Canadian Food Inspection Agency | Agence canadienne d'inspection des aliments

Canadian Food Inspection Agency



Our vision:
To excel as a science-based regulator, trusted and respected by Canadians and the international community.

Our mission:
Dedicated to safeguarding food, animals and plants, which enhances the health and well-being of Canada's people, environment and economy.

The Seed Health Toolbox II

Molecular Seed Testing Methods and Processes: What are the sources of variability?

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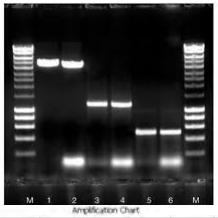
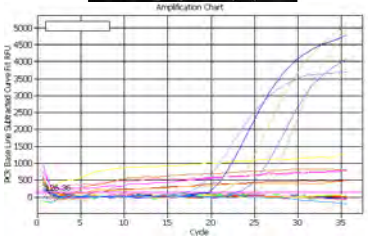
PCR Methodology: Sources of Variability

Conventional PCR





Realtime PCR

Seed Sample

Sources of Variability
- Sample splitting
- Sample prep
- Sample integrity
- Quality Assurance

Overall Sources of Variability
- Proper Laboratory Workflow
- Contamination
- Analyst Variability
- Maintenance and Verification of Equipment
- Method Validation
- Quality Assurance

DNA Extraction

Sources of Variability
- Manual vs Automated
- DNA Yield
- Minimize inhibitors
- Validate Extraction SOP
- Quality Assurance

Sources of Variability
- Standardize DNA concentration
- Choice of Master Mix
- Manual vs Automated Prep
- Validated SOP
- Quality Assurance

PCR Reaction Plate

Sources of Variability
- Equipment Accuracy
- Detection Threshold
- Cross Reactivity
- Detection Threshold
- Multiplexing
- Quality Assurance

Overall - **Sources of Variability**

Proper laboratory workflow

- Proper laboratory layout with designated areas

Contamination

- Proper work habits will greatly reduce sample-to-sample and general laboratory contamination

Analyst variability

- All analysts need to be properly trained, read the SOPs, pass proficiency panels (DNA extraction and PCR)

Maintenance and Verification of Equipment

- Proper maintenance and verification of pipettes, robots, PCR thermocyclers, fridges, freezers, etc.

Method Validation

- Sensitivity
- Specificity
- Cross reactivity
- Reference material

Quality Assurance (QA)

Seed Sample Processing - **Sources of Variability**

Sample splitting

- Proper use of seed sample dividing and splitting techniques help assure uniformity of seed samples

Sample prep

- Assay should target likely source of pathogen DNA by "concentrating" infected seed. (ie. sieving of seed or wash material)
- Sample grinding for oilseeds

Sample integrity

- Must maintain sample integrity throughout testing process
- Cleaning/sterilizing tools/equipment between samples will help reduce false positives

QA

- Equipment monitoring and maintenance, reagents, SOPs, analyst proficiency training/panels

DNA Extraction - **Sources of Variability**

Manual vs automated liquid handling/DNA extraction

- Manual pipetting by individual and/or between various staff is one of the highest sources of variability; increases as volume decreases (5ul or lower)

DNA yield

- DNA extraction kits vary widely in the total DNA that may be extracted which will affect PCR sensitivity and detection thresholds

Minimize inhibitors

- Environmental samples such as direct seed assays will have PCR inhibitors that can adversely affect PCR reactions and result in failures or false negatives. Cleanup columns or specialized DNA extraction kits will help reduce potential inhibitors

