI	100	0.02	0.4
3.	Primer 2 – GOI	100	0.02	0.4
4.	FAM-Probe -GOI	100	0.01	0.2
5.	Primer 3 – Internal Std	100	0.02	0.4
6.	Primer 4 – Internal Std	100	0.02	0.4
7.	VIC-Probe – Internal 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	

PCR protocol

Step No.	Cycle No.	Settings
1	1	95°C 20 seconds
2	Usually 35, sometimes 40	95°C 3 seconds
3	Usually 35, sometimes 40	60°C 20 seconds
4	1	10°C hold until retrieved

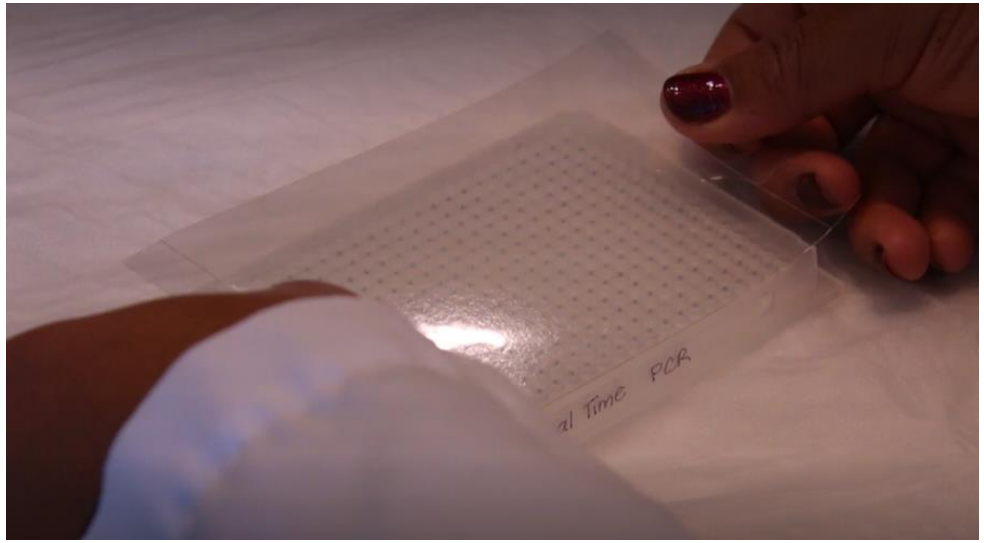
Preparing PCR reactions

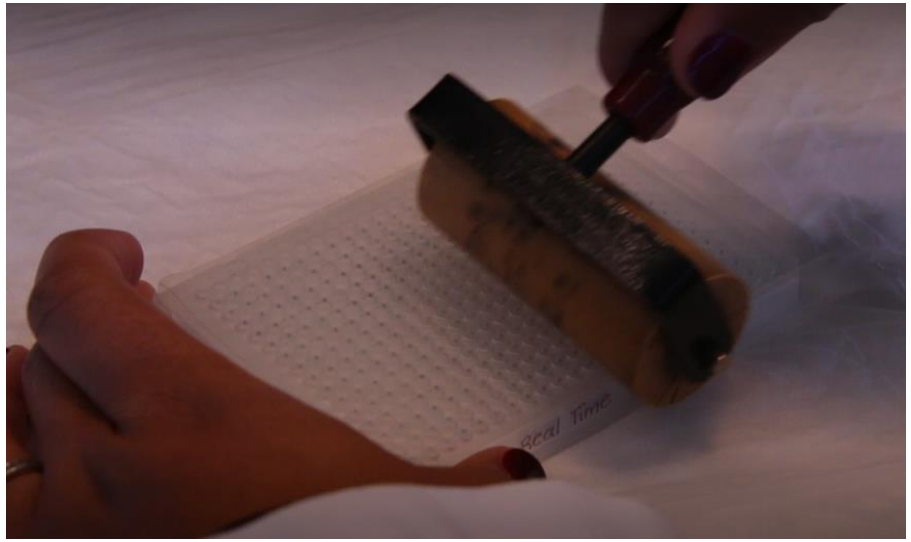




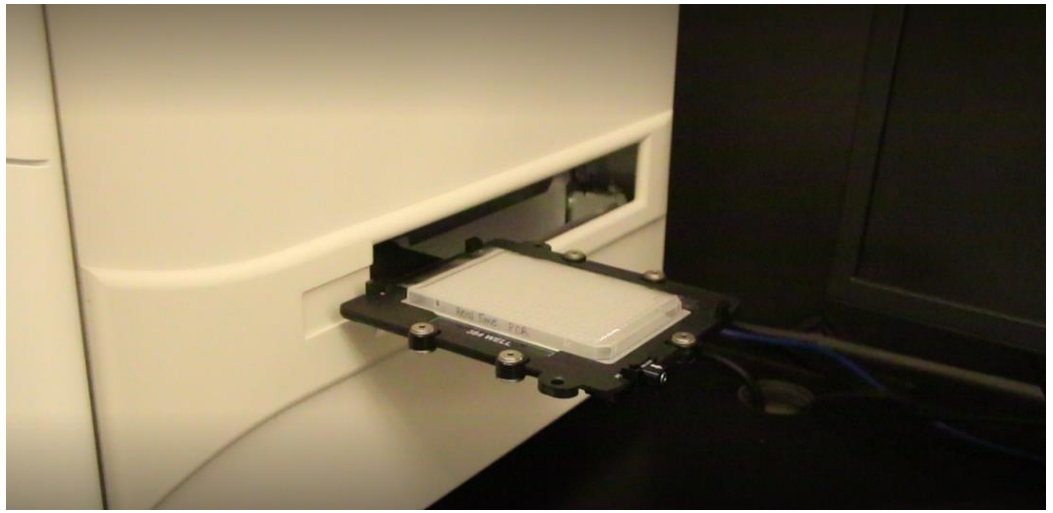
- Add DNA to plates for PCR
- Add PCR mix to plates (primers, probes, and enzyme)

