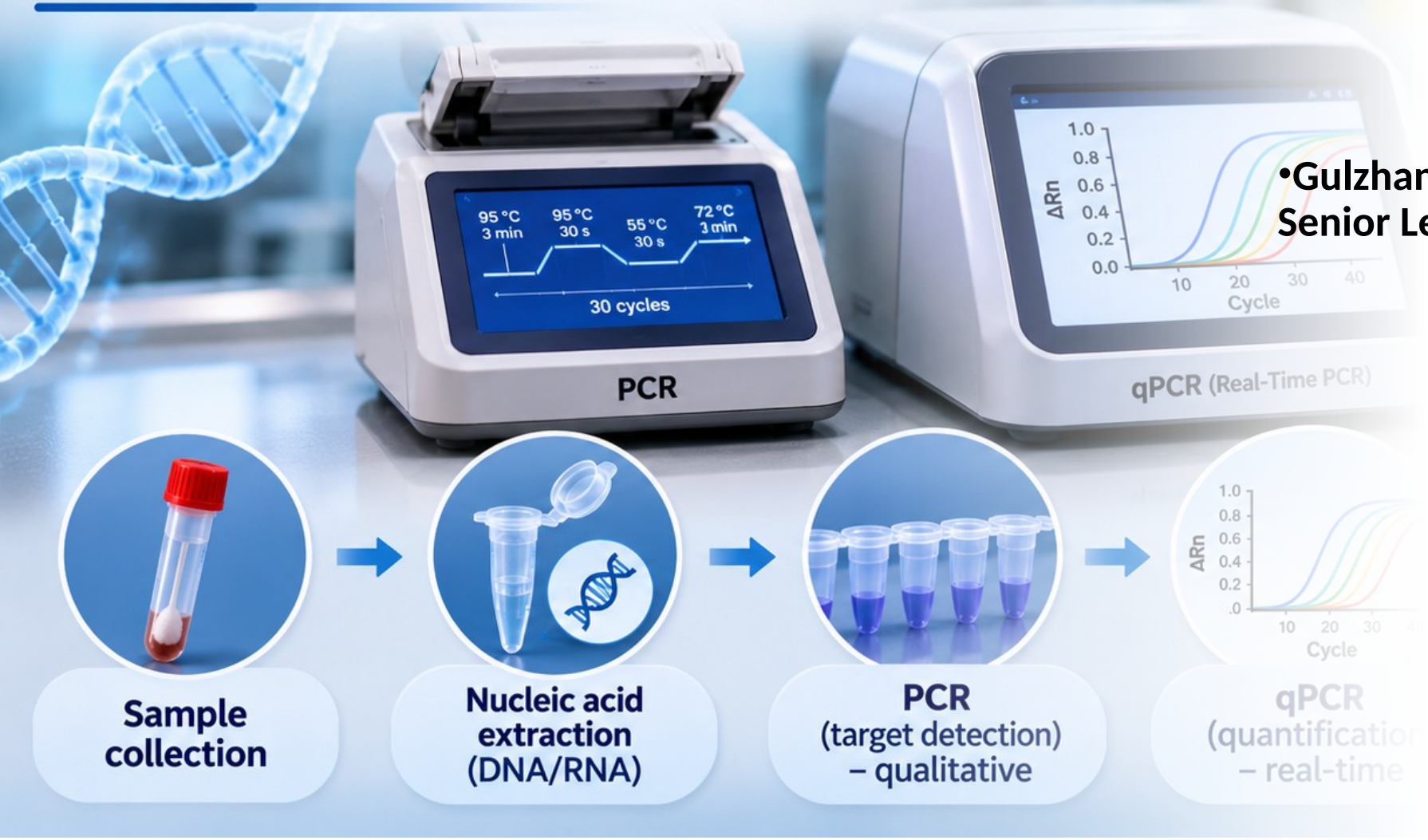
Lecture

Molecular Diagnostics:

PCR and qPCR in Real Diagnostic Workflows



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Why this continuation?

We already know the methods. Now we learn to use them in diagnostic reasoning.