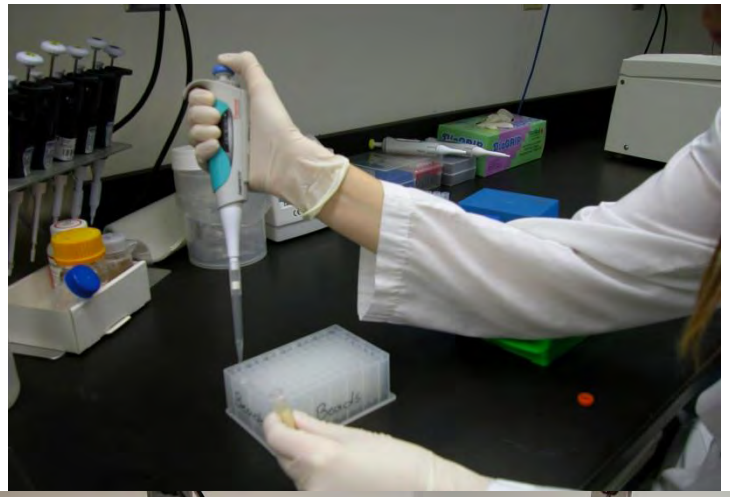
Validate extraction SOP

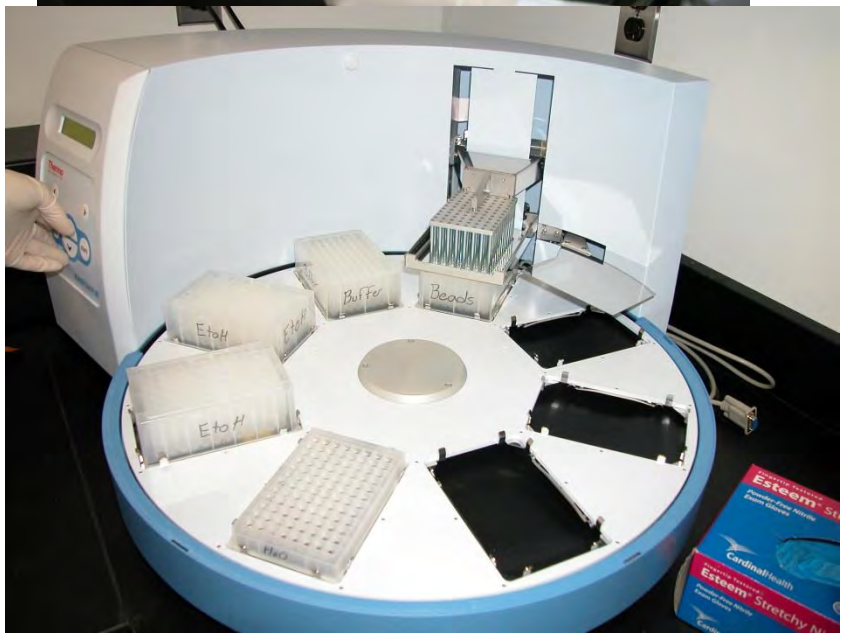
- Important to properly validate and verify DNA yields

QA

- Quality and expiry dates of reagents, pipet tips, etc.

Tissue Maceration and DNA Extraction





Preparing PCR Reaction Plate/Tubes - Sources of Variability

Standardize DNA concentration

- PCR assays should be verified with DNA dilution series to both note optimal DNA concentrations and limits of detection
  - Samples should be run in duplicate, undiluted and 1/5 or 1/10 (also reduces PCR inhibitors)

Choice of master mix

- All PCR DNA polymerases (master mix) are not the same and can vary by several CT values in sensitivity and affect thresholds

