

PCR Detection of DNA and RNA Sequence Alterations

Principles, analytical readouts, and diagnostic applications

Molecular genetic diagnosis

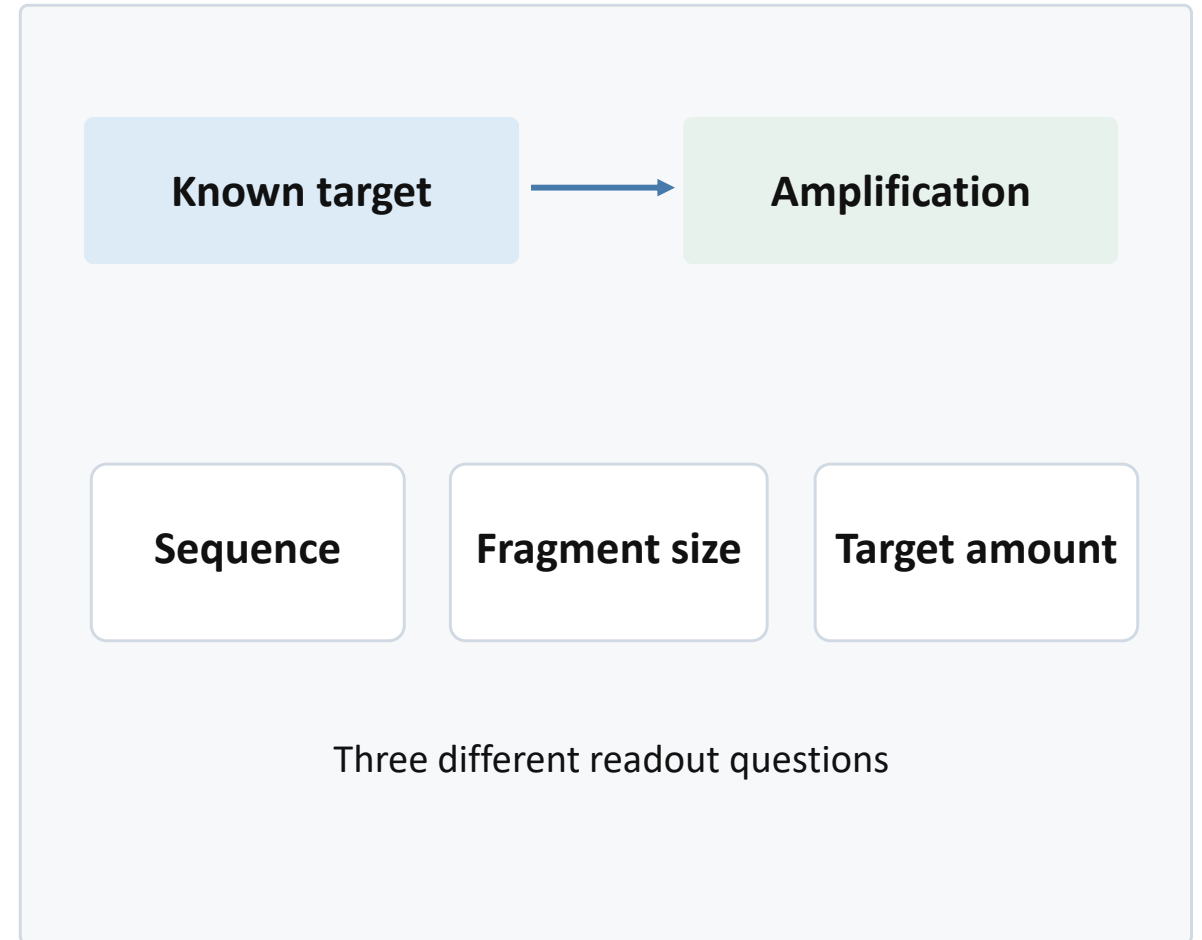
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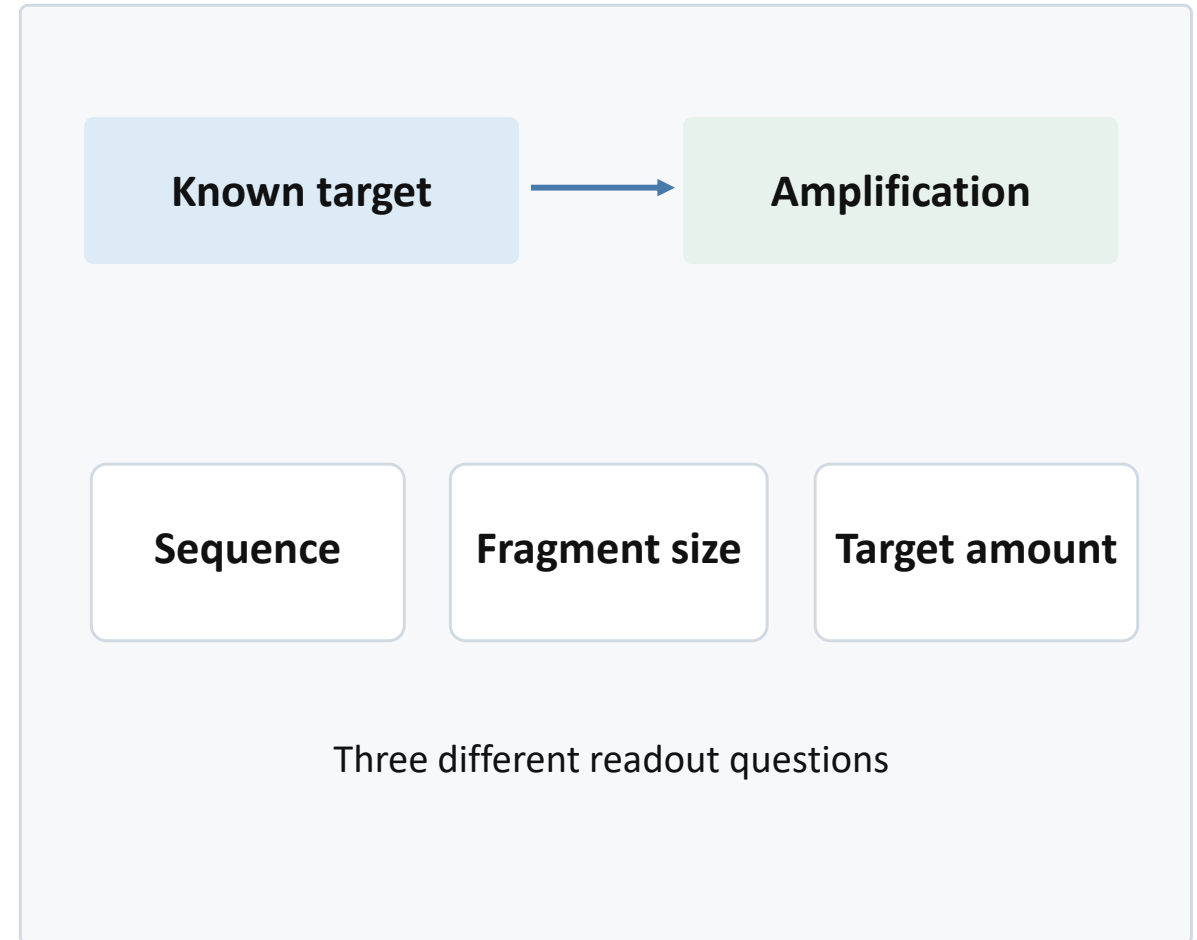
Learning outcomes

- Explain how primers and repeated thermal cycles amplify a defined DNA target.
- Distinguish sequencing, restriction analysis, amplicon sizing, and quantitative PCR.
- Select a PCR-based readout for known sequence variants, repeat alterations, or molecular burden.
- Recognize assay limitations and interpret negative findings in clinical context.



PCR as a platform for molecular diagnosis

- PCR amplifies a selected DNA fragment before further molecular analysis.
- A defined target can be enriched from a very small quantity of starting DNA.
- Amplification is a preparation step; The readout determines which alteration is detected.
- Many molecular diagnoses combine PCR with a second analytical technique.



The clinical question determines the assay

- Identify whether the desired result is sequence identity, fragment size, or target quantity.
- Ask whether the lesion is known and localized or potentially large and complex.
- Choose an appropriate specimen containing the genetic material of interest.
- A technically successful test is useful only if it addresses the suspected abnormality.

What is the lesion?



What material is available?



Which readout is needed?

Genetic alterations occur at different scales

- Single-nucleotide substitutions alter base identity without necessarily changing product length.
- Small insertions, deletions, and repeat expansions may change amplicon size.
- Large deletions and complex rearrangements may lie outside a practical PCR assay.
- No single readout detects every class of molecular lesion equally well.

Single-base change

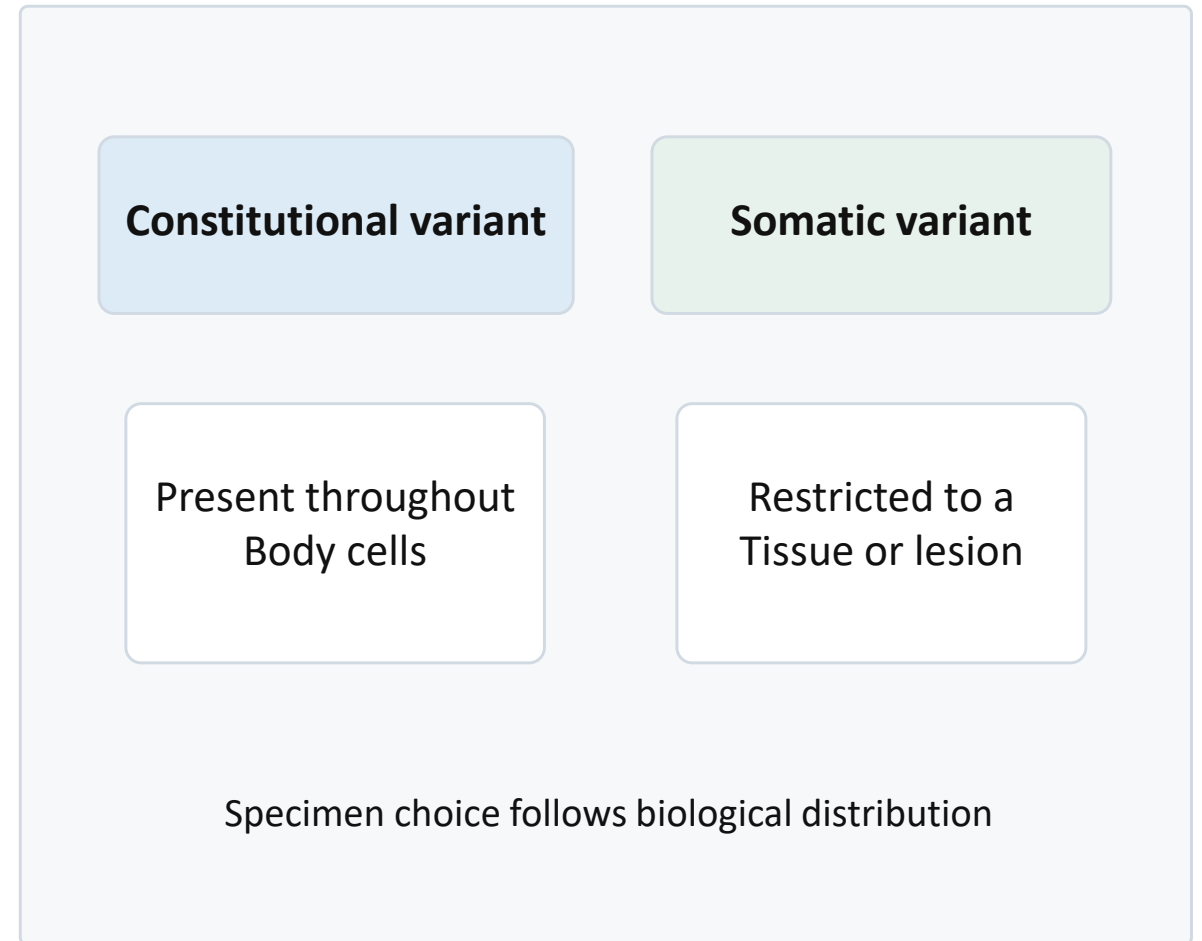
**Short insertion /
deletion**

Large rearrangement

PCR is most straightforward for a defined short target

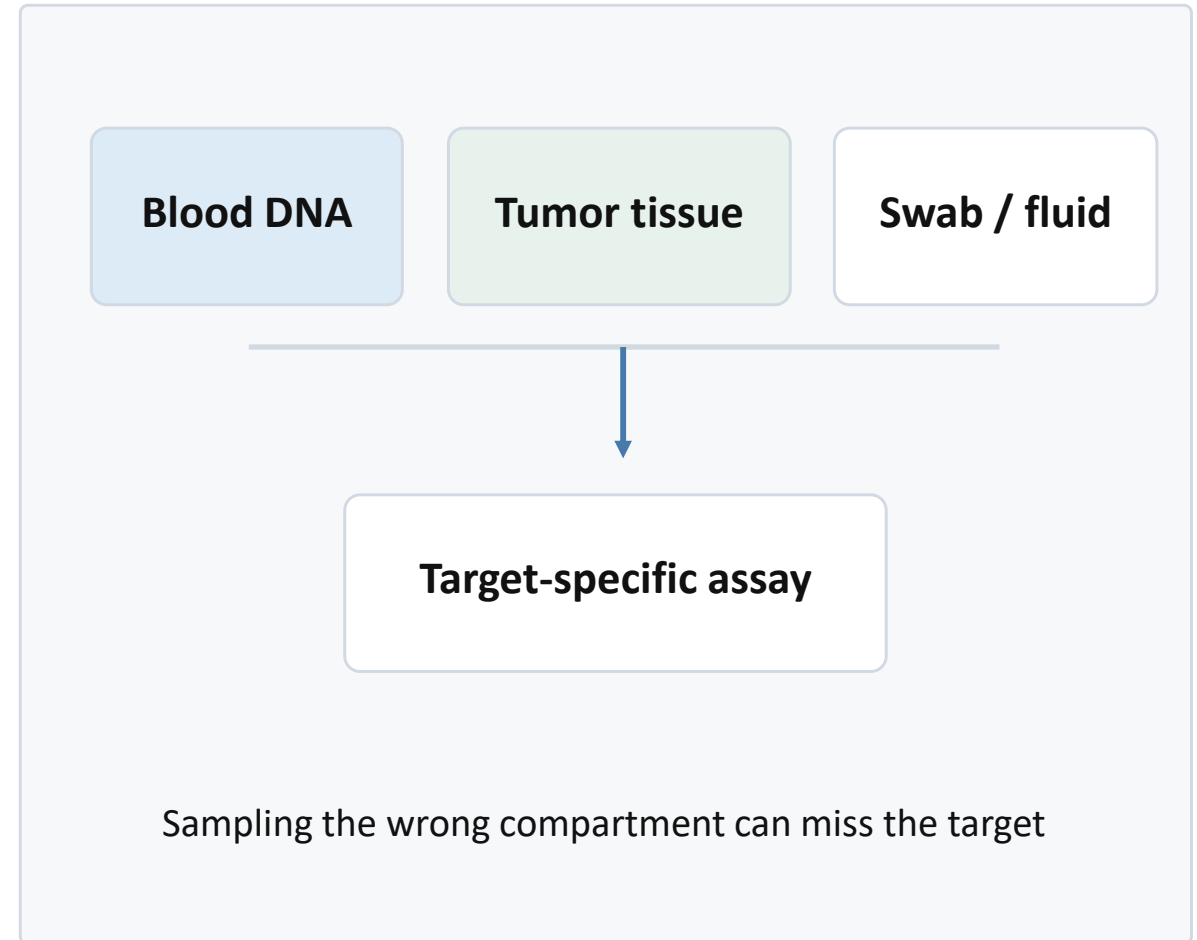
Constitutional and somatic alterations

- Constitutional variants are present throughout the patient's body.
- Somatic variants may be confined to tumor tissue or another affected compartment.
- The same assay principle can apply to inherited and acquired disease.
- A sample lacking the altered cell population can yield an uninformative result.