Method knowledge

PCR components
qPCR curves and Cq
Gel bands and controls

Diagnostic question

Is the target present?
Is the result valid?
Could it be a false result?

Decision

Accept
Repeat
Troubleshoot
Request confirmation

Learning outcomes

By the end of the session, students should be able to:

Build	a sample-to-result diagnostic workflow
Evaluate	positive, negative and internal controls
Interpret	gel and qPCR case data
Recognize	false-positive and false-negative patterns
Choose	when to repeat or troubleshoot an assay

PART I

Diagnostic reasoning before the result

A technically correct PCR can still lead to a wrong conclusion if sampling, controls or interpretation are weak.

What diagnostic question are we asking?

Presence / absence

Is a specific DNA or RNA target detectable?

Amount

How much target is present relative to a standard or control?

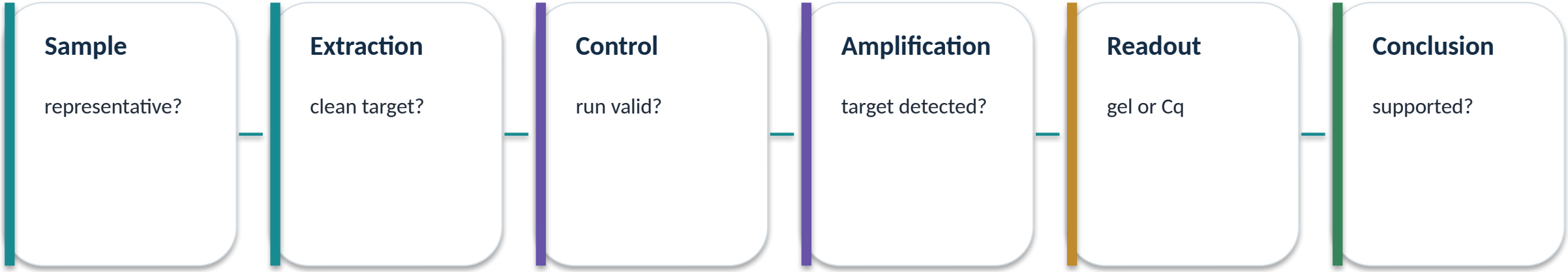
Expression

Is an RNA transcript higher or lower between conditions?

Resistance marker

Is a resistance-associated gene present?

A diagnostic workflow is a chain of evidence



One weak link can invalidate the final conclusion.

Choose the target molecule first

DNA target

Stable genetic target
Pathogen gene
Resistance gene
Transgene
Genomic marker

RNA target

RNA virus
Gene-expression marker
Transcript detection
Requires reverse transcription before qPCR

DNA question → PCR/qPCR | RNA question → RT-qPCR

PCR, qPCR or RT-qPCR?

Question	PCR + gel	qPCR	RT-qPCR
DNA presence	✓	✓	—
Approximate product size	✓	not primary	—
Quantitative signal	limited	✓	✓
RNA target	—	—	✓
Real-time control review	—	✓	✓

Pre-analytical errors can create false molecular results

Wrong sample

target not represented

Poor transport

target degrades

Cross-contamination

false positive

Low biomass

target below detection

Inhibitory matrix

false negative

Labeling error

correct result assigned to wrong sample

Different sample matrices bring different inhibitors

Plant tissue

Polyphenols
Polysaccharides
Secondary metabolites

Soil / environment

Humic substances
Salts
Low target biomass

Clinical / food matrix

Proteins
Complex biological material
Extraction carry-over

No amplification can mean “target absent” or “reaction inhibited”.

An extraction control tests more than the PCR mix

PCR control only

Checks whether amplification reagents and cycling conditions can work.

Extraction control

Passes through extraction and can reveal extraction failure, reagent contamination or strong inhibition.

Good diagnostic control design follows the whole workflow.

Internal amplification control: is the reaction capable of amplifying?

Target signal

Answers the biological question: is the diagnostic target detected?

Internal control signal

Checks whether nucleic acid extraction / amplification worked and whether strong inhibition is present.