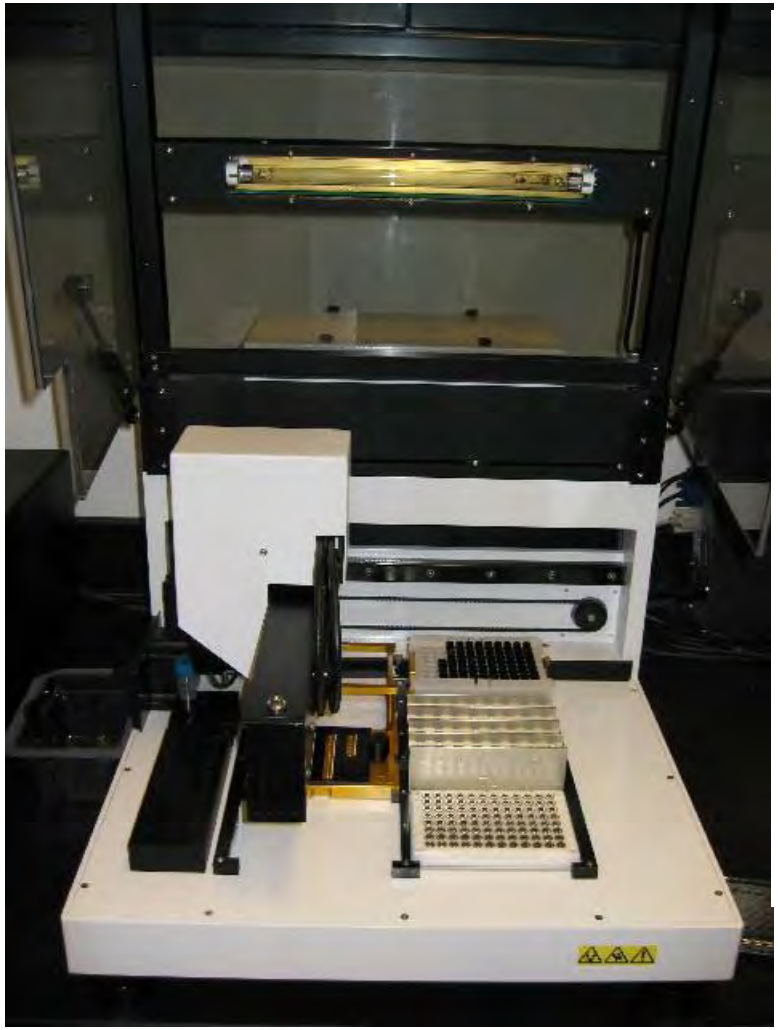
Manual vs. automated prep

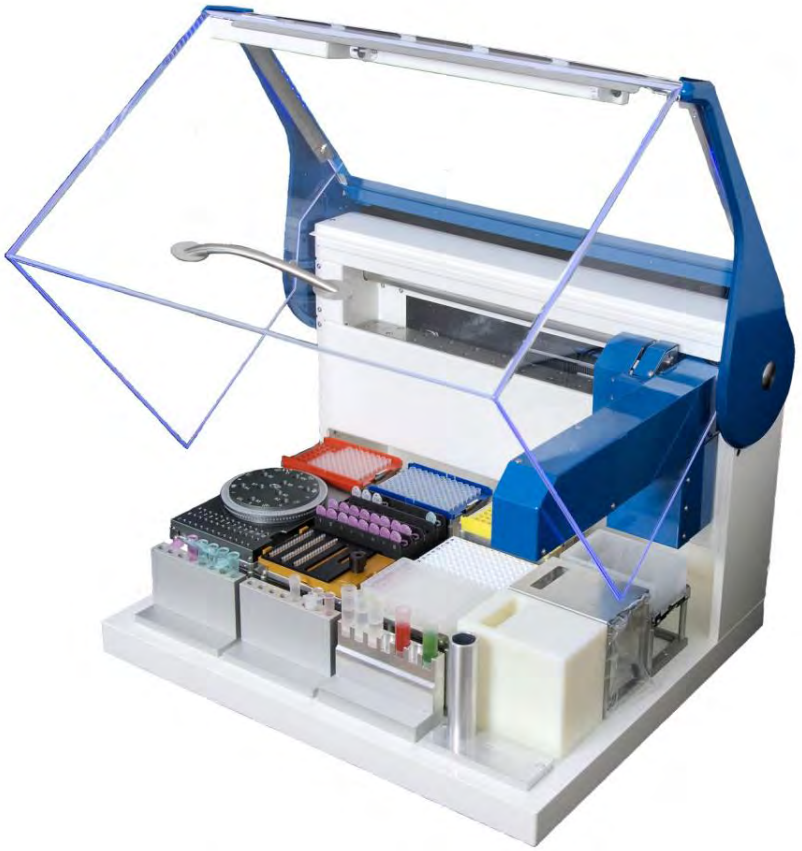
- Automated prep will reduce error and variability mostly due to pipetting of low volumes smaller than 5ul
- Greatly reduces cross contamination, sample mix-ups and pipetting errors