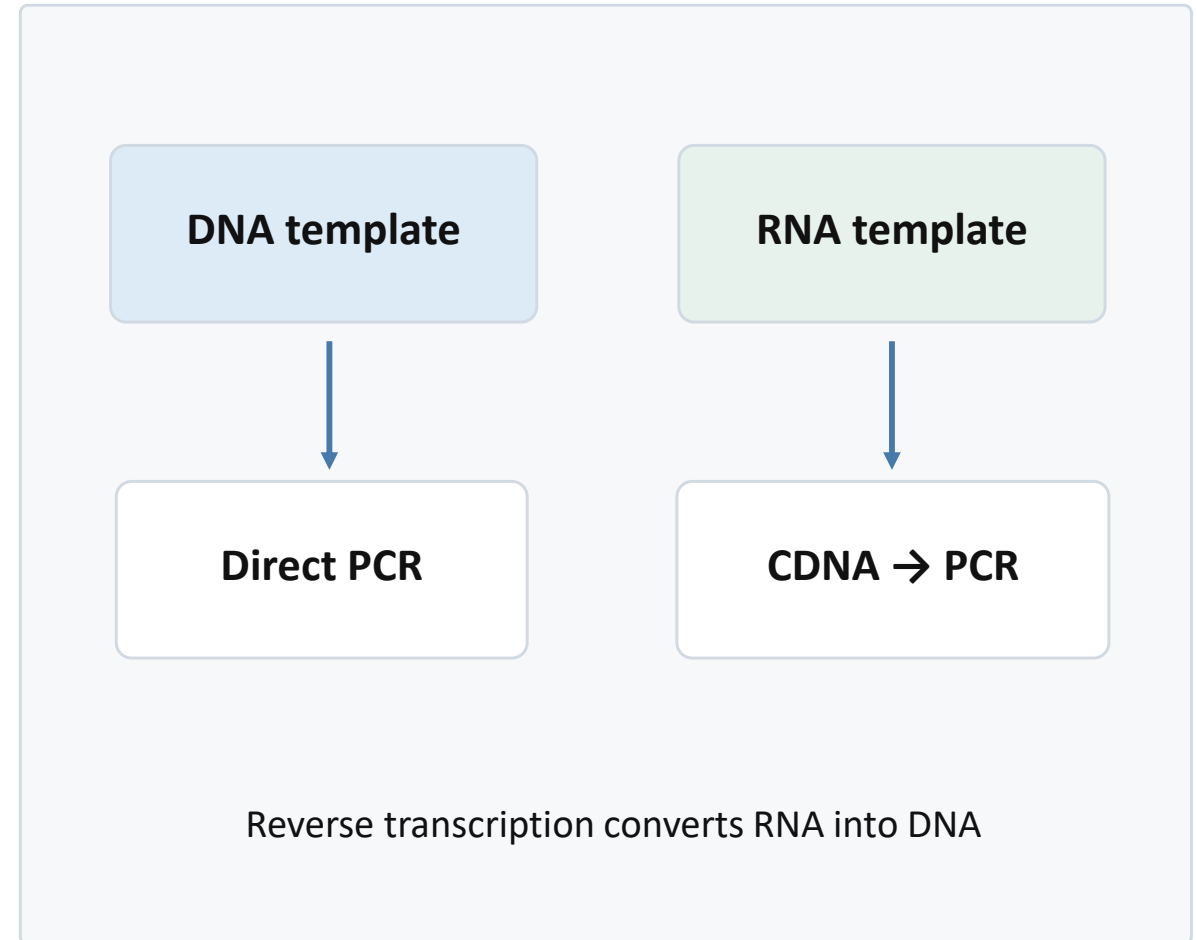
Specimen selection is part of test design

- Peripheral blood is commonly used when a constitutional genetic disorder is suspected.
- Tumor tissue can provide the somatic alteration sought in a cancer assay.
- Swabs and other infected specimens can be used to identify pathogen nucleic acid.
- Assay selection and specimen choice must match the biology of the lesion.



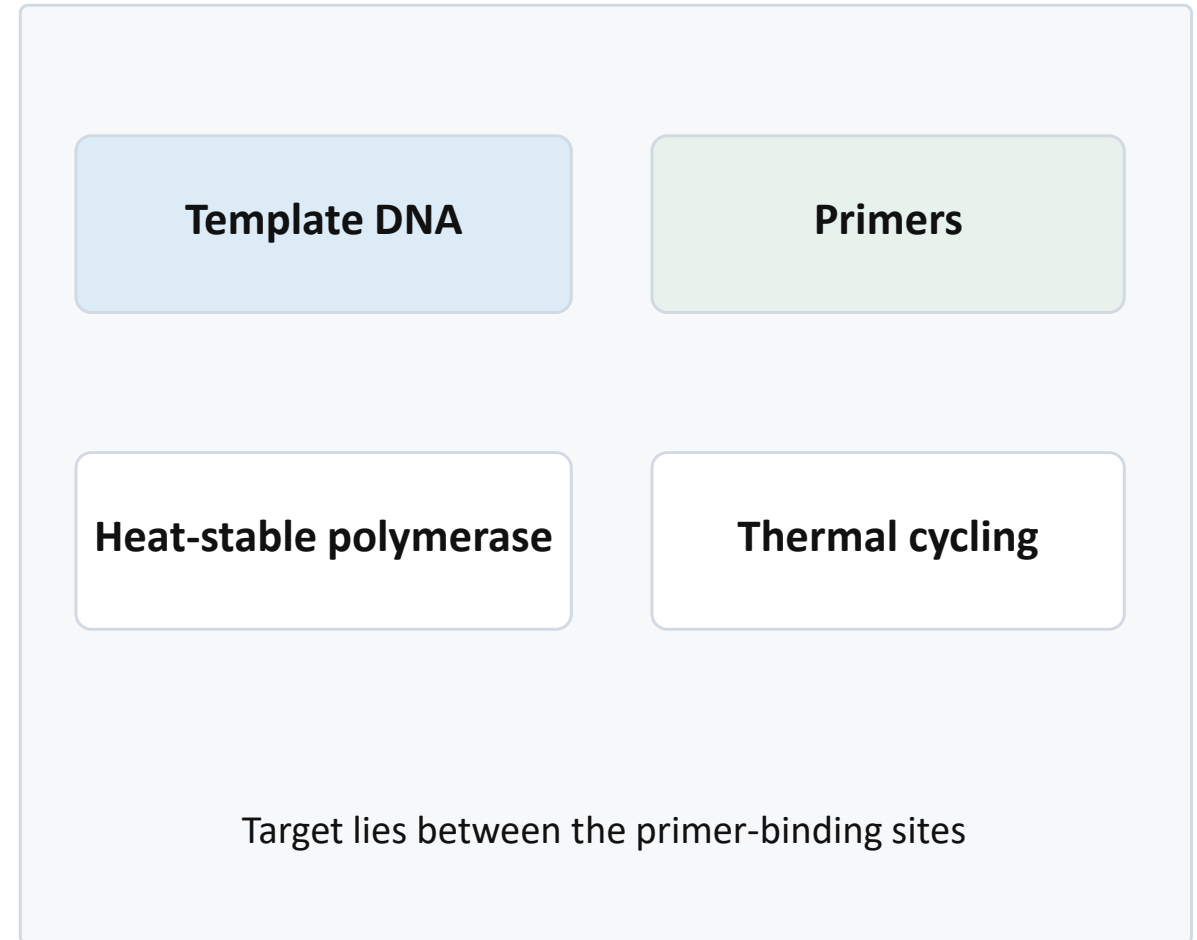
PCR begins with a DNA template

- Conventional PCR amplifies DNA—not RNA directly.
- Genomic DNA can serve as template when the target is encoded in the genome.
- RNA measurements require reverse transcription into complementary DNA (cDNA).
- The distinction matters when detecting transcripts rather than genomic lesions.



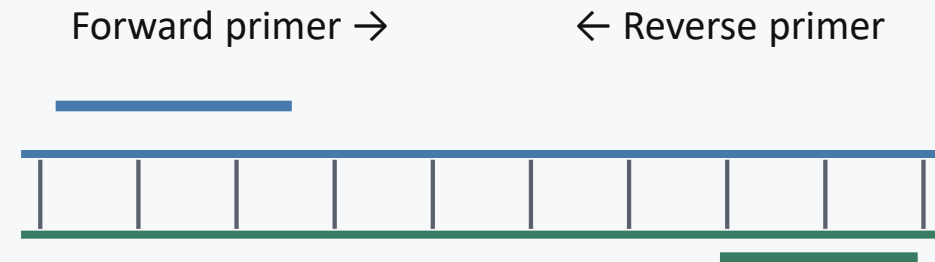
Core components of a PCR reaction

- Template DNA contains the sequence to be amplified.
- A pair of synthetic primers defines the flanking sequences of the target.
- A heat-stable DNA polymerase extends complementary DNA strands.
- Repeated temperature changes coordinate strand separation and primer binding.



Two primers define the amplicon

- One primer anneals on each side of the selected target region.
- Primer orientation allows synthesis across the intervening sequence.
- The region between primer-binding sites is enriched during cycling.
- A mutation affecting primer binding can compromise target recovery.

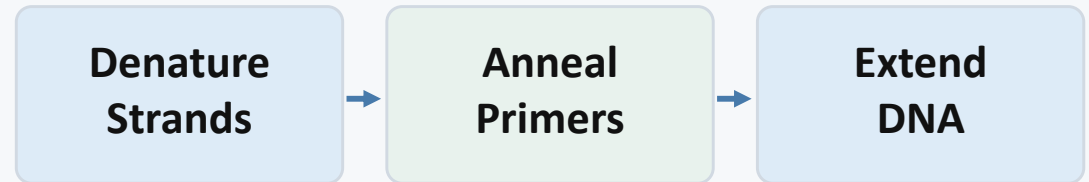


Amplicon

Illustrative primer positions; Not genomic coordinates

Thermal cycling has three logical stages

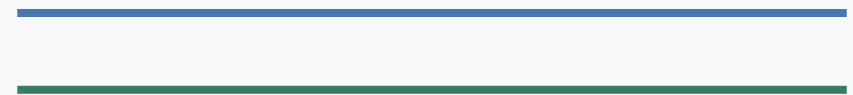
- Denaturation separates the paired DNA strands.
- Annealing permits each primer to bind its complementary template.
- Extension produces a new complementary strand from the bound primer.
- Repetition produces progressively more copies of the target region.



Repeated thermal cycling enriches the defined segment

Denaturation makes templates accessible

- Double-stranded DNA is separated into single strands by heating.
- Both original and newly synthesized strands become templates in later cycles.
- The process is necessary before primers can access their complementary sites.
- Denaturation is an enabling step rather than the sequence-detection readout.

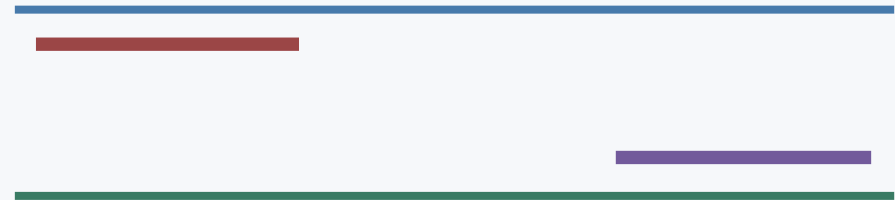


Two strands separate so primers can bind

Annealing specifies the target

- Primers bind to complementary sequences flanking the region of interest.
- Specificity is governed by where these primer sequences hybridize.
- A target outside the primer-defined interval is not interrogated by this amplicon.
- Primer-site alterations can contribute to failure to amplify an allele.

Primer binding identifies boundaries

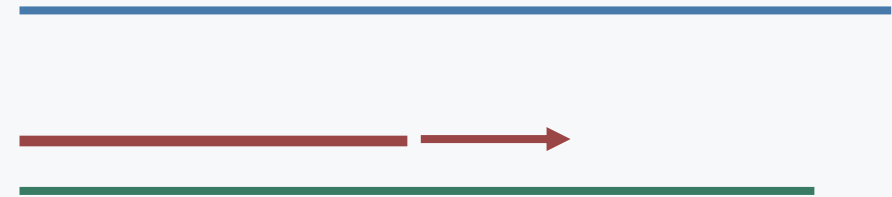


Primers hybridize to complementary target sites

Polymerase extension copies the target

- A heat-stable polymerase remains functional over repeated thermal cycles.
- From a bound primer, the enzyme synthesizes a complementary DNA strand.
- New strands can participate as templates in subsequent cycles.
- Amplification increases the material available for analysis.