Target negative + internal control positive → interpretable negative

A defined positive control can come from the cloning workflow you already know

Plasmid positive control

A plasmid containing the target sequence can provide a stable, defined positive template.

Important caution

High-copy positive controls can become powerful contamination sources. Handle them separately from diagnostic samples.

Positive control validates detection; it must not contaminate the assay.

The NTC asks a simple but essential question

NTC composition

All PCR/qPCR reagents
No sample template

Expected result

No target amplification

Unexpected signal

Investigate contamination, primer-dimer or non-specific amplification

Analytical sensitivity and analytical specificity

Analytical sensitivity

How little target can the assay detect reliably? Related to limit of detection and low-copy performance.

Analytical specificity

Does the assay detect the intended target without reacting strongly with non-target material?

Sensitive ≠ automatically specific | Specific ≠ automatically sensitive

Where do false results come from?

False positive

- carry-over contamination
- cross-reactivity
- non-specific amplification
- sample mix-up
- threshold / interpretation error

False negative

- poor sampling
- low target amount
- nucleic-acid degradation
- extraction failure
- inhibitors
- target mismatch with primers/probe

Contamination control is part of diagnostic accuracy

Unidirectional workflow	clean area → extraction → amplification → post-PCR
Separate materials	dedicated tips, racks and reagents
Aerosol-resistant tips	reduce transfer of droplets
Clean surfaces	routine decontamination
Closed-tube qPCR	reduces post-amplification handling

Interpretation checklist before reporting a result

Was the sample appropriate and labeled correctly?

Did extraction / internal controls perform as expected?

Did the positive control work?

Is the NTC clean?

Is the product / curve consistent with the expected target?

Could inhibition, contamination or low target explain the pattern?

Is the conclusion supported by the complete control set?

CASE CHECKPOINT

A sample is negative, but the internal control is also negative. Is this a valid negative result?

CASE 1

Bacterial pathogen detection by PCR + gel

The simplest diagnostic case: detect a target sequence, verify the expected fragment size, and judge the controls.

Case 1 — sample to DNA

Sample

biological material

Lysis

release DNA

Purification

remove inhibitors

PCR

target-specific primers

Gel

expected band



A diagnostic target should be informative and specific

Good target

Conserved within the organism or group we want to detect

Avoid

Strong similarity to unrelated organisms in the same sample matrix

Assay design

Primers define what DNA can be amplified; specificity begins before the PCR run

Case 1 — control set

Positive control

Known target DNA
Expected band must appear

NTC

No template
No target band expected

Extraction / internal control

Shows that extraction and amplification were technically successful

Case 1 — interpret this gel



Questions

Which samples are positive?
Which sample needs more context?
Are the controls valid?

Case 1 — convert the gel into diagnostic statements

S1

Target detected
Band at expected size
Controls valid

S2

Target not detected
Interpret only if internal / extraction control is valid

S3

Weak target-like band
Repeat or confirm before strong conclusion

Case 1 — hidden false negative caused by inhibition

Sample	Target PCR	Internal control	Interpretation
A	No band	Positive	Target not detected
B	No band	No band	Invalid / inhibited
C	Band	Positive	Target detected

Sample B is not a true negative until inhibition is resolved.

When should a diagnostic PCR be repeated?

Positive control failed

NTC shows target-like contamination

Internal control failed

Weak or borderline sample band

Unexpected fragment size

Replicates disagree

Sample quality or labeling is uncertain

Repeat intelligently

Repeat the step that likely failed. Do not simply rerun the same reaction without changing the suspected problem.

CASE 2

Detection of a resistance-associated gene

A molecular marker can be highly informative—but gene presence is not identical to the full phenotype.

Gene detected ≠ complete phenotype explained

Molecular result

A resistance-associated gene is detected by PCR/qPCR.

Biological phenotype

Expression, regulation, gene integrity and additional mechanisms influence observed resistance.

Molecular marker = evidence, not the entire phenotype.