Seal PCR plates with sealing film

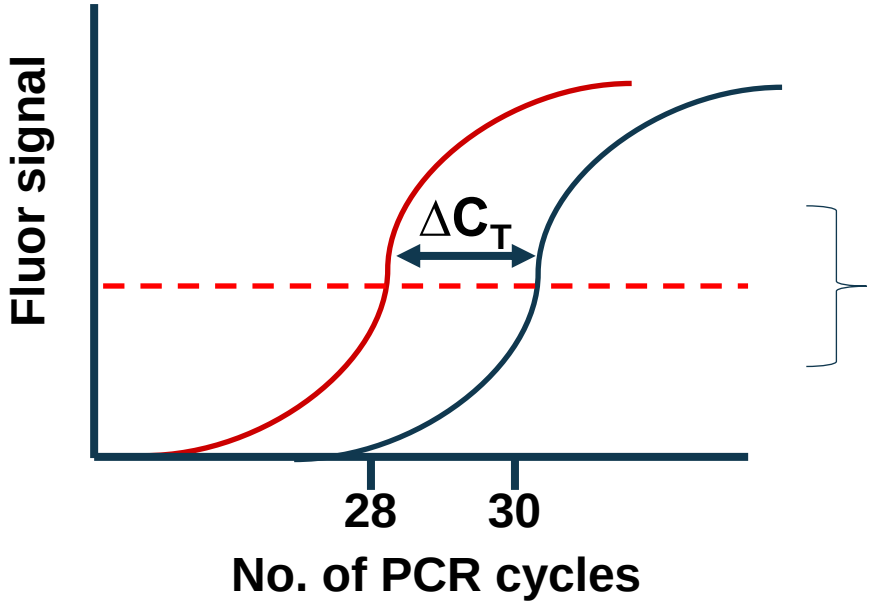




Load PCR plate into Real-time PCR instrument



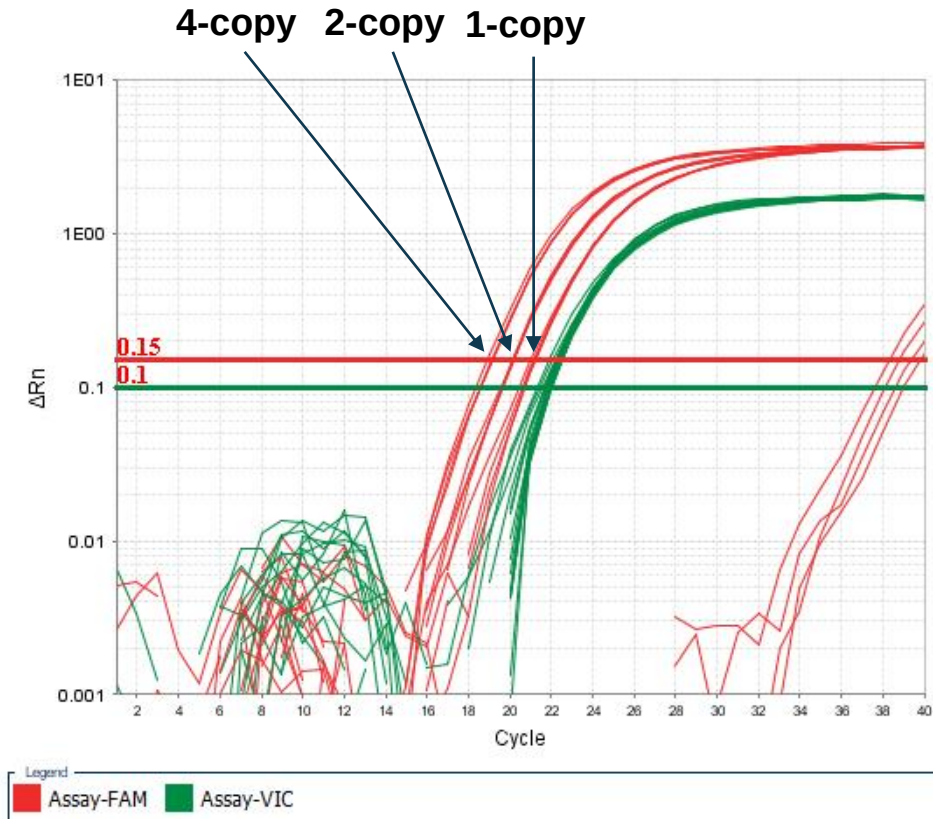
Set the cycle threshold for the run data

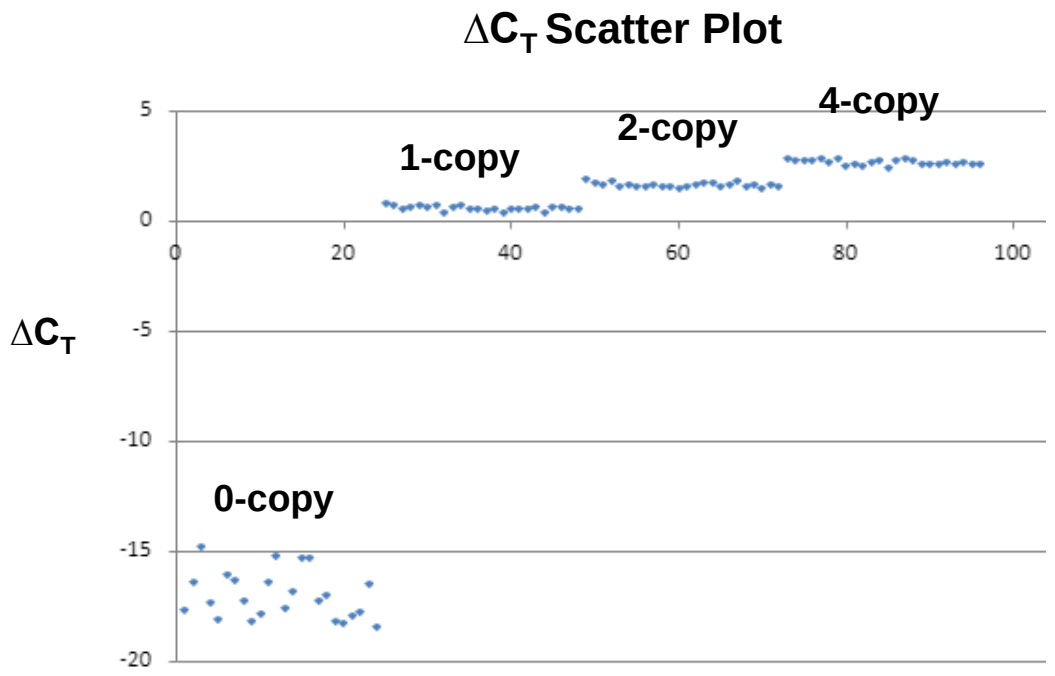


Set the threshold:

- **Above the background of the amplification curve**
- **Below the plateau and linear regions of the curve**
- **Within the exponential phase of the curve**

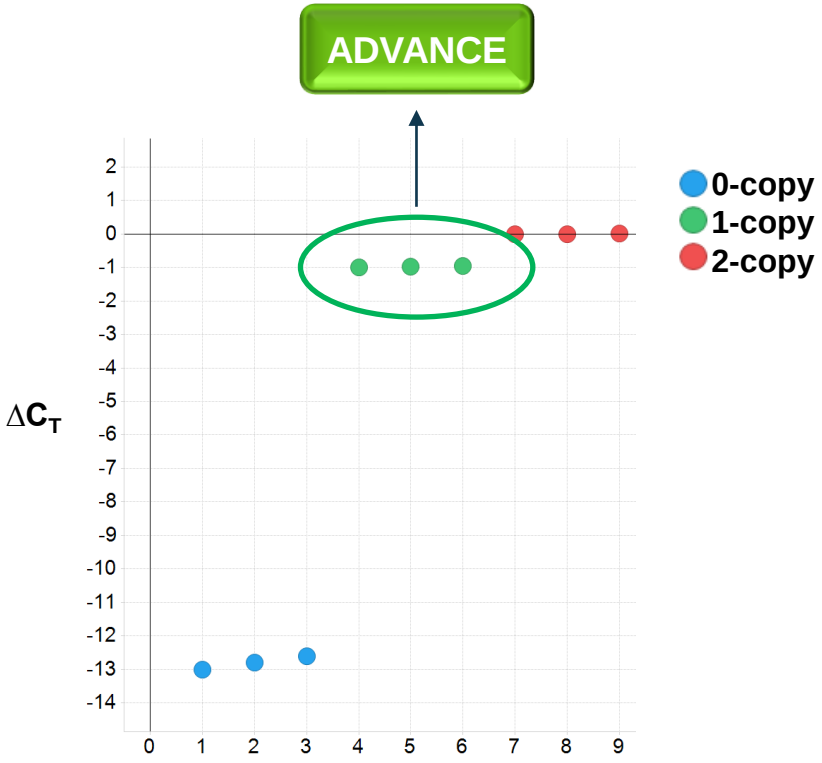
Example plot of validation of a copy number assay