Attention to all aspects related to QA

- Standard validated SOPs
- Assays must be validated and include controlled worksheets

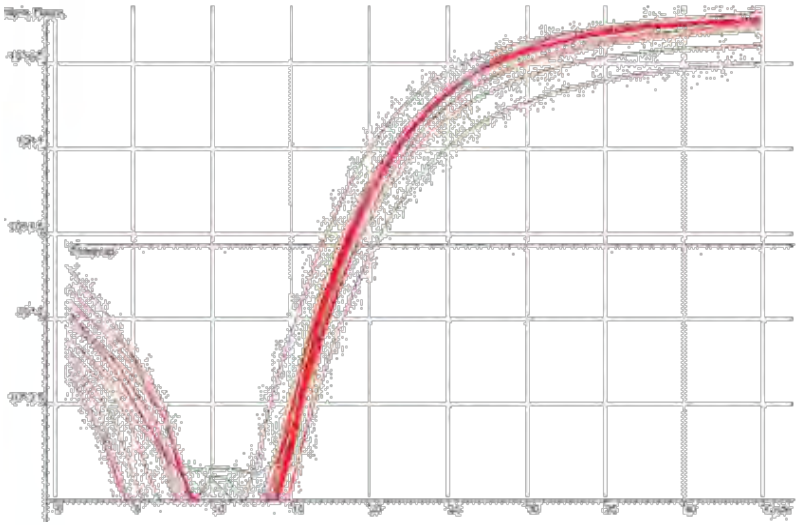
Liquid Handling Robots for PCR Reaction Plate/Tube Prep

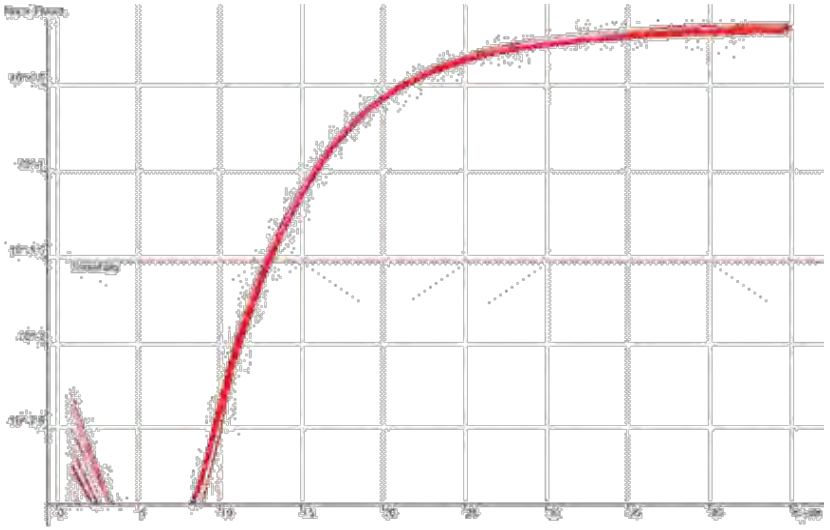




Hand Pipetting vs Liquid Handling Robot

5 µL reaction volume, 18 replicates





Hand pipetting

C_T std dev 0.64

CAS-1200 robot

C_T std dev 0.12

PCR and Data Handling - **Sources of Variability**

Equipment accuracy

- Verification of thermocyclers

Detection threshold

- Standardized procedure for setting a fixed (CT cut-off) or standard curve based detection threshold cross reactivity
- Verifying that the assay will not cross react to related species and other pathogenic organism typically associated with target crop