Case 2 — three isolates, one resistance target

Isolate	Species marker	Resistance gene	Interpretation
1	Positive	Positive	Target organism + resistance marker
2	Positive	Negative	Target organism, marker not detected
3	Negative	Positive	Check identity / contamination / mixed sample

Multiplex logic: several targets in one diagnostic reaction

Target 1

Organism-specific marker

Target 2

Resistance-associated gene

Target 3

Internal amplification control

Multiplexing saves sample, but assay interactions must be validated.

Case 2 — interpret multiplex patterns

Sample	Organism	Resistance	Internal control	Conclusion
A	+	+	+	resistance marker detected
B	+	–	+	organism detected, marker not detected
C	–	–	+	valid negative
D	–	–	–	invalid / inhibited

What a resistance-gene assay can and cannot conclude

Can support

Presence of the tested sequence
Association with a known resistance mechanism
Rapid molecular screening

Cannot prove alone

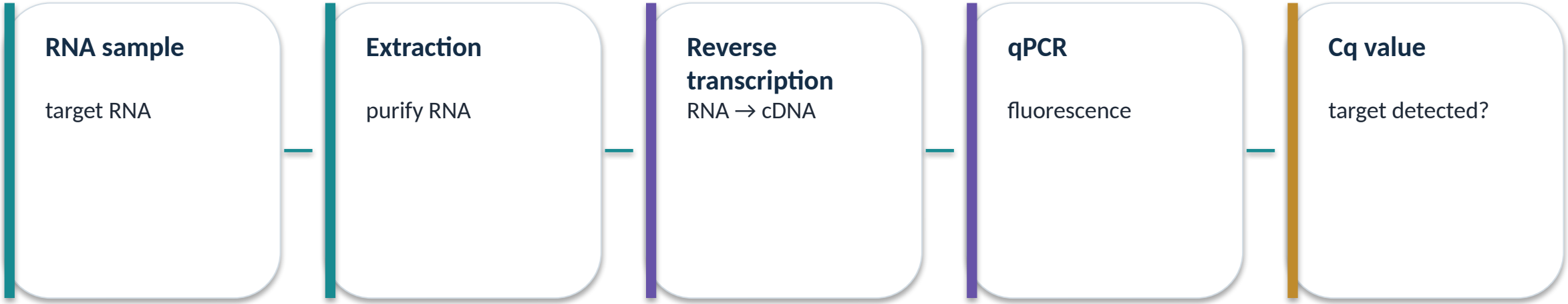
Complete phenotype
Expression level
All possible resistance mechanisms
Clinical significance in every context

PART II

RT-qPCR and difficult diagnostic matrices

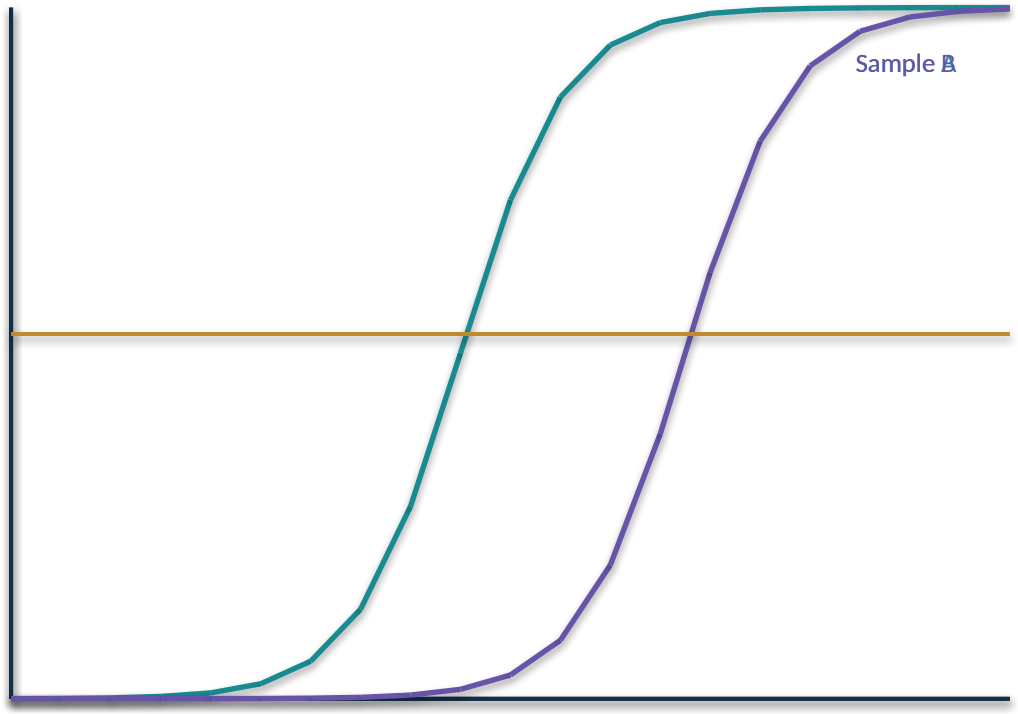
Now we move from visible gel bands to fluorescence curves, internal controls and invalid-result patterns.