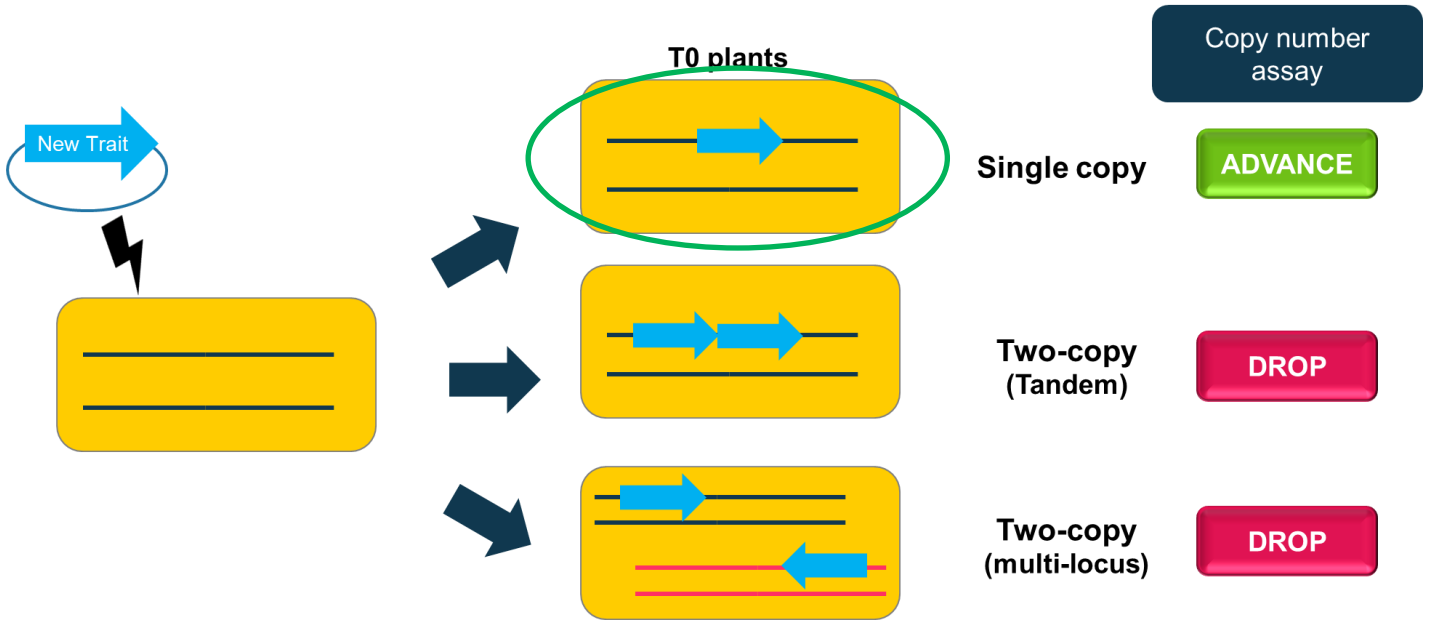




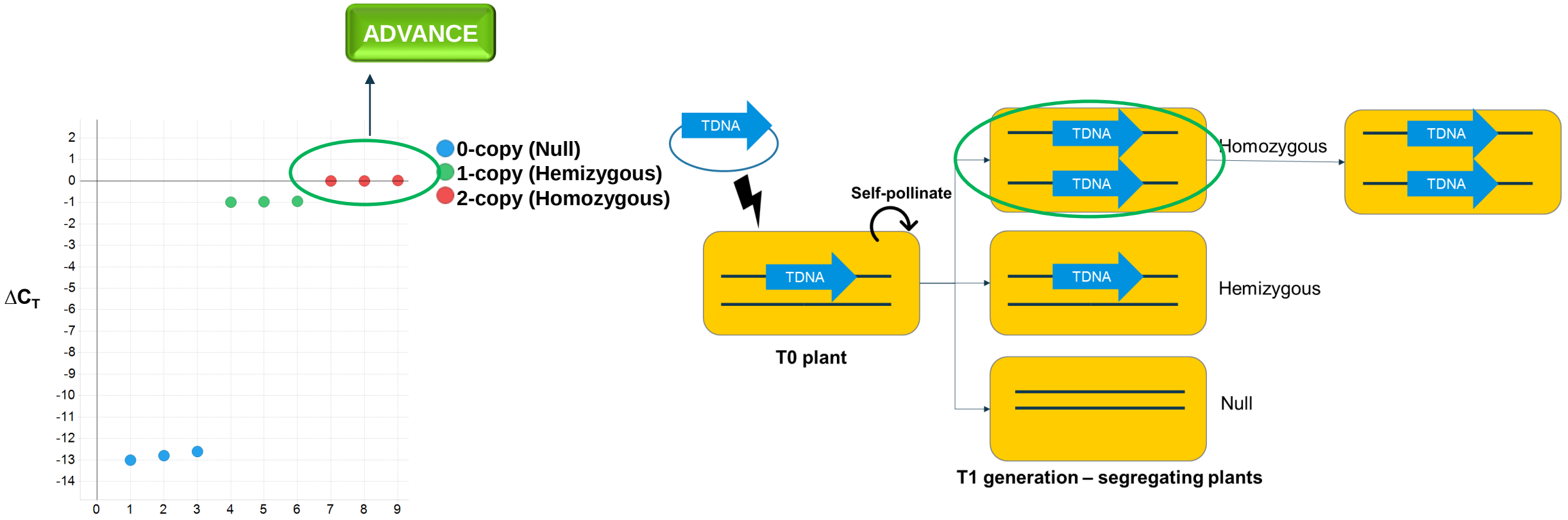
Assay should show clear separation on different copy number populations
For plant selection purposes, separation of 0, 1, and 2 copy populations are most important