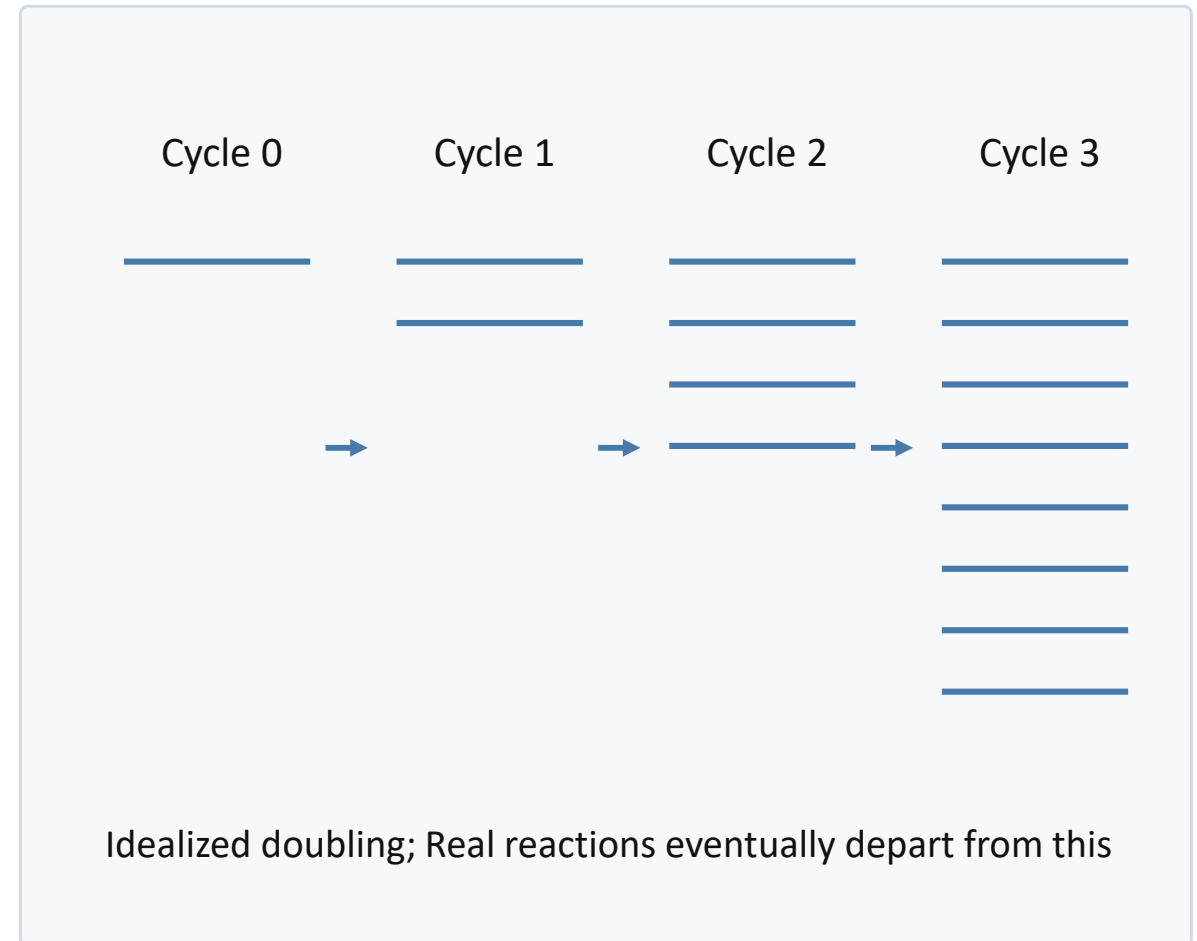
Polymerase extends from primer ends



New complementary strands are synthesized

Repeated cycles lead to large enrichment

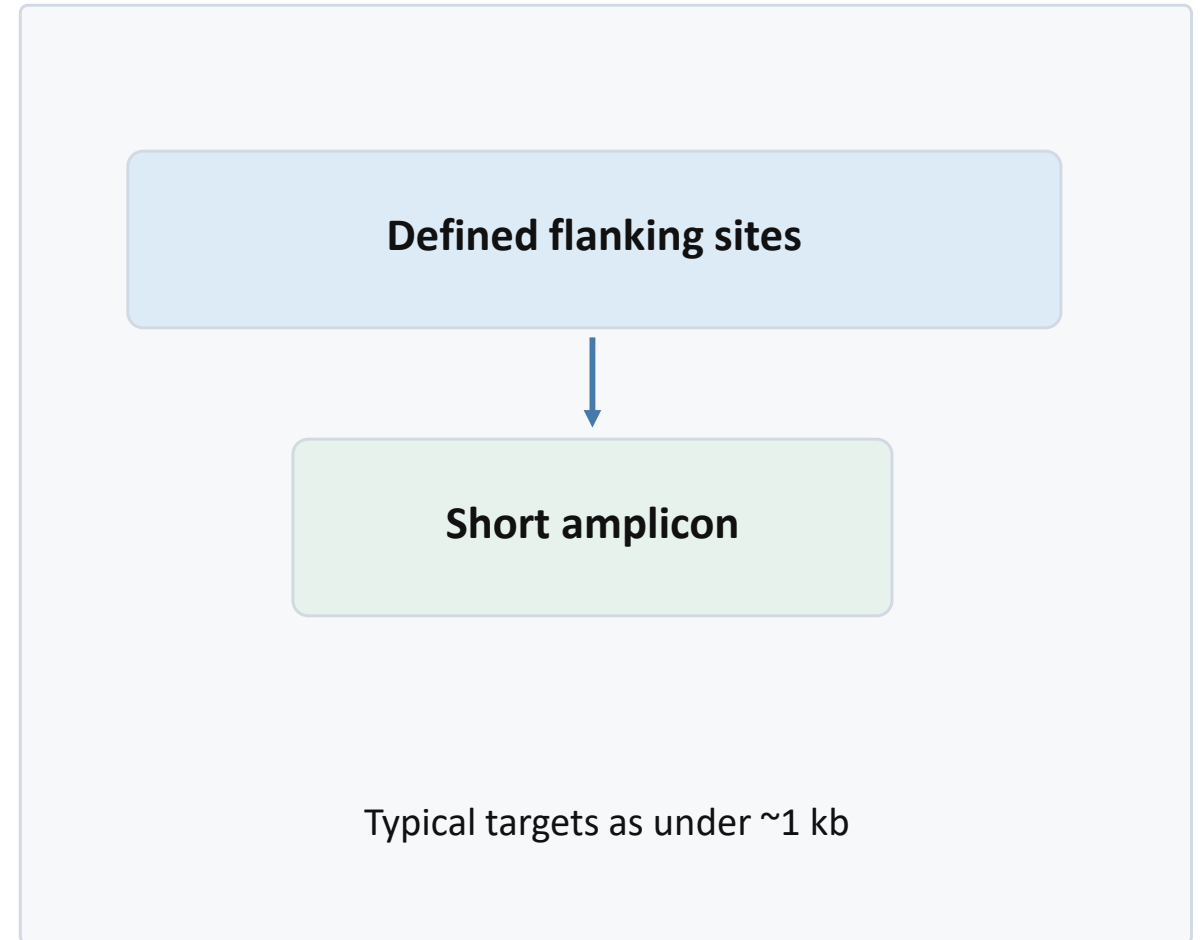
- Each newly created target can contribute template in a later cycle.
- Amplification can be exponential during the efficient early phases.
- Amplification may begin from as little as a single template copy.
- Idealized doubling is a conceptual model, not a guaranteed clinical yield.



Schematic — not patient or laboratory data

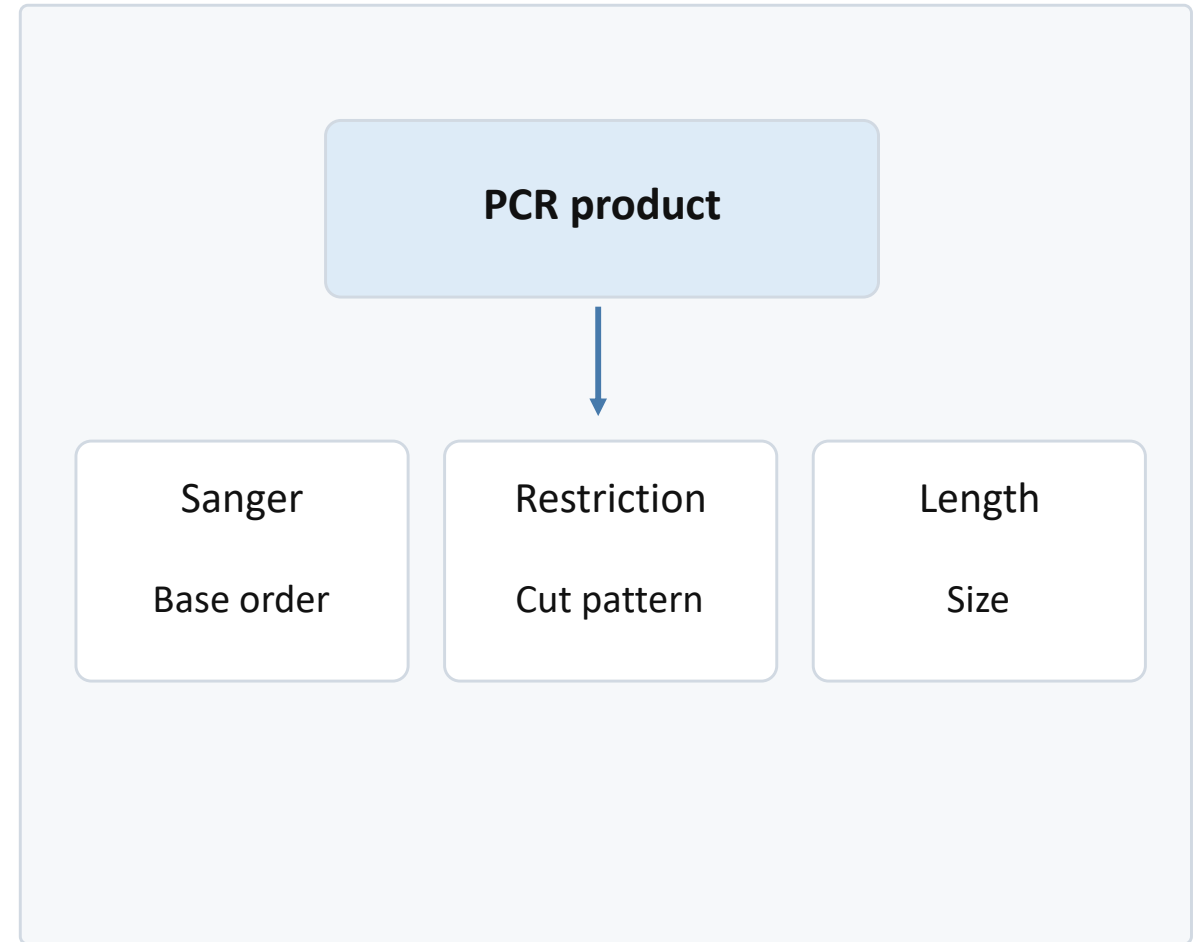
PCR is usually applied to defined short regions

- The Robbins discussion focuses on relatively short DNA targets, usually under about 1000 bp.
- A defined amplicon is well suited to localized sequence interrogation.
- Long or difficult regions may not amplify under standard assay conditions.
- Amplicon design imposes a boundary on what an assay can report.



PCR amplification is not a mutation call

- A visible PCR product establishes that a target-sized fragment was amplified.
- Sequence changes may leave product length unchanged.
- Post-amplification sequencing can reveal the order of nucleotide bases.
- Digestion or length measurements answer different, narrower questions.



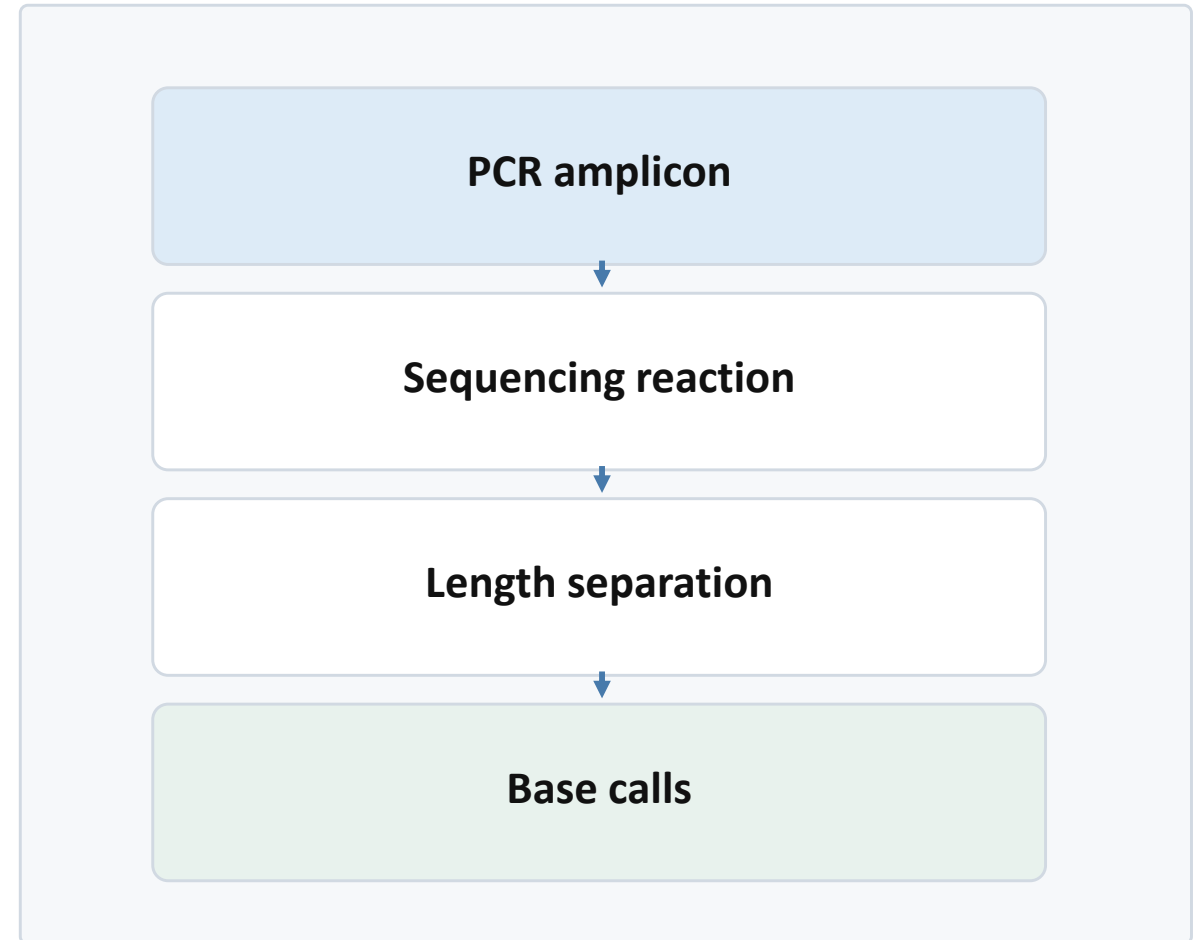
Four readouts highlighted by Robbins

Approach	Primary observation	Useful target
Sanger sequencing	Order of bases in a PCR product	Localized sequence changes
Restriction fragment analysis	Pattern after sequence-specific digestion	Known variant at enzyme cut site
Amplicon length analysis	Size of amplified fragment	Small indels or repeat-length changes
Real-time quantitative PCR	Fluorescent signal during amplification	Target burden / transcript abundance

Readout selection should follow the suspected lesion and clinical purpose.

Sanger sequencing: The central question

- What is the nucleotide sequence within the amplified target?
- A single PCR amplicon can be submitted to a sequencing reaction.
- The measured sequence is aligned with a reference for variant detection.
- This approach is broader than a test asking only about one restriction site.



Sanger sequencing reaction components

- The reaction uses one primer together with DNA polymerase and a DNA template.
- Ordinary nucleotides permit extension of the new DNA strand.
- Four differently labeled dideoxy terminators correspond to A, T, G, and C.
- Terminator incorporation stops extension and labels the ending base.