Multiplexing

- Compatibility probes and primers for multiplexing 2 or more assays into one PCR run

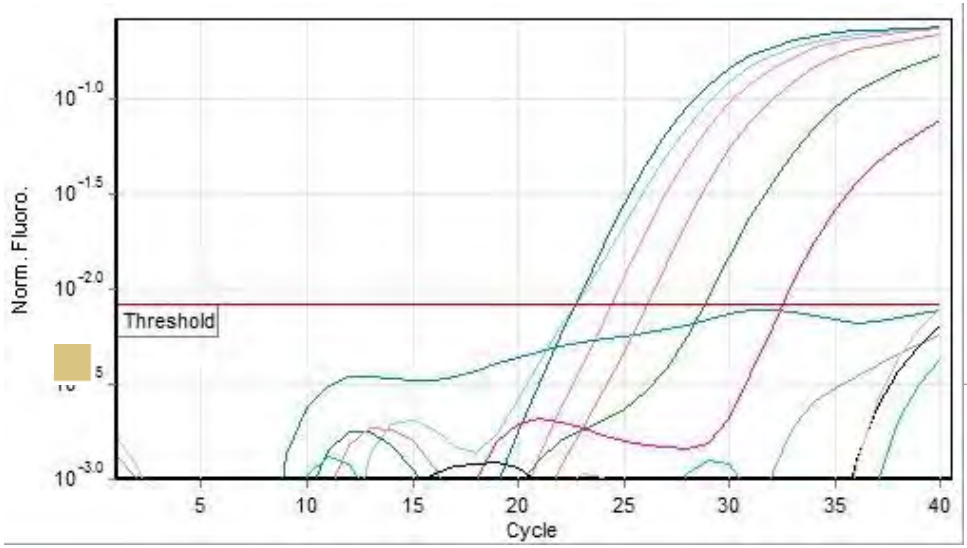
Nested PCR

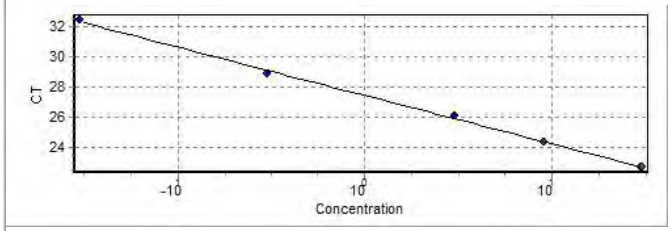
- Risk of laboratory contamination by short amplified DNA fragments from DNA aerosol contamination
- False positives

QA

- Segregation of PCR/Post PCR area to limit aerosol based contamination

Dilution Series of Target DNA to Set Thresholds and Limits of Detection.





No.	Colour	Name	Type	Ct	Given Conc (Copies)	Calc Conc (Copies)	% Var
47		Ep 1	Unknown	22.72		30.237	
48		Eb 1	Unknown				
69		Ep 2	Unknown	24.38		9.123	
70		Eb 2	Unknown				
71		Pos 10-1	Standard	22.71	30.000	30.341	1.1%
72		Pos 10-2	Standard	26.09	3.000	2.674	10.9%
73		Pos 10-3	Standard	28.86	.300	.365	21.7%
74		Pos 10-4	Standard	32.46	.030	.027	8.8%
75		Pos 10-5	Standard		.003		
76		Neg 10-1	Unknown				
77		water	Unknown				

Harmonization of PCR Based Detection Assays will **Reduce Variability**

Development, adoption and inter-laboratory validation of PCR based assays

- Uniformity of QA practices and procedures
- Standard method SOP
- List of approved reagents and equipment
- Specific controls, thresholds and data analysis
- Proficiency panels and inter-laboratory comparisons

It will not reduce variability caused by…

- Analysts – lack of proper training, proficiency testing
- Lack of equipment maintenance and verification
- Improper setting of thresholds for positive detection
- Improper sample handling and processing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