T0 plant selection: Selecting the single-copy plants using ΔC_T scatter plots



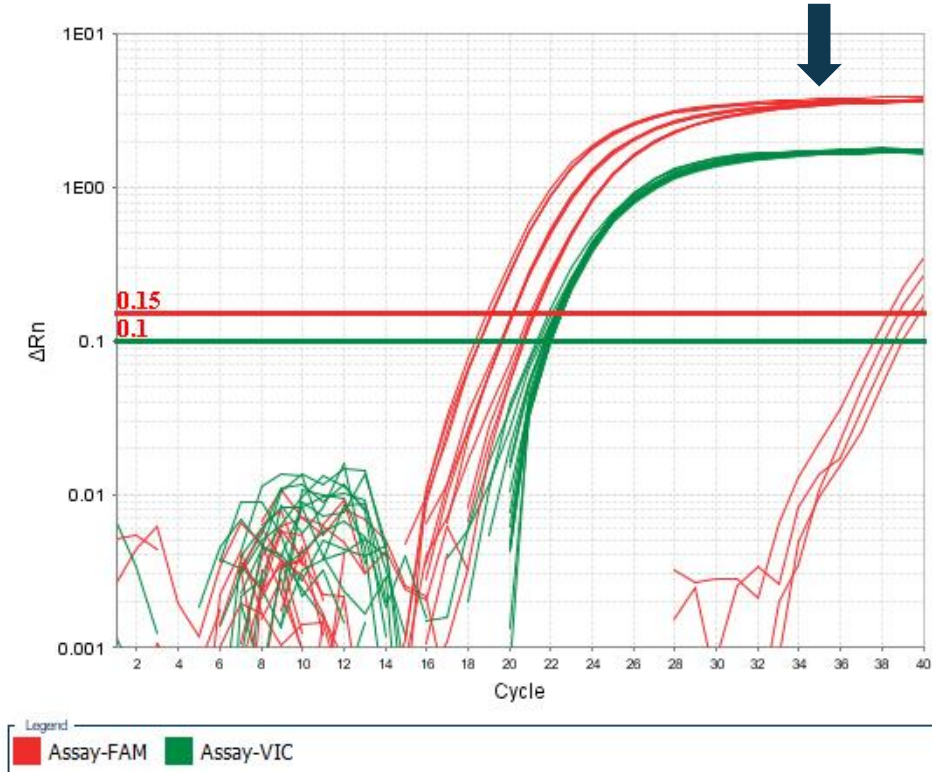


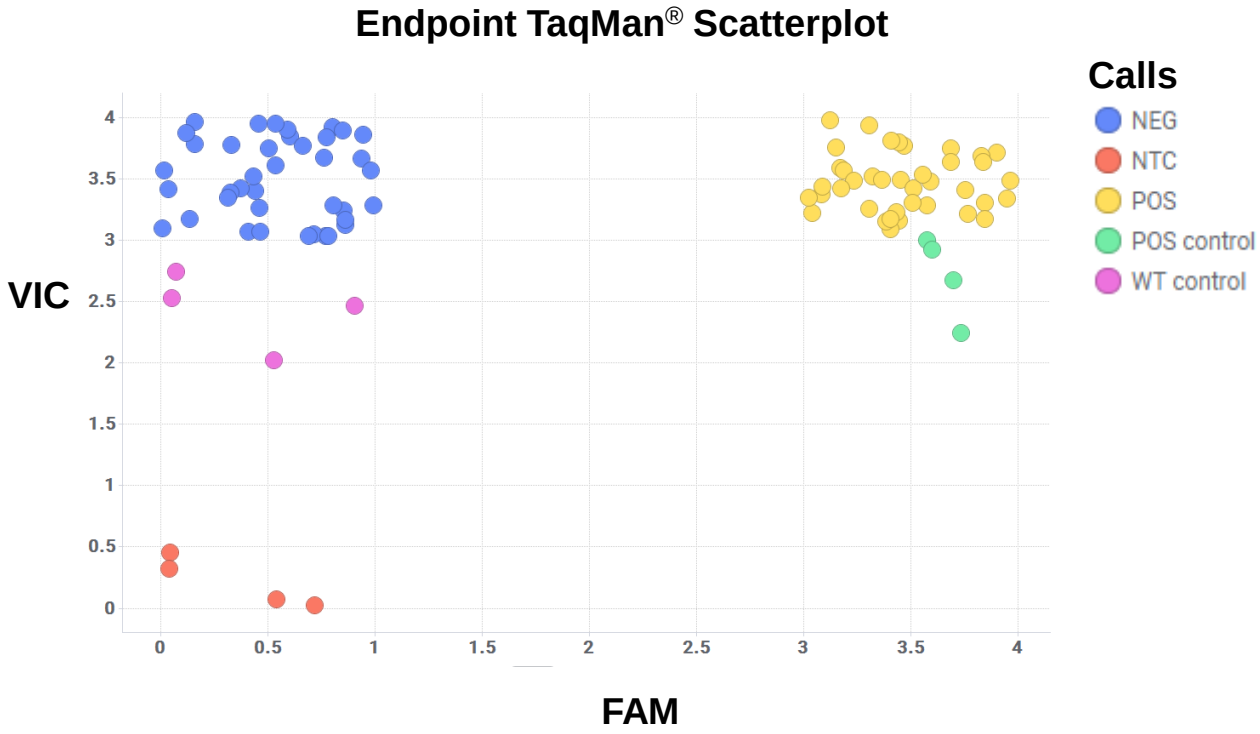
T1 plant selection: Selecting the homozygous 2-copy plants



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

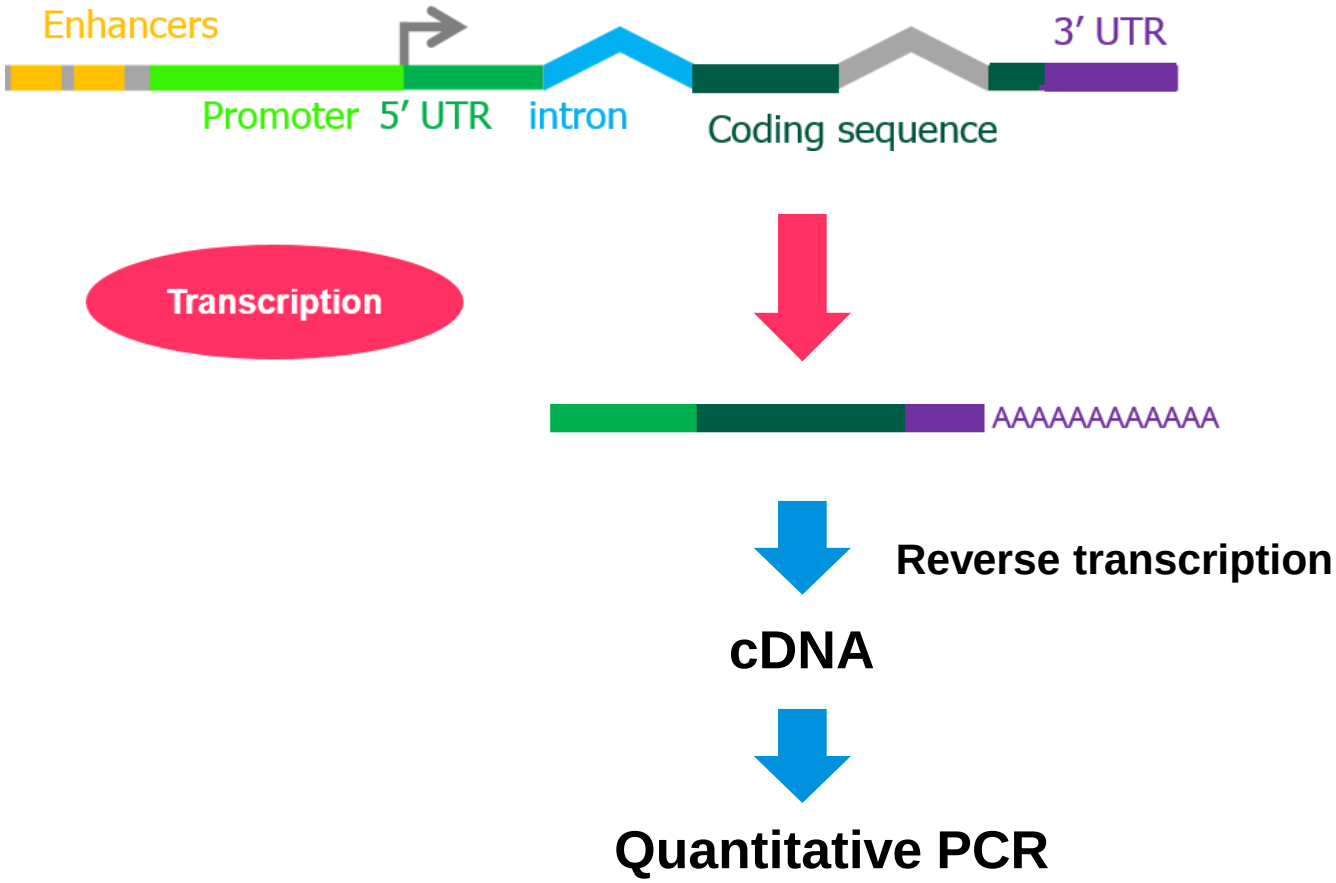




Real-time and Endpoint PCR instrumentation and automation considerations

- 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 PCR can also be determined using combinations of thermocyler and fluorescence readers
  - Having separate thermocylers and fluorescence plate readers gives flexibility for qualitative TaqMan® assay throughput
- There are many automated liquid handlers that can used to automate liquid transfer steps
  - Example manufactures: Agilent, Hamilton, Tecan, Formulatrix, SPT Labtech