Single primer

Polymerase

Ordinary nucleotides

Fluorescent ddNTPs

All components interrogate one defined template

Dideoxy terminators interrupt synthesis

- A dideoxy nucleotide can be incorporated during DNA synthesis.
- Once incorporated, the terminated molecule cannot be extended further.
- Different reaction molecules terminate at different positions.
- The terminal fluorescent label identifies the base at that stopping site.

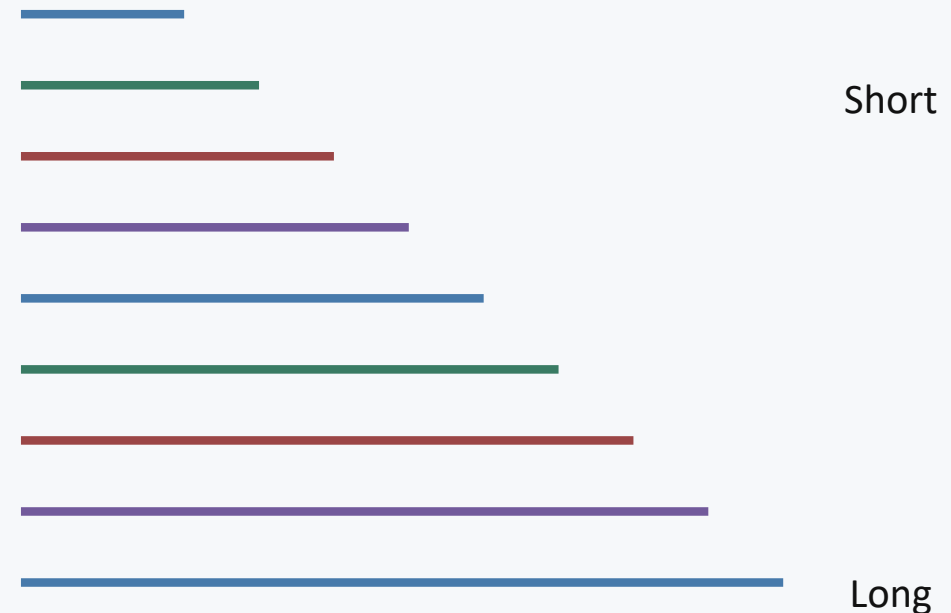


A terminator stops extension at its
incorporated base

Colors represent the labeled terminator identities

The reaction generates a length ladder

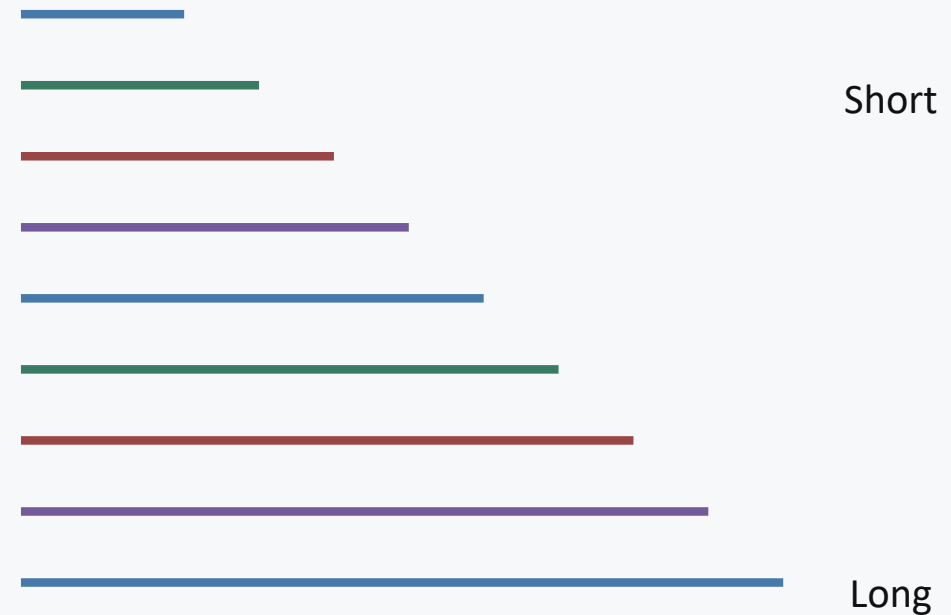
- Products are generated at a range of lengths from the same starting template.
- Each terminated product carries a fluorescently labeled final nucleotide.
- The size ladder preserves positional information about the base sequence.
- This mixture must be ordered by length to reconstruct the nucleotide sequence.



Schematic — not patient or laboratory data

Electrophoretic separation orders fragments

- Sequencing products are separated according to their lengths.
- Reading labels in the resulting length order recovers the base calls.
- The output describes one defined amplified region, not the whole genome.
- Quality depends on a usable amplicon and an interpretable sequencing reaction.

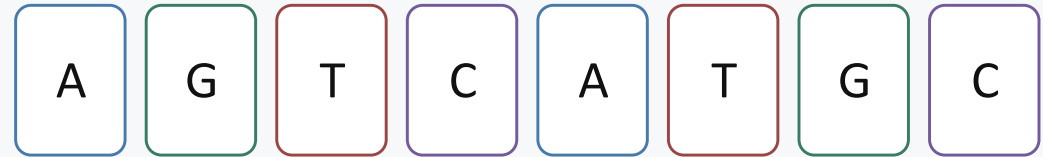


Illustrative length-ordered fragment mixture

Schematic — not patient or laboratory data

A sequence is read one base at a time

- Each length-position is associated with an A, T, G, or C fluorescence label.
- Together, these labels allow reconstruction of a nucleotide sequence.
- Base order—not fragment length alone—is the useful diagnostic observation.
- The resulting sequence must be interpreted against a reference.



Example base-order display; Not a sequencing trace

Schematic — not patient or laboratory data

Reference comparison reveals a substitution

- A base substitution can be recognized when sample and reference differ at one position.
- The illustrative C-to-T change does not alter total amplicon length.
- Consequently, simple fragment sizing would not reveal this example.

Reference ATG C T A A G

Sample ATG T T A A G

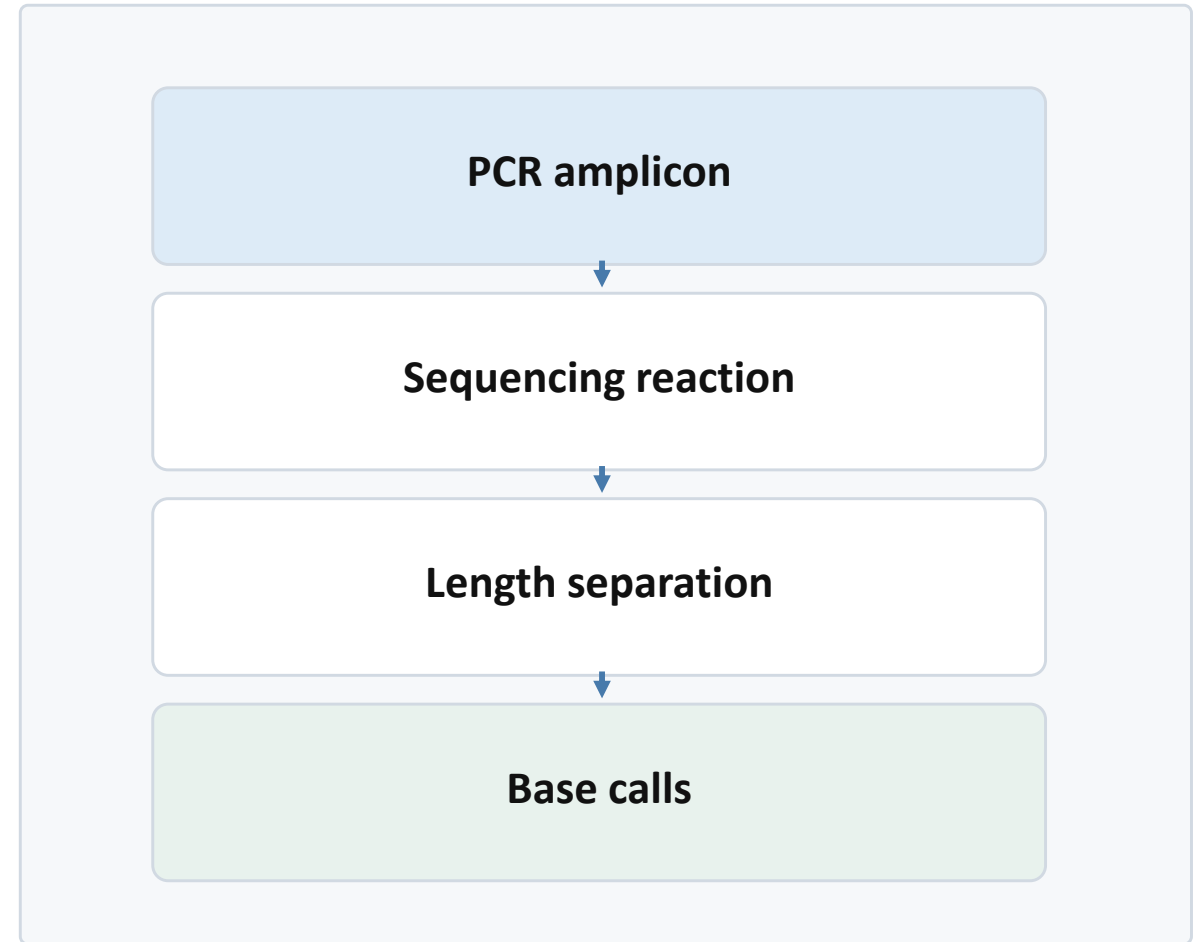
C → T

Hypothetical substitution; No patient sequence implied

Schematic — not patient or laboratory data

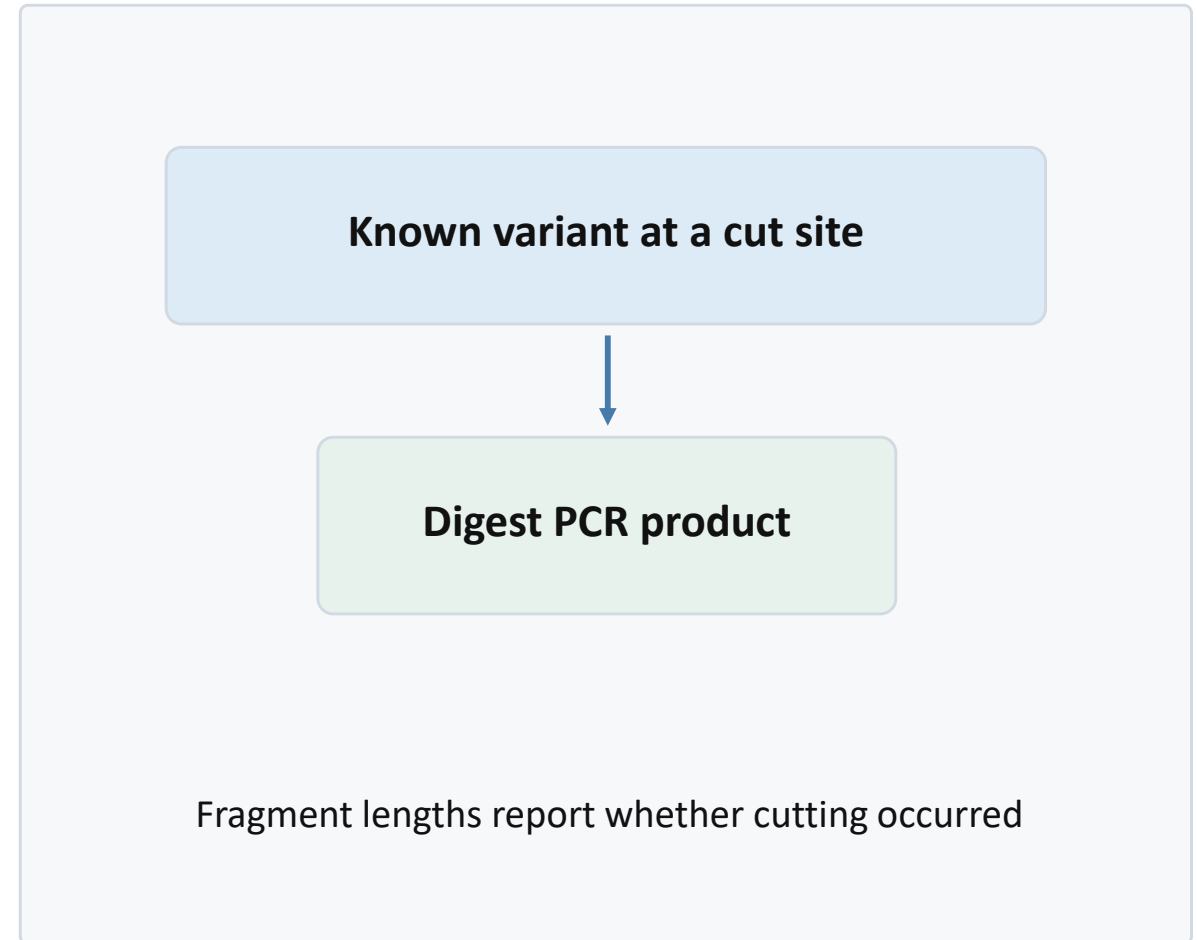
What Sanger sequencing can and cannot answer

- It directly describes nucleotide order across the amplified region.
- It can identify a localized sequence change not predicted in advance.
- It is limited by the region amplified and submitted for sequencing.
- Large or complex genomic changes require consideration of other methods.



Restriction fragment analysis: Principle

- Restriction endonucleases cut DNA at particular recognition sequences.
- A known variant may create or disrupt a recognition site.
- Digestion can then yield a different set of product lengths.
- The diagnostic signal is an electrophoretic fragment pattern.



A restriction enzyme recognizes a sequence

- Enzyme binding is determined by a specific nucleotide sequence.
- When the recognition site is present, the DNA product can be cut.
- A change in that sequence may prevent the normal cut.
- Whether a variant affects the site must be established before using this assay.

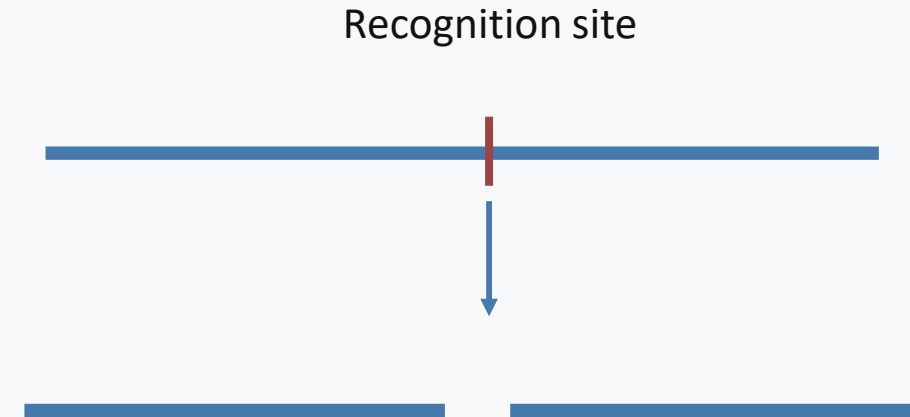


Diagram is conceptual, not a specific restriction enzyme

Restriction analysis workflow

- Amplify the locus containing the relevant recognition site.
- Expose the amplified material to the chosen restriction enzyme.
- Separate digested fragments by gel electrophoresis.
- Compare observed band patterns with the patterns expected from the alleles.