Case 3 — detecting an RNA target by RT-qPCR



RNA integrity and reverse transcription are additional points of failure.

Case 3 — amplification curves and Cq values



Interpretation

Sample A crosses threshold earlier → more starting target than Sample B, assuming comparable assay conditions.

Cq is not a direct biological conclusion by itself.

Case 3 — small RT-qPCR diagnostic dataset

Reaction	Target Cq	Internal control Cq	Interpretation
Sample 1	22.4	28.1	Target detected
Sample 2	34.9	28.5	Late / borderline target
Sample 3	Undetermined	28.2	Target not detected
Sample 4	Undetermined	Undetermined	Invalid

Case 3 — when the run itself is invalid

Positive control fails

Do not trust negative samples; assay performance is not demonstrated.

NTC amplifies

Investigate contamination or non-specific signal before reporting positives.

Internal control fails

Sample may be inhibited or extraction may have failed.

Borderline / late signal: do not force a binary answer too early

Check replicates

Are repeated wells consistent?

Check curve shape

Does the signal look like real amplification?

Check controls

Are NTC and internal controls acceptable?

Borderline result → review → repeat if required by the validated assay

CASE 4

Plant pathogen molecular diagnosis

Plant tissue is a useful teaching matrix because inhibitors can produce apparently negative PCR results.

Plant tissue can challenge extraction and amplification

Polysaccharides

Can co-purify and interfere with downstream reactions.

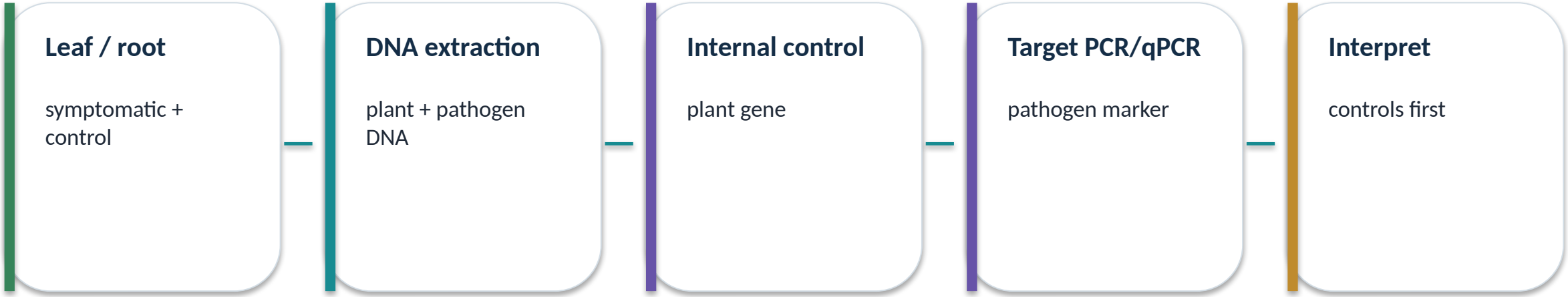
Polyphenols

Can oxidize and interact with nucleic acids or enzymes.

Low pathogen load

Target may be rare relative to abundant plant DNA.