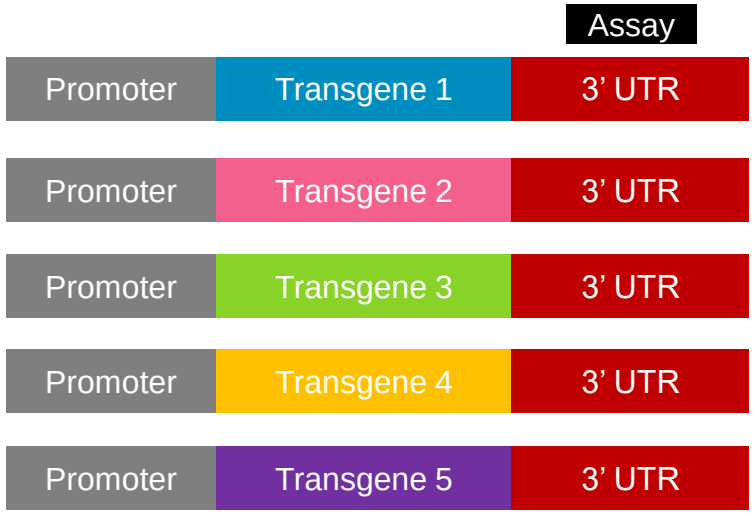
Gene expression level can be measured by Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) method



Assay design and selection can aid in expression evaluation in different vectors

A single assay can be used to do direct comparisons for mRNA expression

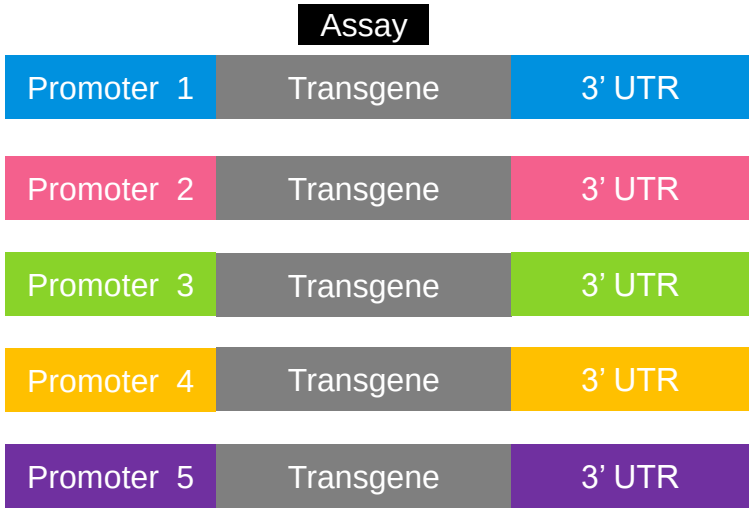
Scenario 1



Assay can be designed to 3' Untranslated regions (UTR)

- Useful where different transgenes are being evaluated

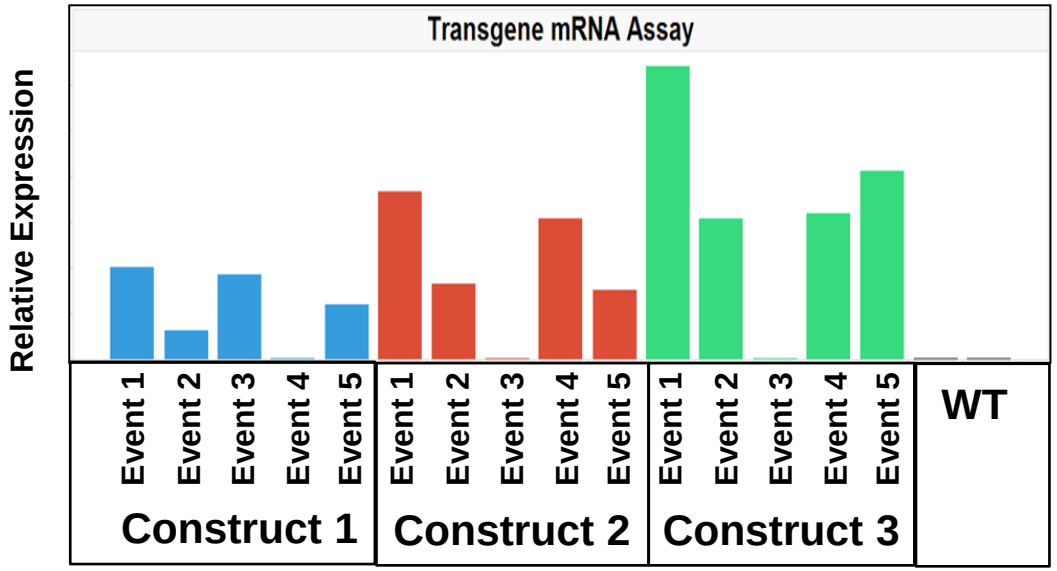
Scenario 2



Assay can be designed to transgene

- Useful where different promoters or 3' Untranslated regions (UTR) are evaluated

Gene expression level can be used to construct comparison and event selection for transgenes



- Compare gene expression across constructs and events at T0 generation
- Compare gene expression of hemizygotes (1-copy) to homozygotes (2-copy) at T1 generation

Real Time – quantitative PCR

Gene expression methodology considerations

- Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) requires RNA that is high quality and high purity to accurately quantitate messenger RNA
  - Care must be taken to avoid DNA and RNAse contamination
- Use commercially-available extraction kits and follow manufacturer's protocol
  - Example: DirectZol96 from Zymo Research (Irvine, CA USA)
  - Sample collection of leaf must be on dry ice or liquid nitrogen
- Use commercially-available cDNA synthesis kits
  - Example: High capacity cDNA kit by Life Technologies (Thermo Fisher Scientific)
- Liquid handlers can be used to automate large samples numbers

Gene expression methodology

1. Extract RNA from leaf using DirectZol96 from Zymo Research (Irvine, CA USA)
2. Perform cDNA synthesis step with either of three primer options:
    1. Random Primers (consider this for screening as more robust for different targets)
    2. Oligo dT primers
    3. Gene-specific primers

    - Check RNA concentration as it should not exceed the input maximum for the reverse transcriptase reaction in order to be in the linear range of enzyme processivity
3. Perform PCR assay on Real-time PCR instrument
    1. Both TaqMan and SYBR green assays can be used
    2. Consider running assay as singleplex with GOI target for second singleplex with internal standard gene
4. Calculate Relative Quantity (RQ) based on transformation of delta Ct to linear scale