Known variant at a cut site

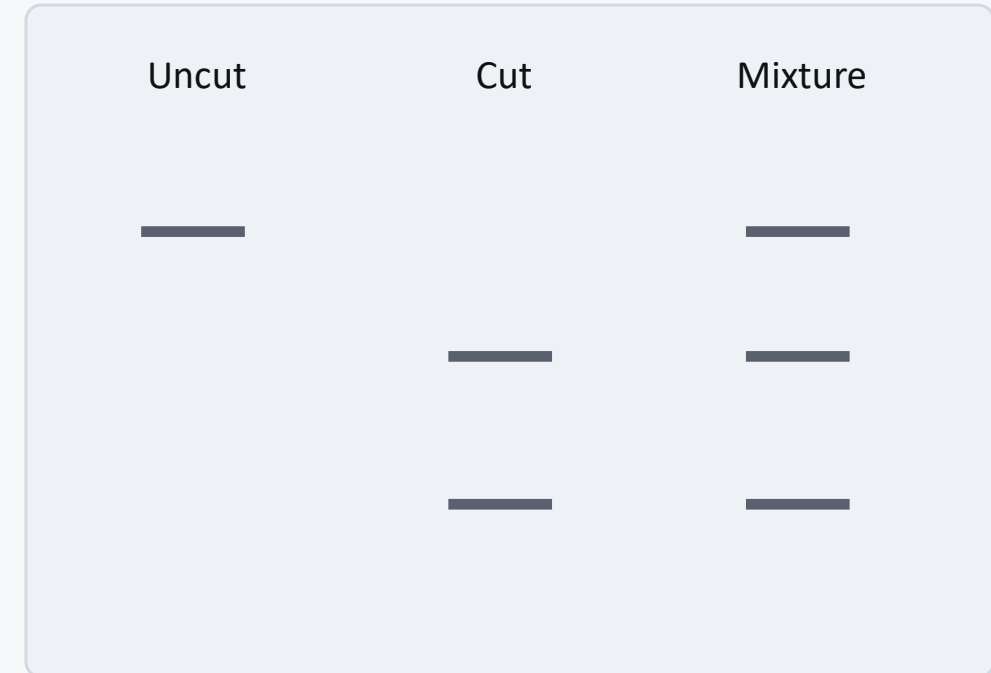


Digest PCR product

Fragment lengths report whether cutting occurred

Cut versus uncut products yield different bands

- Cut DNA produces shorter fragments than an otherwise uncut product.
- Mixtures of product classes may yield multiple bands in a lane.
- Fragment sizes depend on the actual assay design and restriction site.
- The diagram illustrates logic only and contains no laboratory measurements.



Conceptual gel bands; Not measured fragment lengths

Schematic — not patient or laboratory data

Restriction analysis is deliberately targeted

- It is useful when a mutation is known to alter a specific cut site.
- The assay asks whether a predicted restriction pattern is present.
- Other variants that leave the site unchanged may go undetected.
- This narrower scope explains why restriction analysis is less comprehensive than sequencing.

Sequence change



Cut site altered

Different fragment pattern

Best for a known, recurrent variant

Sanger versus restriction analysis

Feature	Sanger sequencing	Restriction analysis
Direct output	Nucleotide base sequence	Cut / uncut fragment pattern
Prior variant knowledge	Not always needed for any variation in target	Known relevant cut-site change
Readout	Fluorescence by fragment length	Gel bands after digestion
Important limitation	Only the amplified region	Only sequence changes affecting cut pattern

Both approaches usually begin with targeted amplification.

Amplicon length analysis: Principle

- Some deletions, insertions, and repeat expansions change the distance between primers.
- The resulting amplicons differ in length if each form can be amplified.
- Size separation can distinguish products without sequencing every base.
- A single-base substitution may produce no length difference.

Shorter allele



Longer allele



Length contrasts are illustrative; No scale is implied

Length analysis cannot determine base identity

- Two sequences can have the same length yet differ in nucleotide composition.
- Sizing is therefore suitable for size-altering lesions, not arbitrary substitutions.
- The method is particularly useful when the length change is the diagnostic signal.
- The expected sizes must be interpreted using the assay design.

Shorter allele



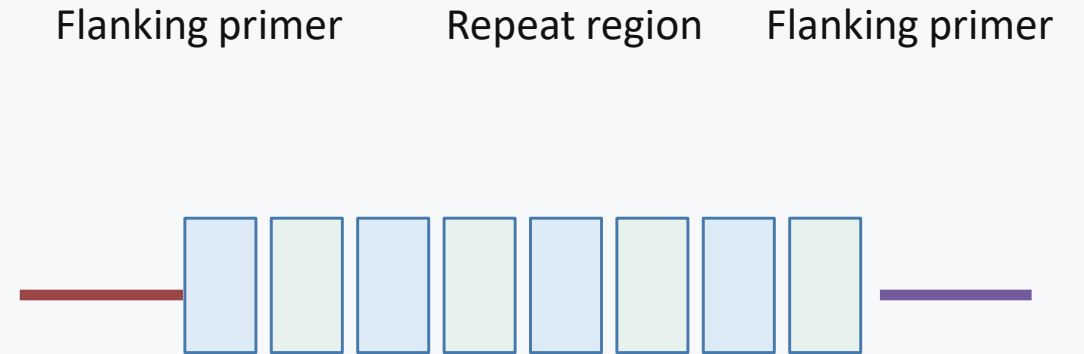
Longer allele



Length contrasts are illustrative; No scale is implied

Repeat-length variation changes product size

- Tandem repeats contain recurring sequence units arranged end to end.
- A different number of repeat units changes the distance between flanking primers.
- An amplifiable repeat interval therefore generates a different-sized fragment.
- Repeat copy number is inferred from the size relationship, not from a displayed micrograph.

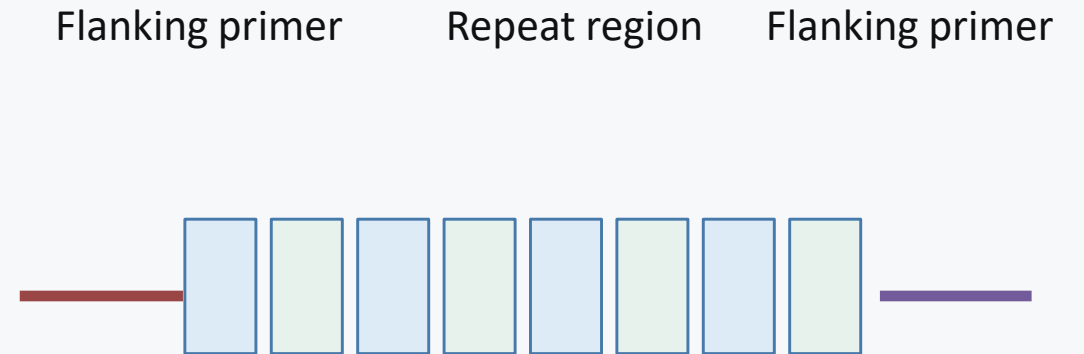


Each repeat contributes to amplified length

Schematic — not patient or laboratory data

Primer design around a repeat tract

- The primers must lie outside the variable-length repeated region.
- Each amplified allele includes the intervening repeat tract.
- Changes in the tract alter product length while primer positions remain fixed.
- Amplification failures complicate interpretation of very large expansions.



Each repeat contributes to amplified length

Schematic — not patient or laboratory data

FMR1 is a clinical example of repeat analysis

- A trinucleotide-repeat region at the 5' end of FMR1.
- Different repeat counts in normal and premutation alleles can alter PCR product length.
- Gel electrophoresis may distinguish products from alleles that amplify.
- PCR alone may not resolve the largest expansions relevant to fragile X syndrome.

FMR1 5' repeat region

Different repeat counts

Products from amplifiable alleles differ in size

Fragile X repeat analysis has a PCR blind spot

- Very large repeat expansions may not amplify with conventional PCR.
- Failure to recover an expanded allele can make a size-only result incomplete.
- Southern blot analysis as a possible complementary method.
- A missing product must not automatically be interpreted as a normal-sized repeat.

Very large repeat expansion

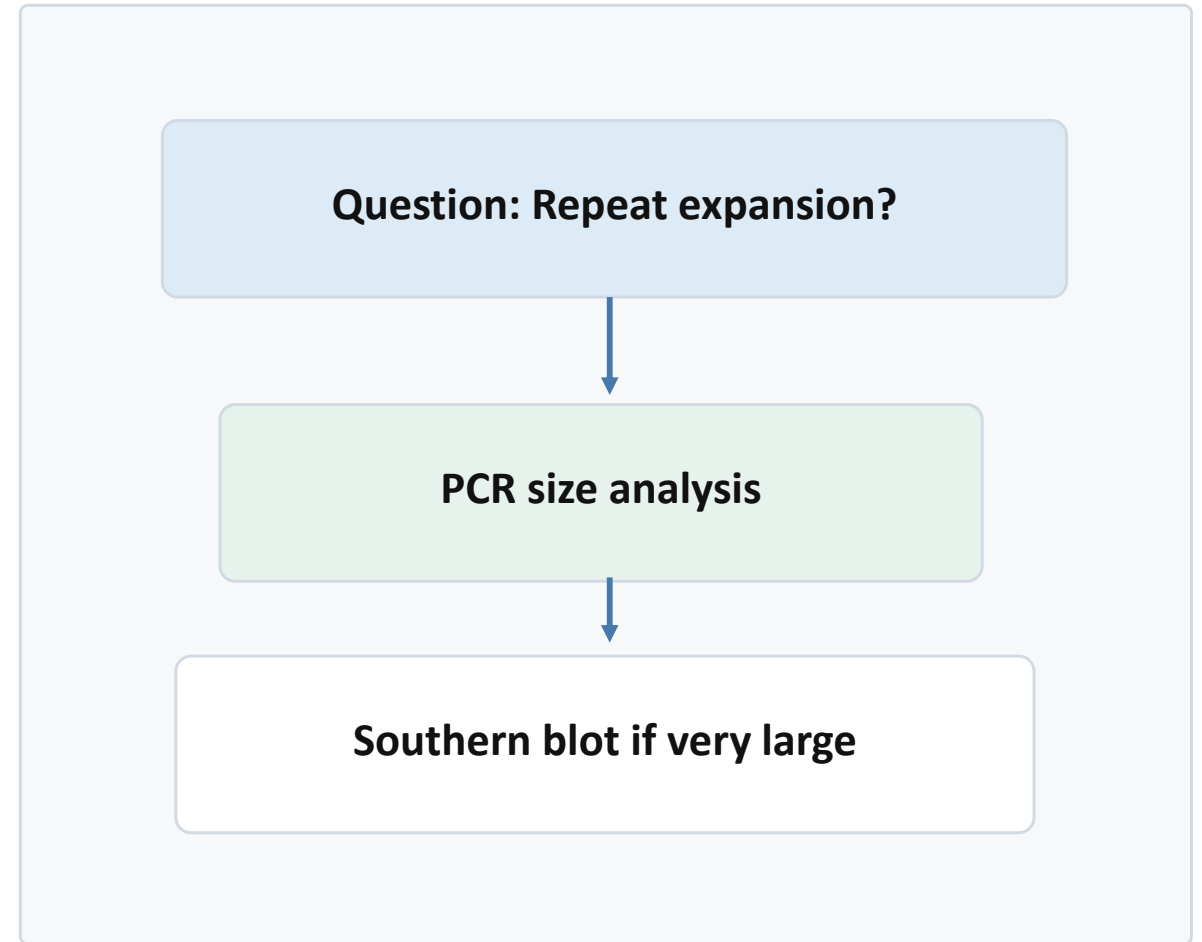


Conventional PCR may fail

Absence of product is not proof of absence of expansion

When complementary testing is needed

- Start from the clinical question and expected lesion size.
- Conventional amplicon sizing works only when relevant allele forms amplify.
- If a very large FMR1 expansion is suspected, Southern blot may be required.
- Interpret an apparently negative result in the context of assay reach.



Deletion and expansion can both alter length

- Deletion of sequence between primers can shorten the amplified product.
- An insertion or repeat expansion may lengthen the amplified interval.
- Allele sizes do not, by themselves, identify exact deleted or inserted bases.
- Sequencing provides different information from the length readout.

Shorter allele



Longer allele



Length contrasts are illustrative; No scale is implied

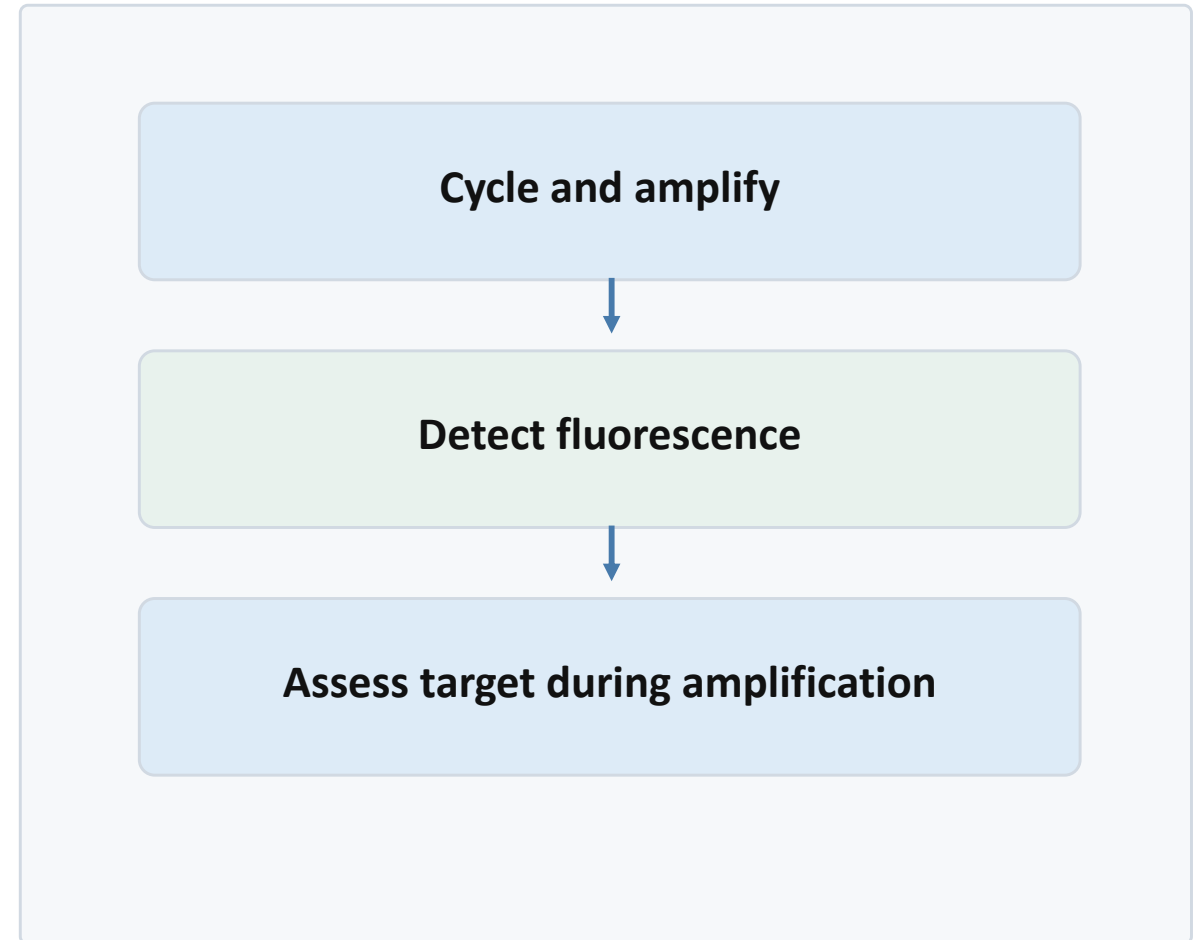
Length analysis versus sequence analysis

Diagnostic question	Fragment sizing	Sanger sequence
Is the product shorter or longer?	Directly informative	May be informative
Which base was substituted?	Usually not informative	Directly informative
How many repeat units?	Can infer if alleles amplify	May be challenging for large repeats
What lies outside PCR primers?	Not interrogated	Not interrogated

Targeted methods inherit the boundaries established by their primers.