Case 4 — plant pathogen workflow



Case 4 — interpret plant diagnostic results

Plant	Pathogen target	Plant control	Interpretation
P1	Positive	Positive	Pathogen detected
P2	Negative	Positive	Valid negative
P3	Negative	Negative	Invalid / inhibition or poor extraction
P4	Weak positive	Positive	Repeat / confirm borderline signal

CASE 5

Food and environmental molecular detection

Heterogeneous samples, low target abundance and inhibitors make sampling strategy as important as PCR chemistry.

Sampling error can dominate the molecular result

Heterogeneity

Target organisms may be unevenly distributed.

Low abundance

The extracted aliquot may contain zero target molecules by chance.

Matrix inhibition

Fats, proteins, salts or environmental compounds may interfere.

Representative sampling is part of assay sensitivity.

Sometimes the workflow increases target availability before molecular detection

Direct molecular detection

Fast, but may struggle with very low target abundance or difficult matrices.

Pre-analytical enrichment / concentration

Can improve target recovery before PCR, but adds time and changes workflow interpretation.

Pre-analytical design changes what “negative” means.

Case 5 — qPCR results from a difficult matrix

Sample	Target Cq	Internal Cq	Interpretation
E1	25.1	29.0	Target detected
E2	Undetermined	29.2	Valid negative
E3	Undetermined	36.8	Possible inhibition / weak control
E4	Undetermined	Undetermined	Invalid

A simple inhibition test: does dilution improve amplification?

Undiluted extract

Target: no signal
Internal control: very late

Diluted extract

Internal control shifts earlier
Target may become detectable

Dilution lowers both inhibitors and target concentration.

PART III

Can we trust the diagnostic assay?

Before routine use, the assay must demonstrate reproducible detection, appropriate specificity and reliable control behavior.

Limit of detection is a probability concept

High target

Nearly all replicates detected

Near the LoD

Some replicates positive, some negative

Below LoD

Detection becomes unreliable

Near the detection limit, replicate variability is expected.

Reproducibility: does the assay behave consistently?

Within-run

replicate wells agree

Between-run

different days give similar results

Operator

different users reproduce performance

Instrument / lot

changes do not break interpretation

Specificity testing must include relevant non-target material

Expected target panel

Different strains / variants of the intended target should be detected if the assay claims broad coverage.

Non-target panel

Closely related organisms and common matrix DNA should remain negative.

Specificity is tested, not assumed from primer names.

Diagnostic sensitivity and specificity: simple 2 × 2 logic

	Reference positive	Reference negative
Test positive	True positive	False positive
Test negative	False negative	True negative

Sensitivity

How often positives are detected

Specificity

How often negatives remain negative

Choose the assay from the diagnostic question

Need visible confirmation of DNA fragment size	Conventional PCR + gel
Need rapid real-time target detection	qPCR
Starting target is RNA	RT-qPCR
Need resistance-marker screening	Target-specific PCR/qPCR
Matrix is difficult / inhibition likely	Include internal control and extraction-quality checks

Integrated final case — what would you report?

Sample	Target	Internal	NTC	Your conclusion
A	Cq 23	Cq 29	clean	?
B	Undet.	Cq 29	clean	?
C	Undet.	Undet.	clean	?
D	Cq 36	Cq 30	clean	?

Discuss

A = clear detection; B = valid negative; C = invalid; D = borderline/late signal requiring assay-specific review or repeat.

Take-home messages

Molecular diagnostics begins with representative sampling and good extraction.

Controls are part of the result, not an optional extra.

A negative target is valid only when the workflow shows that amplification was possible.

Weak or late signals require cautious, assay-specific interpretation.

PCR/qPCR detects the target it was designed to detect—not every biological mechanism.

False positives and false negatives can originate at multiple stages.

Diagnostic conclusions should match the strength of the molecular evidence.

Selected sources and guidance

MIQE principles for qPCR reporting and interpretation.

General PCR/qPCR application guidance from established molecular biology manufacturers and laboratory manuals.

Molecular diagnostic textbooks and validated laboratory SOPs for assay-specific cutoffs and decision rules.

Use local validated protocols for exact controls, acceptance criteria, concentrations and reporting thresholds.