For further reading on gene expression

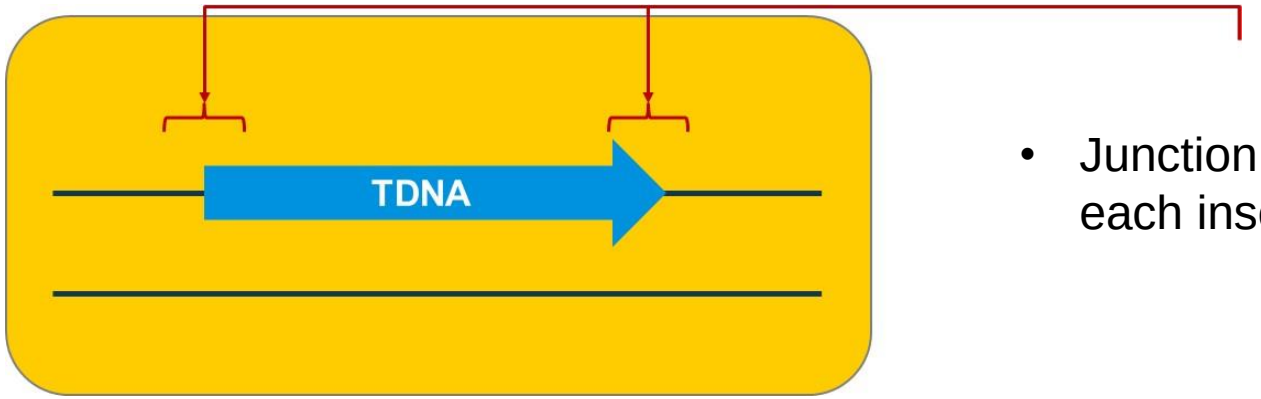
- Millipore-Sigma
  - https://www.sigmaaldrich.com/US/en/technical-documents/technical-article/genomics/qpcr/quantitative-pcr
- Thermo-Fisher
  - https://assets.thermofisher.com/TFS-Assets/LSG/manuals/cms_042380.pdf

Molecular Assays

Inverse PCR for TDNA junction sequencing

Genomic flank DNA sequence and TDNA junction sequences of transgenic are used in multiple ways

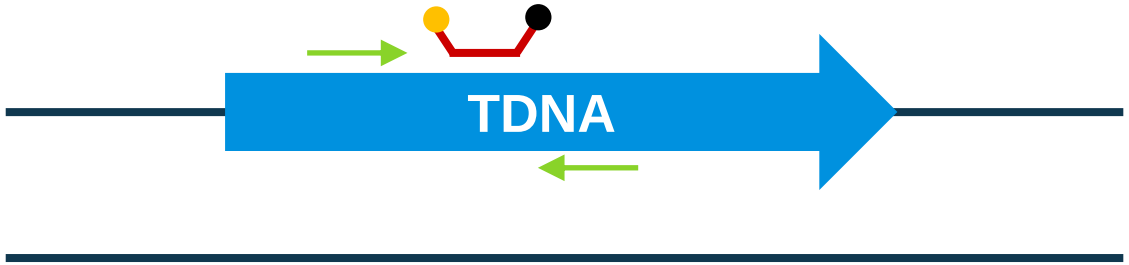
- Determines the context of the insertion
  - Is it inserted in or near an existing gene or trait?
  - Is it in a repetitive sequence in the genome?
- Used to identify and track specific transgenic events
  - Where is it located in the genome?



- Junction sequences can uniquely identify each insertion

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

Genetic element-based assay

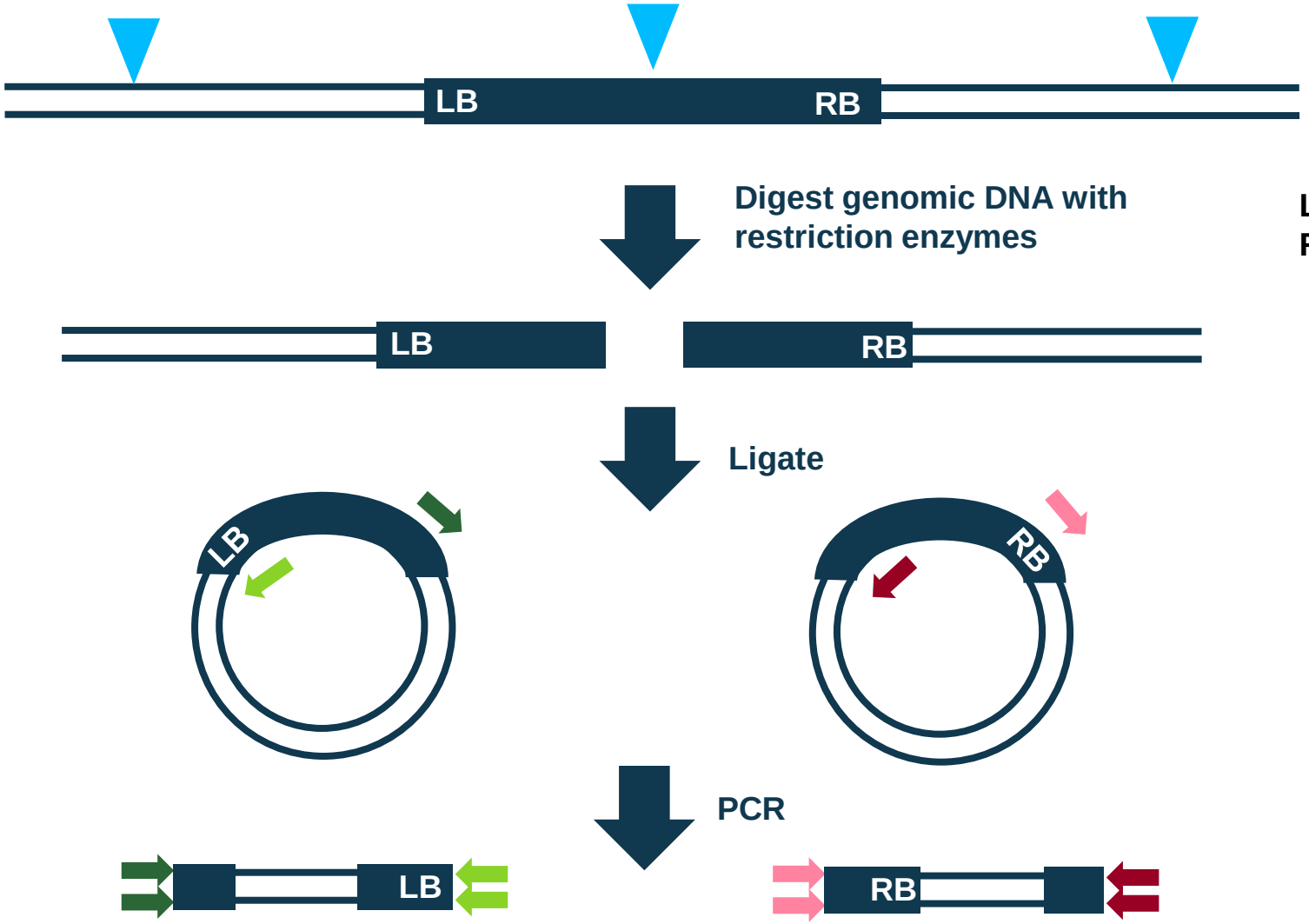


- Detects all events from construct with the element sequence
- Can detect events from different constructs if constructs contains the same element sequence
- Used to determine copy number of TDNA
- Does not require genomic flank DNA sequence
- For example: NPTII assay in this training is an element-based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