Real-time PCR asks a quantitative question

- The objective is to detect and quantify a defined nucleic acid target.
- Fluorescent indicators report product accumulation during cycling.
- Assessment during the exponential amplification phase.
- Monitoring molecular burden is a central clinical use.



Endpoint PCR versus real-time PCR

- Endpoint analysis examines the products after the reaction is completed.
- Real-time analysis interrogates fluorescent signal during amplification.
- This allows quantitative assessment of the target rather than size alone.
- Readout choice should reflect whether the clinical question is presence or burden.

Endpoint PCR

Analyze after cycling

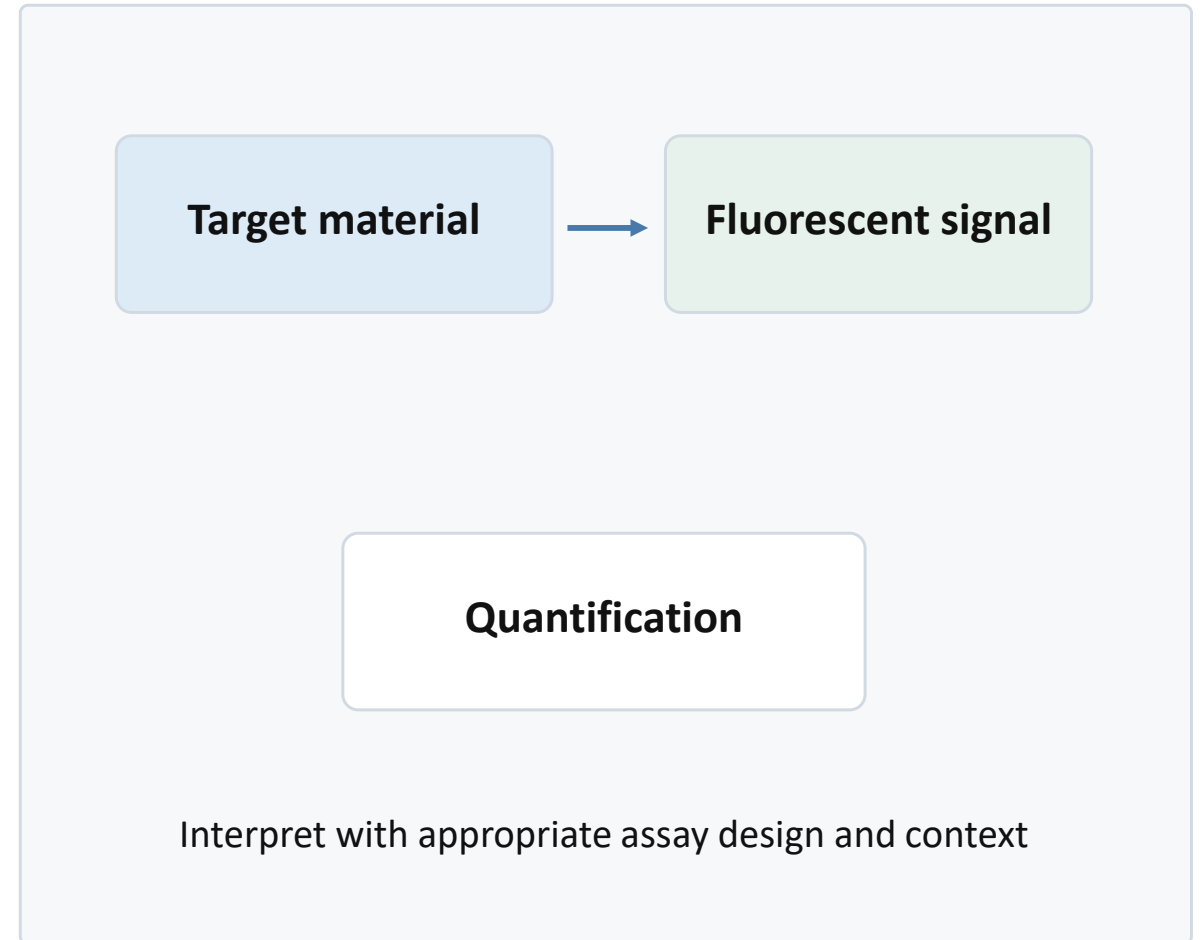
Real-time qPCR

Measure during cycling

Readout timing changes what is measured

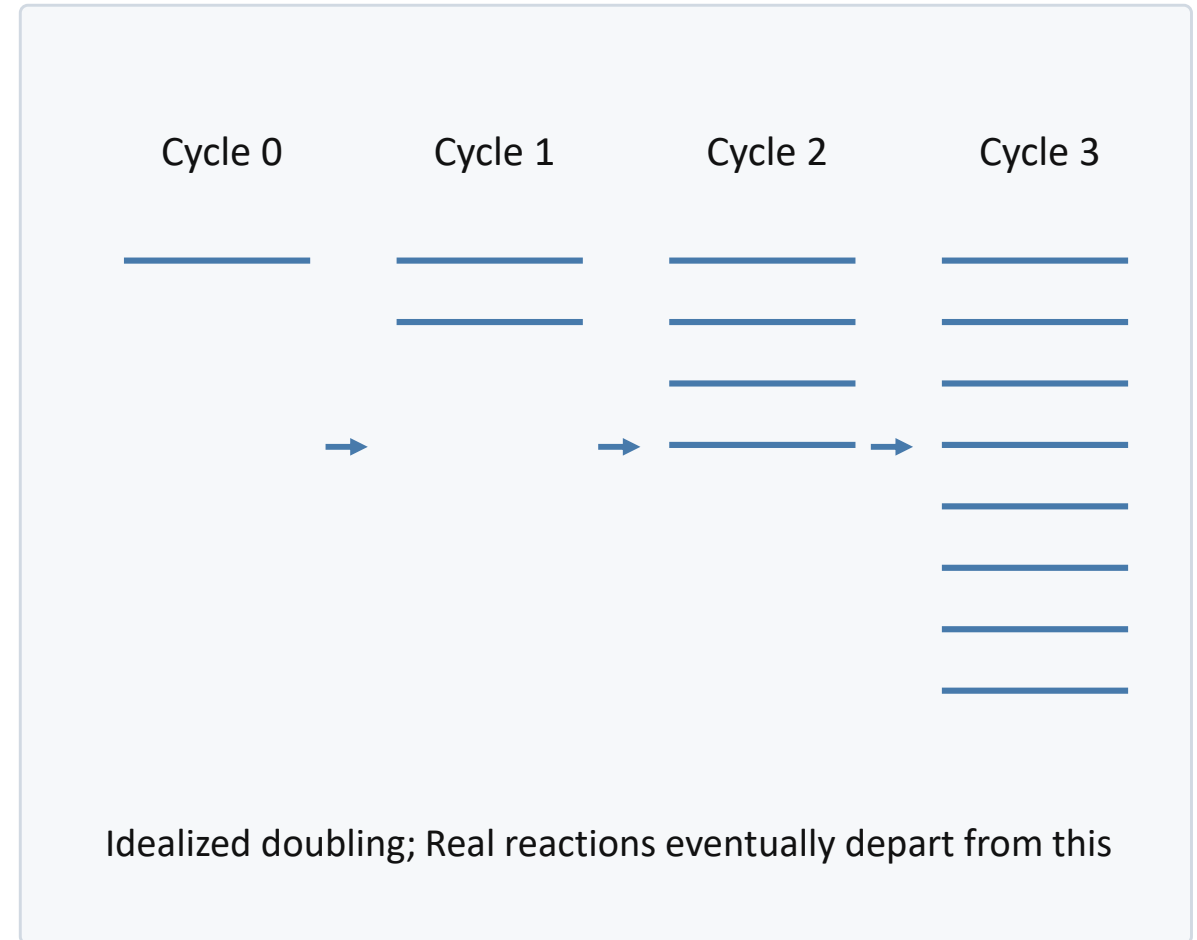
Fluorescence provides the qPCR signal

- Fluorophore-based technologies make target-associated amplification detectable.
- Signal acquisition occurs during repeated PCR cycles.
- The measured signal is interpreted using the assay's quantitative framework.
- The fluorescence mechanism should not be confused with Sanger base labeling.



The exponential phase is analytically important

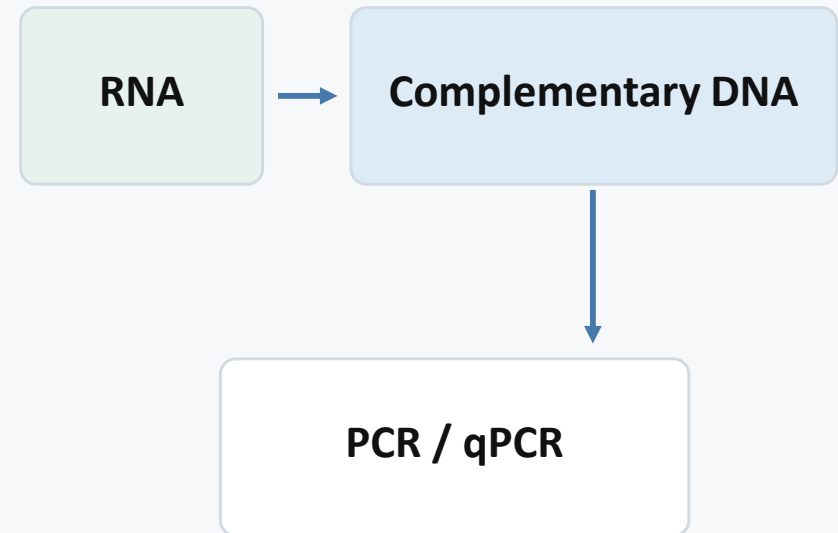
- The exponential phase as the period for real-time assessment.
- Amplification products accumulate across successive cycles.
- QPCR relies on the relationship between product accumulation and fluorescent detection.
- Clinical quantitative results depend on validated assay design and interpretation.



Schematic — not patient or laboratory data

RNA targets require reverse transcription

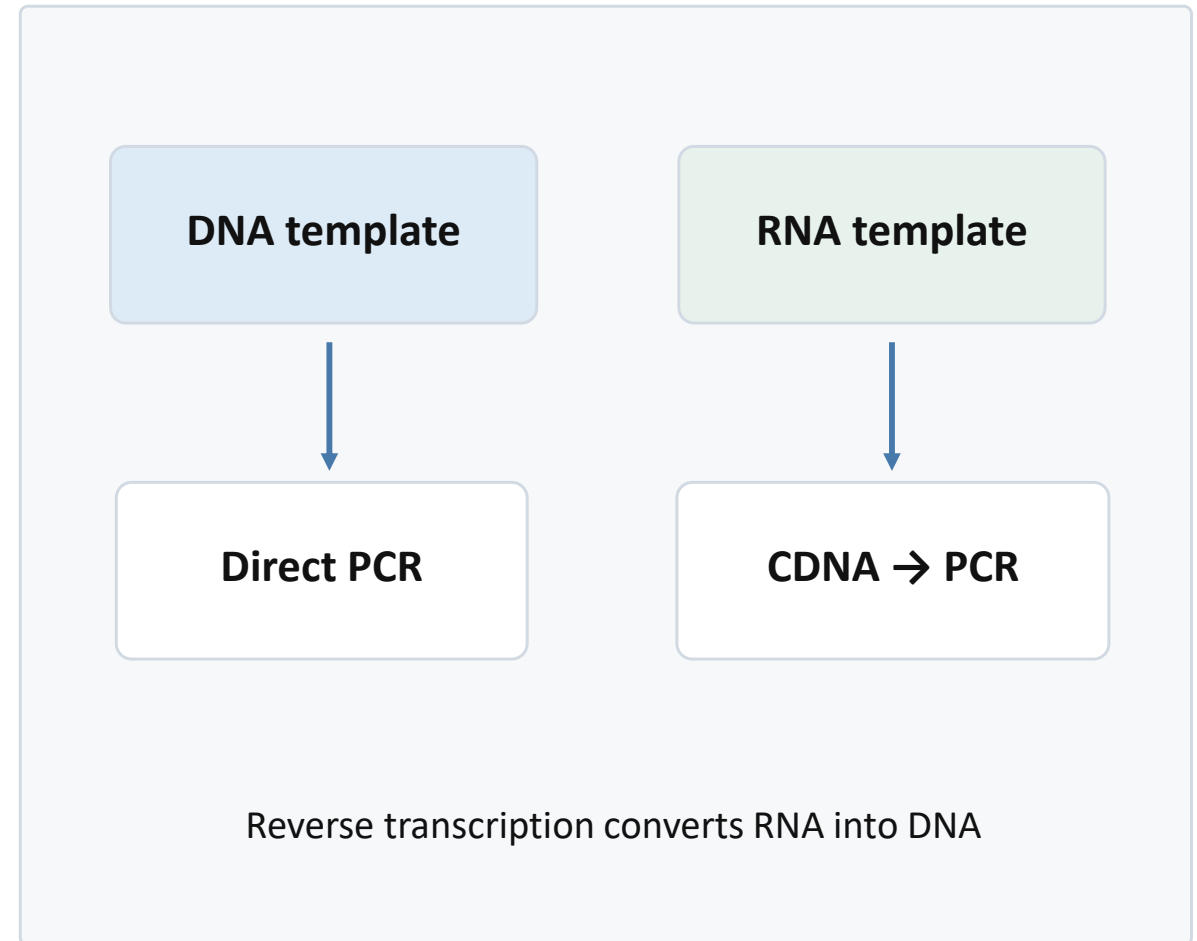
- RNA is converted to complementary DNA before conventional DNA amplification.
- The resulting cDNA can then serve as PCR or qPCR template.
- This enables analysis of RNA species, including disease-associated transcripts.
- Reverse transcription changes template type; It does not by itself sequence RNA.