Event-specific junction assay

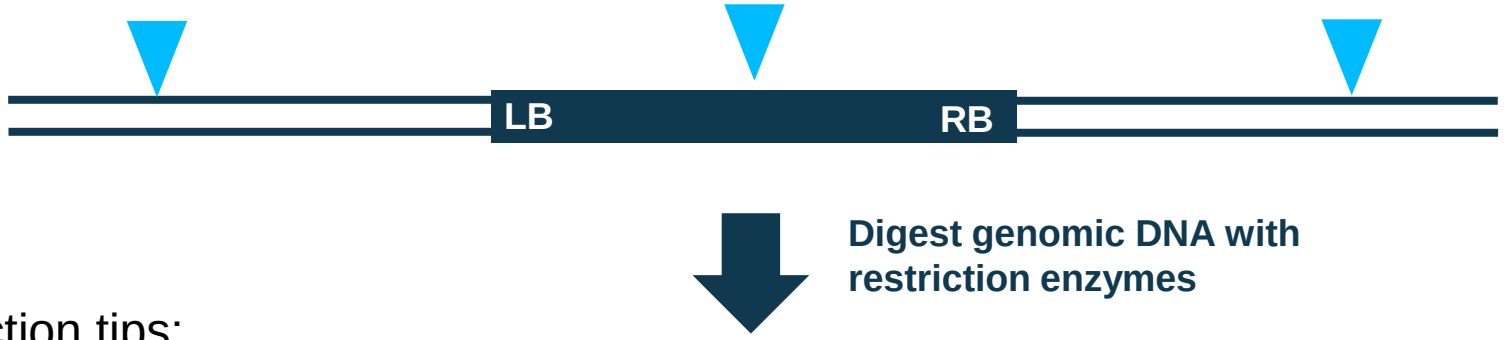
- Typically designed to detect one TDNA insertion
- Often used when the same event will be used or grown many times (as in commercial traits)
- Will not detect multiple events or events from a different construct
- Can be designed as RT-PCR assay or endpoint TaqMan® assay
- Requires isolation of genomic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

Inverse PCR can be used to obtain DNA sequence of genomic flank of transgene inserts



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

Inverse PCR can be used to obtain DNA sequence of genomic flank of transgene inserts



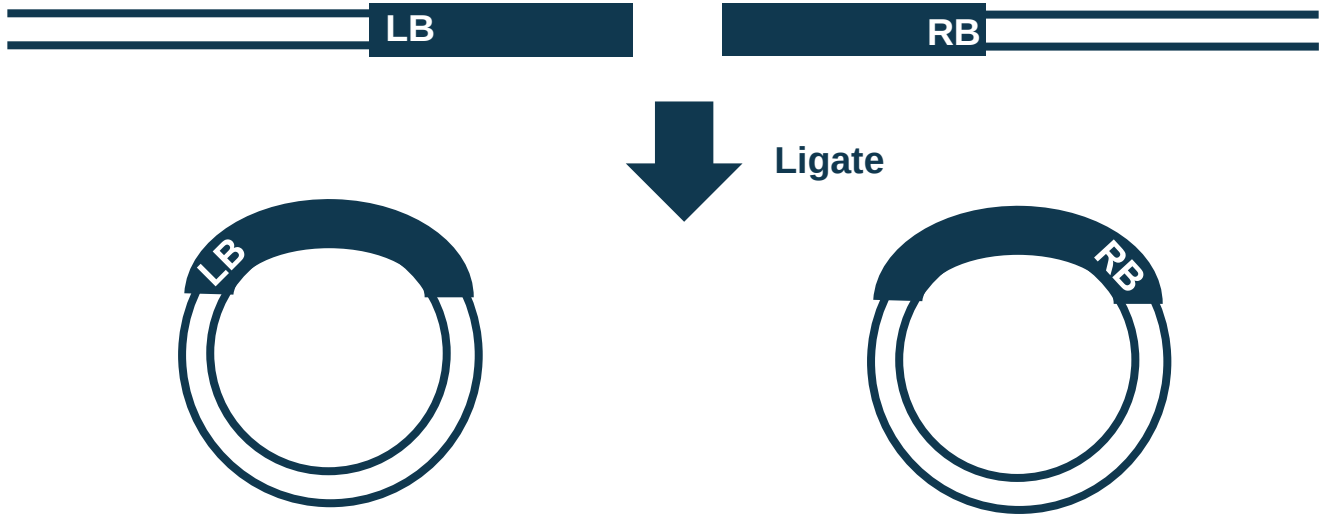
Enzyme selection tips:

- Choose ‘sticky end’ restriction enzymes that can be heat inactivated (6 or 8 base cutter)
- Avoid enzymes that recognize degenerate bases
- A single cutting enzyme in the TDNA may be used to isolate both flanks with two independent PCR reactions
- An enzyme that does not cut in TDNA may be used to isolate both flanks within one reaction

Procedure:

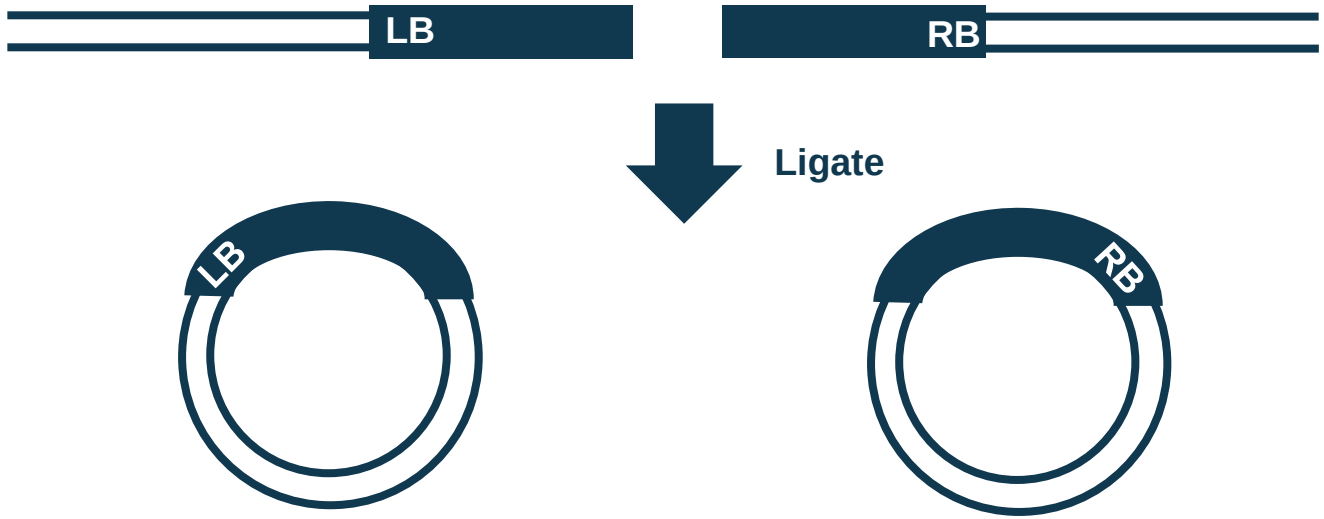
- Digest 200 ng of genomic DNA with restriction enzyme in 20 microliters for 1 hour at recommended temperature for enzyme
- Heat inactivate at recommended temperature and time for enzyme used

Ligation of restricted genomic DNA



- Perform separate ligation reactions for each restriction enzyme used
- Set up ligation reactions with 5 ng/µl of restricted DNA in a volume of 40 microliters using Fast-Link™ DNA ligation kit (Lucigen LK0750H)
- Incubate at room temperature for 1 hr
- Store at 4C until ready to use (-20C for long-term storage if needed)

Ligation of restricted genomic DNA



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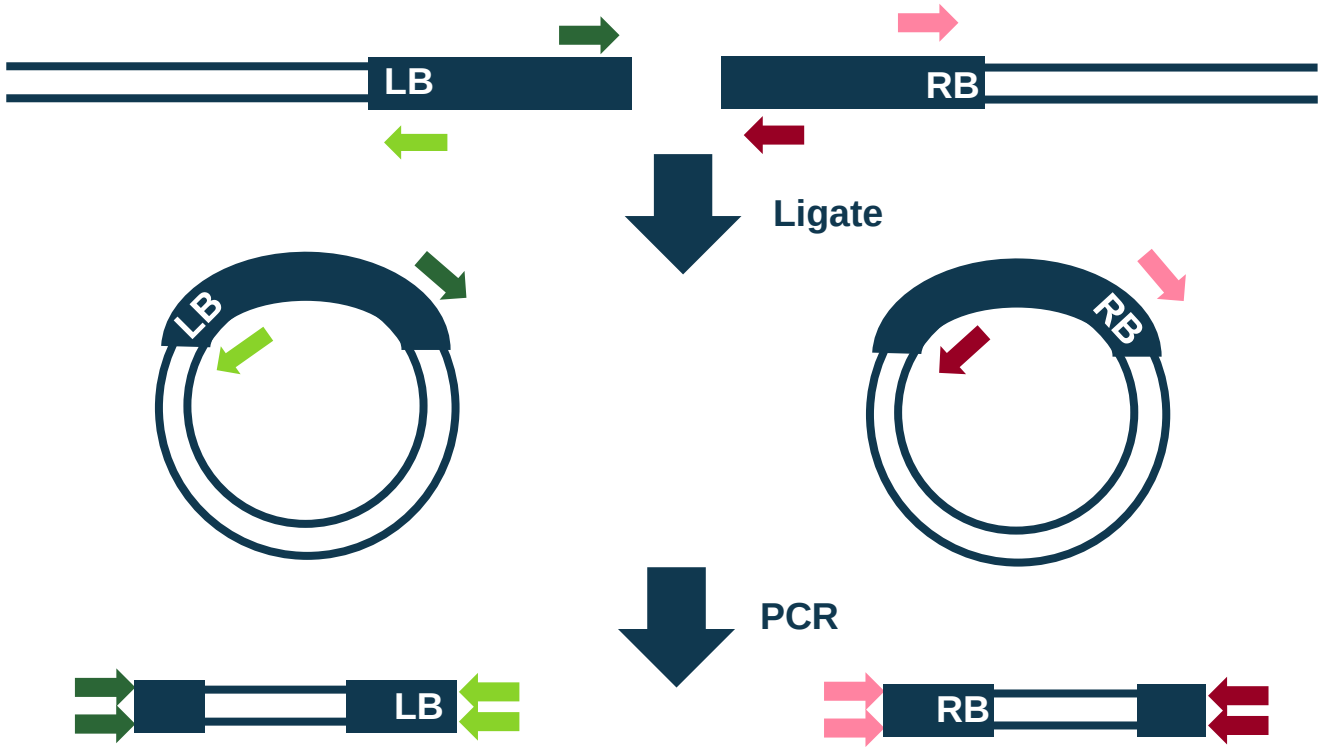
PCR from ligated products



- Design primers on upper and lower strands of known sequence in TDNA in opposite directions rather than toward each other in typical PCR
- After ligation perform PCR to generate amplicons

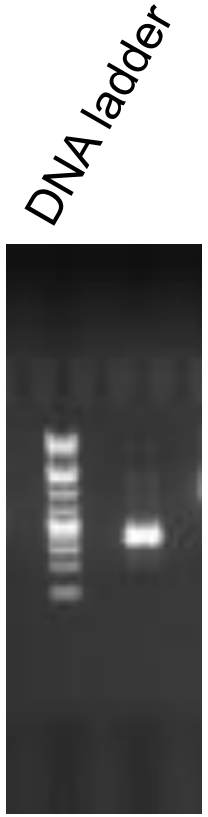
Example PCR amplification protocol

Component	per reaction (µl)
Ligation reaction	2
Primer 1 (2 µM)	1.5
Primer 2 (2 µM)	1.5
Phusion 2x master mix	5
Total	10

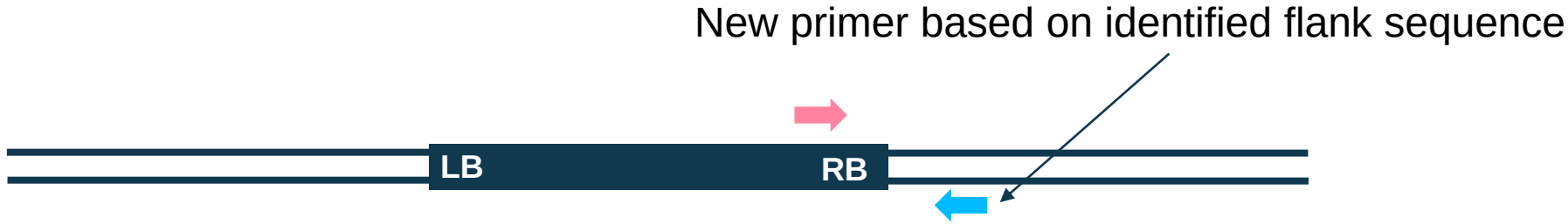
Step	Temperature	Time
1	98°C	30 sec
2	98°C	5 sec
3	60°C*	5 sec
4	72°C	2 min
Go to step 2 30 times		
5	72	5 min

*according to T_m of designed primers

PCR analysis and sequencing of flanking sequences



- Run a portion of the PCR on an agarose gel
- For samples with clear bands, perform sequencing analysis on PCR products by Sanger Sequencing on Applied Biosystems 3730xl DNA analyzer or equivalent sequencer or sequencing methodology
- Genomic flank can be confirmed using design of new PCR based on the identified sequence
- Match genomic flanking sequence to a reference genome using BLAST or similar method and choose best match



Further reading for Inverse PCR

Kim, SR., Jeon, JS., An, G. (2011). Development of an Efficient Inverse PCR Method for Isolating Gene Tags from T-DNA Insertional Mutants in Rice. In: Pereira, A. (eds) Plant Reverse Genetics. Methods in Molecular Biology, vol 678. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60761-682-5_11

Jong, A.Y., T'ang, A., Liu, DP., Huang, SH. (2002). 31 Inverse PCR. In: Chen, BY., Janes, H.W. (eds) PCR Cloning Protocols. Methods in Molecular Biology™, vol 192. Humana Press. https://doi.org/10.1385/1-59259-177-9:301

Offringa, R., van der Lee, F. (1995). Isolation and Characterization of Plant Genomic DNA Sequences via (Inverse) PCR Amplification. In: Jones, H. (eds) Plant Gene Transfer and Expression Protocols. Methods in Molecular Biology™, vol 49. Springer, Totowa, NJ. https://doi.org/10.1385/0-89603-321-X:181

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