Reverse transcription must precede DNA polymerase amplification

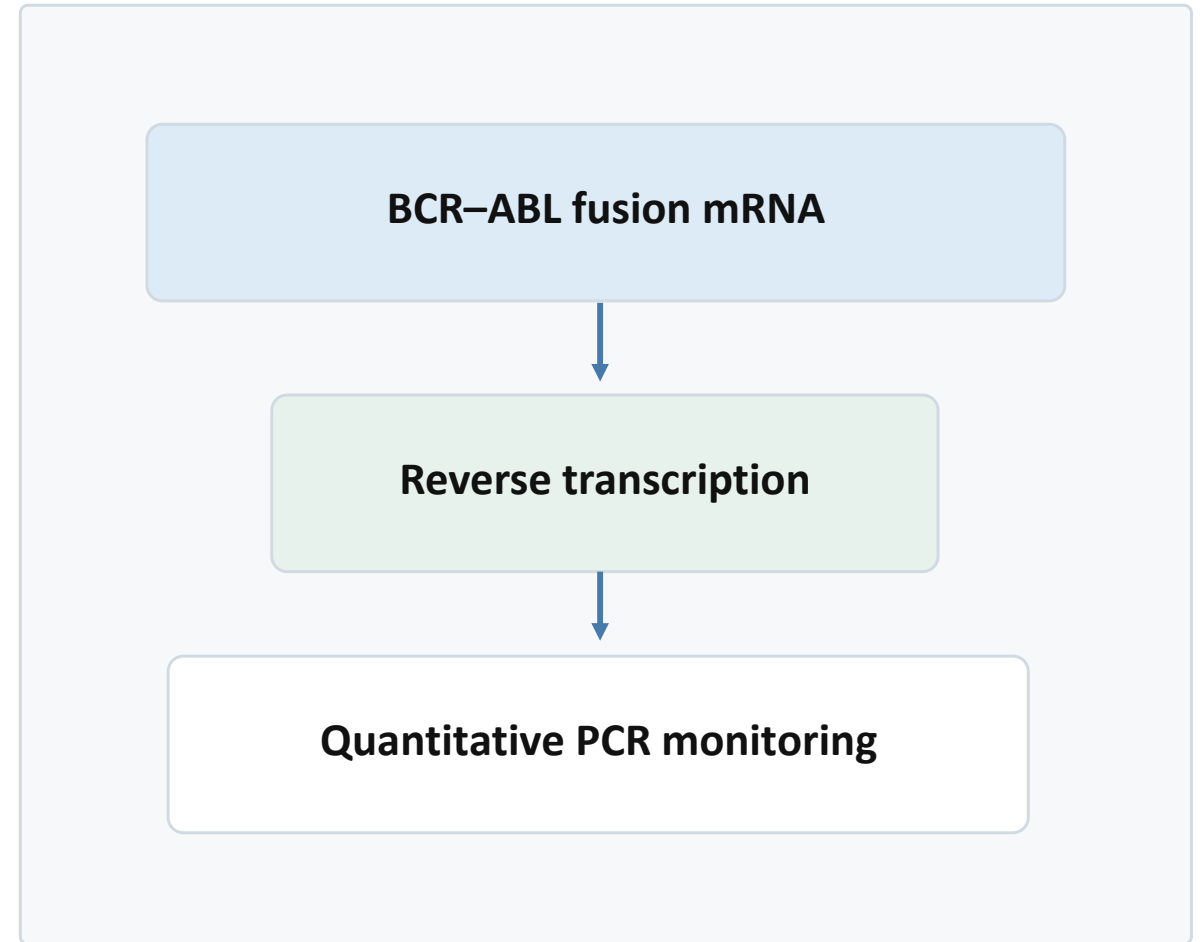
RT-PCR and qPCR describe different steps

- RT-PCR denotes PCR preceded by reverse transcription of RNA into cDNA.
- QPCR denotes real-time quantitative measurement during amplification.
- The same workflow may combine reverse transcription with quantitative PCR.
- Precise naming avoids conflating RNA template preparation with quantification.



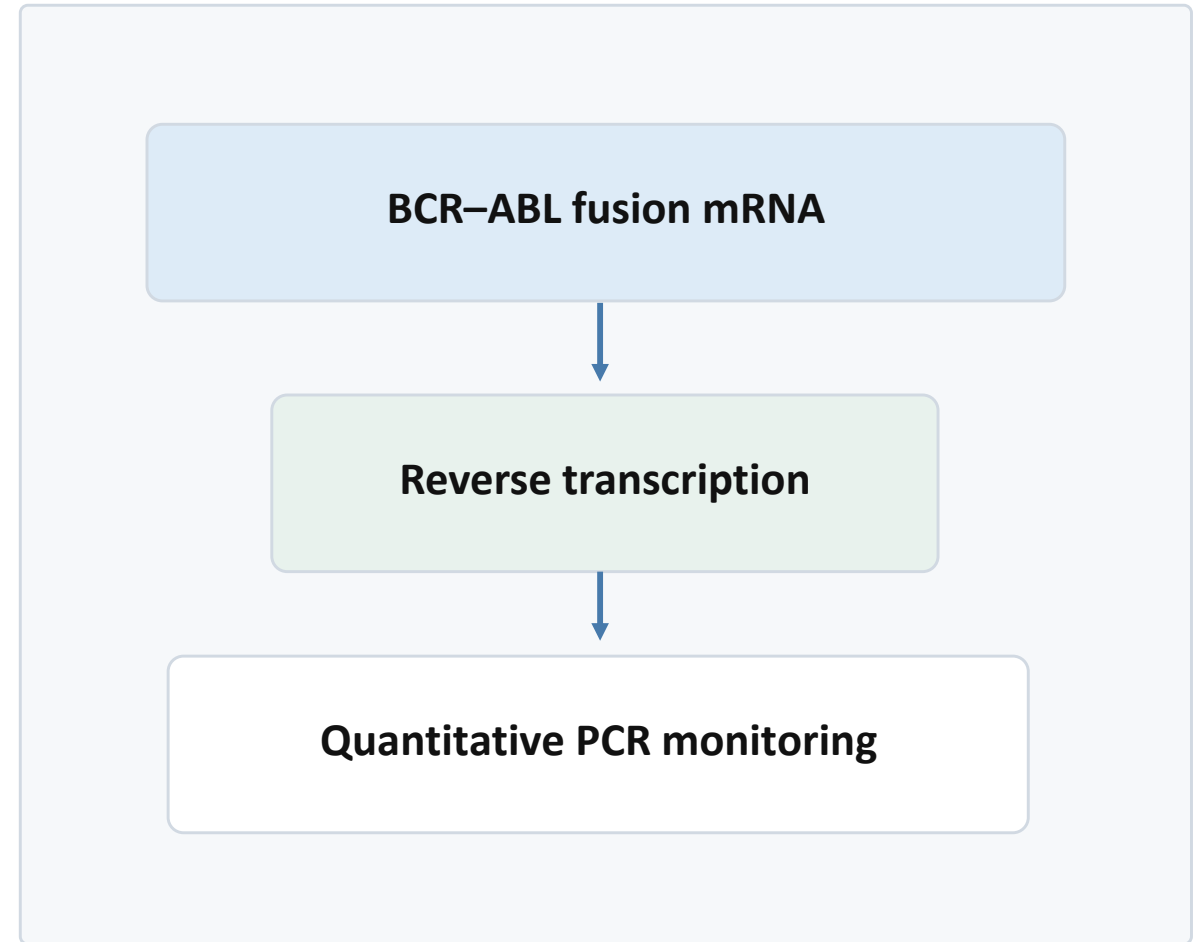
BCR–ABL transcript monitoring in CML

- Chronic myeloid leukemia has a characteristic BCR–ABL fusion lesion.
- BCR–ABL fusion mRNA as an example of a quantitative RNA target.
- Reverse transcription produces a cDNA template for quantitative analysis.
- Serial molecular measurement can inform treatment efficacy and disease burden.



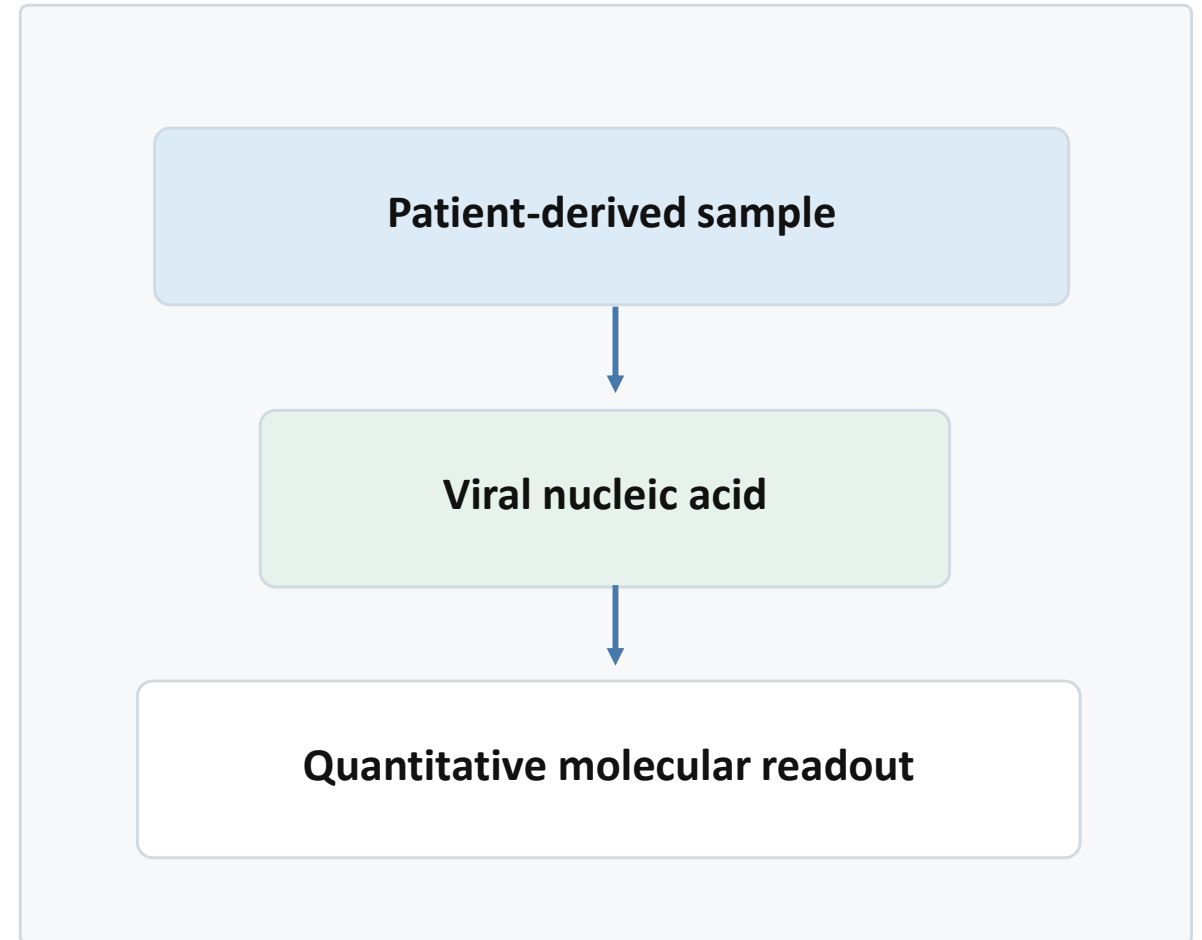
PCR-based monitoring differs from diagnosis

- At diagnosis, the question may be whether a disease-associated lesion is present.
- During follow-up, the question may be how much of a molecular target persists.
- The same target can therefore support more than one clinical decision.
- A quantitative trend should be interpreted within the defined disease context.



Viral nucleic-acid burden is another qPCR use

- HIV and Epstein–Barr virus as clinical examples of viral-load assessment.
- The assay targets microorganism-associated nucleic acid in an appropriate specimen.
- Quantitative monitoring can contribute to evaluation of infection or treatment.
- Whether reverse transcription is required depends on whether the target is RNA.



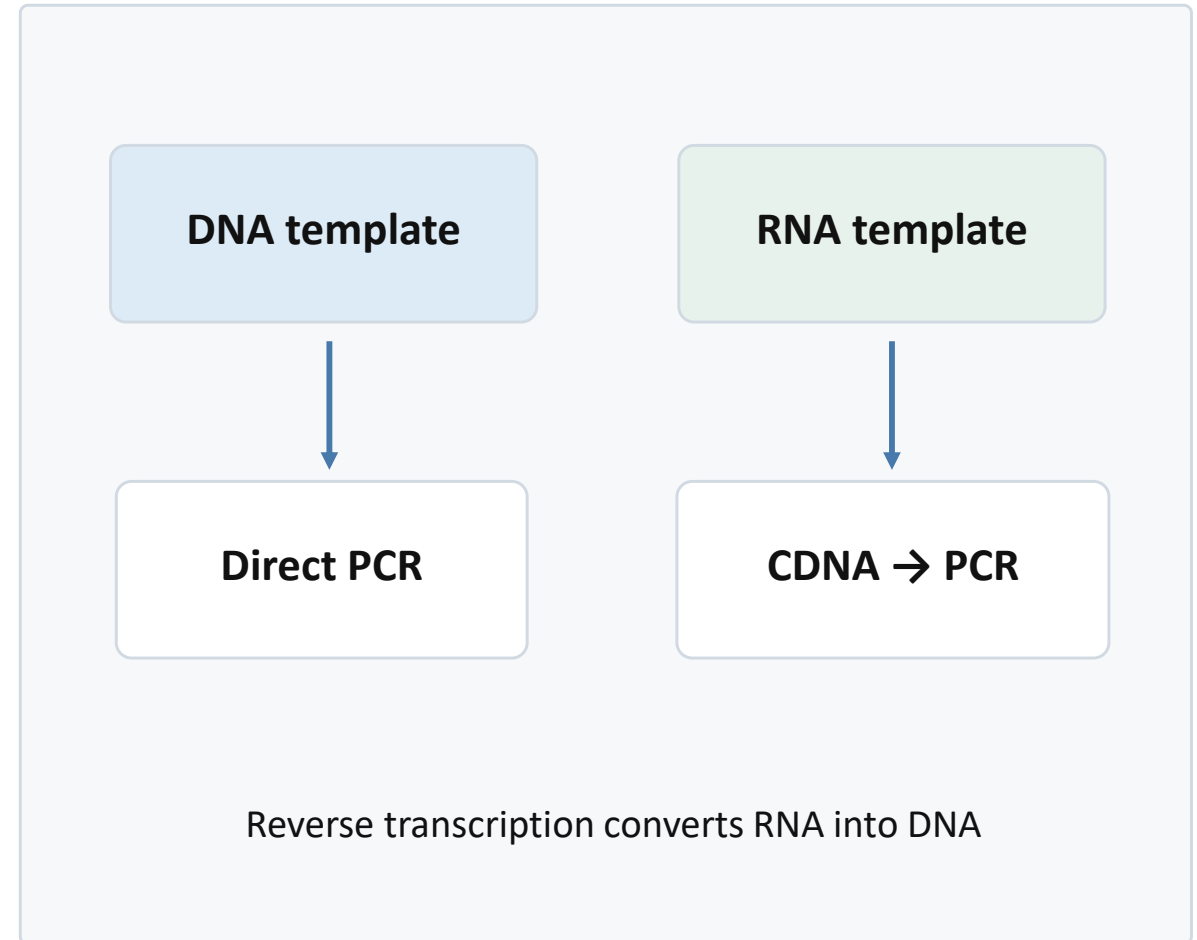
Cancer and infection share the PCR framework

Clinical setting	Target example	Question addressed
CML	BCR–ABL fusion mRNA	How much molecular disease signal?
Infectious disease	HIV-related viral nucleic acid	What is the viral burden?
Infectious disease	Epstein–Barr virus DNA	What is the viral burden?
Inherited disease	FMR1 repeat-containing DNA	How large is the repeat amplicon?

These are textbook examples of PCR applications, not universal testing guidelines.

DNA targets and RNA targets are not interchangeable

- Genomic DNA assays analyze material encoded in DNA.
- RNA assays interrogate a transcribed or viral RNA target only after reverse transcription.
- BCR–ABL fusion mRNA and genomic lesions may answer different questions.
- Assay wording should specify the measured molecule and intended clinical use.



Every method has a defined detection scope

- Sanger sequencing describes sequence within one amplified region.
- Restriction assays depend on an informative cut-site change.
- Size assays require both length change and successful amplification.
- Quantitative PCR measures abundance of a selected target, not every possible mutation.

Question: Identity / length / quantity?

Sequence → Sanger

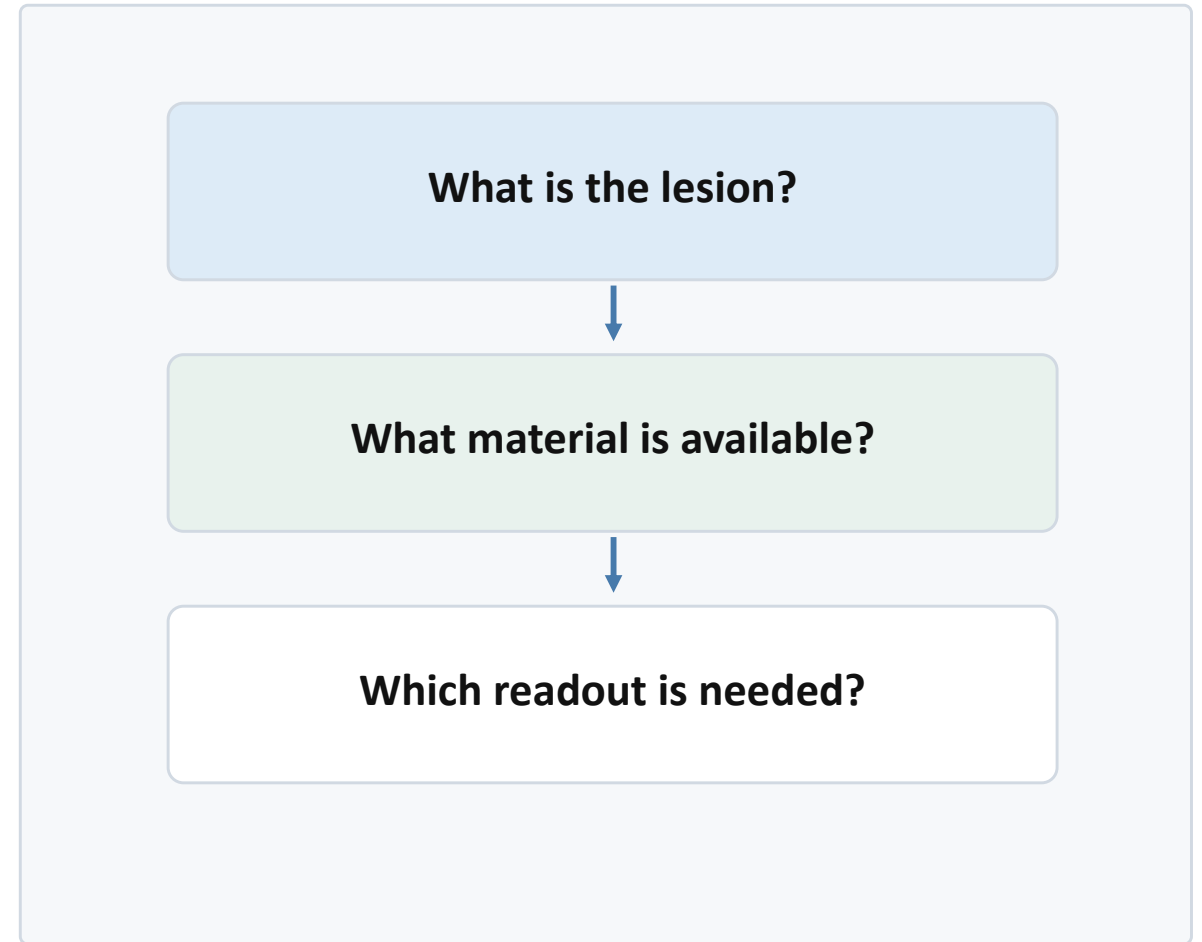
Cut site → restriction

Length → sizing

Abundance → qPCR

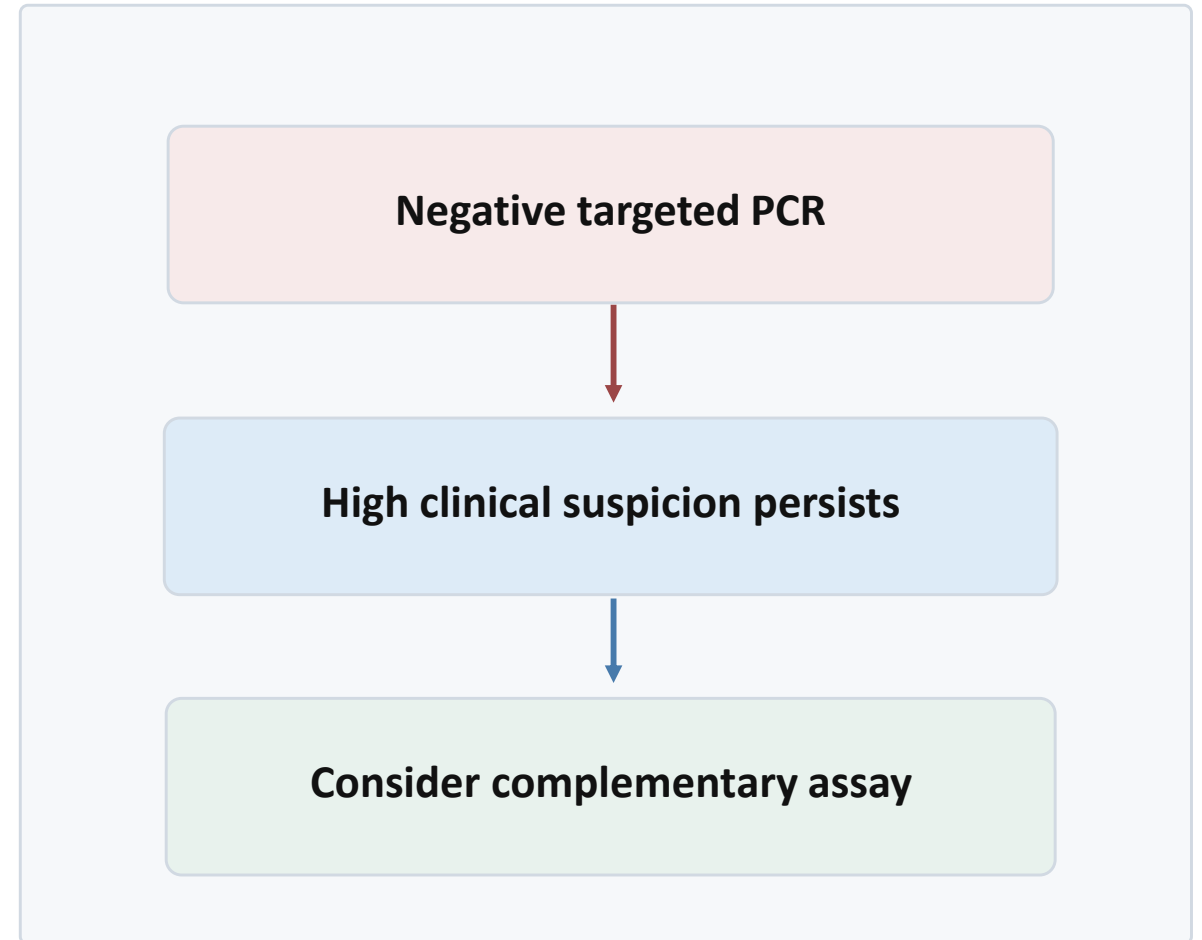
Analytical performance influences method choice

- Sensitivity, specificity, accuracy, and reproducibility.
- Cost, labor, reliability, and turnaround time also shape assay choice.
- A method must match the spectrum of lesions in the population tested.
- Performance characteristics are assay-specific rather than guaranteed by the term PCR.



A negative targeted assay does not exclude disease

- A patient may harbor a lesion outside the amplified interval.
- A suspected repeat expansion may be too large for conventional PCR.
- Large deletions and rearrangements may require other technologies.
- Persistently high clinical suspicion justifies reconsidering test selection.



PCR does not readily resolve every structural lesion

- Large deletions, duplications, and complex rearrangements.
- Such lesions may have variable breakpoints that are poorly suited to targeted PCR.
- Hybridization-based or other genomic methods can address complementary questions.
- The practical limitation is locus coverage, not a universal failure of PCR chemistry.

Short defined locus

Large complex lesion

Often PCR-friendly

May need another
method

Assay reach follows genomic alteration scale

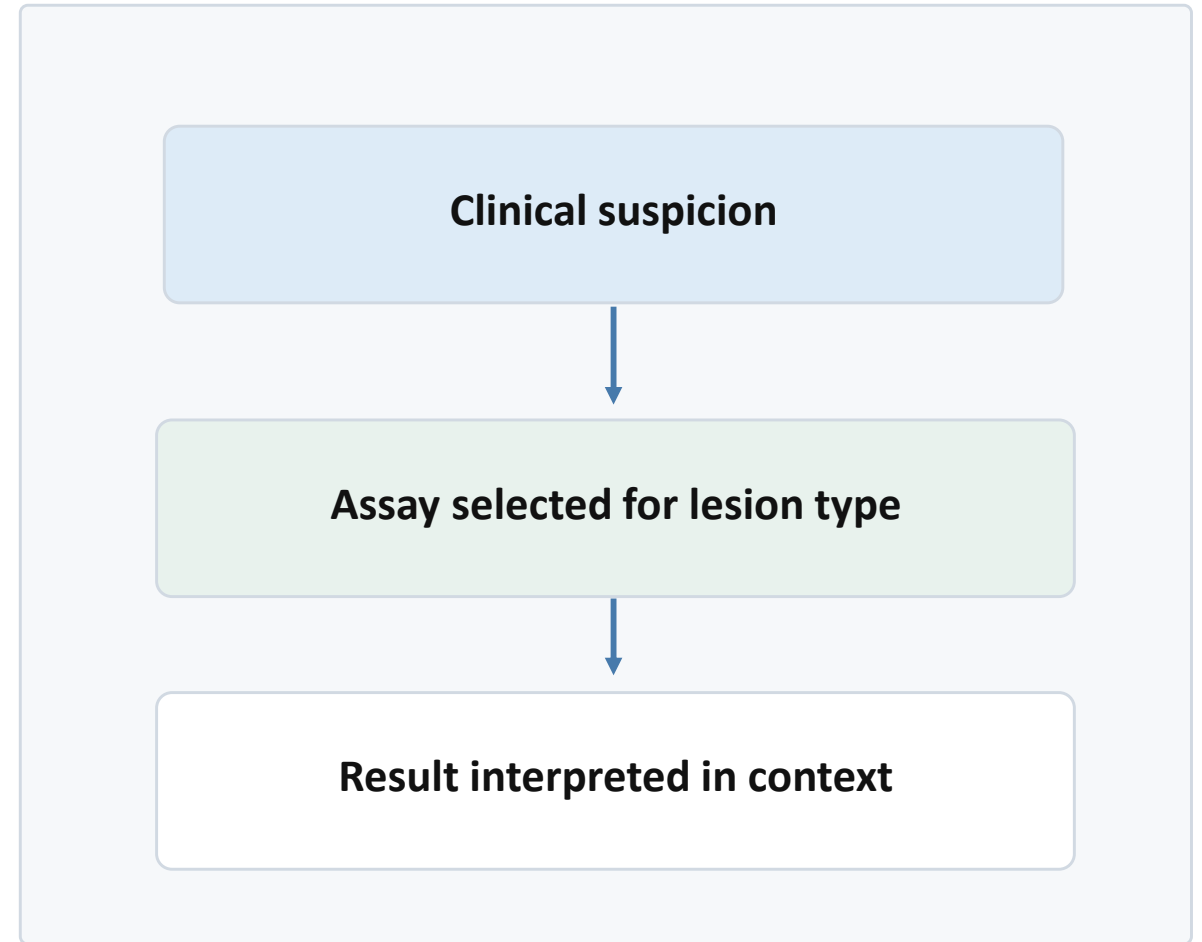
Selecting a PCR readout by abnormality

Suspected alteration	Appropriate initial readout	Key limitation
Localized unknown base change	Sanger sequencing	Only sequenced amplicon
Known cut-site variant	Restriction fragment pattern	Variant must alter enzyme recognition
Amplifiable repeat-size change	Amplicon size analysis	Large allele may fail amplification
Transcript / viral burden	Real-time qPCR (RT step for RNA)	Target- and assay-specific quantification

The table maps the molecular question to the textbook-described assay principle.

Integrated clinical molecular workflow

- Define the suspected disease and likely class of genetic alteration.
- Obtain material likely to contain the constitutional, somatic, or microbial target.
- Choose primer-defined amplification and an informative analytical readout.
- Review test limitations before translating findings into clinical conclusions.



Case: An invariant pathogenic cut-site variant

- A familial disorder is known to involve a fixed nucleotide change affecting a restriction site.
- The laboratory needs an efficient targeted assay for the known alteration.
- Which post-PCR method directly exploits altered enzyme cleavage?
- Why would this method not serve as a general screen for all variants?

Sequence change



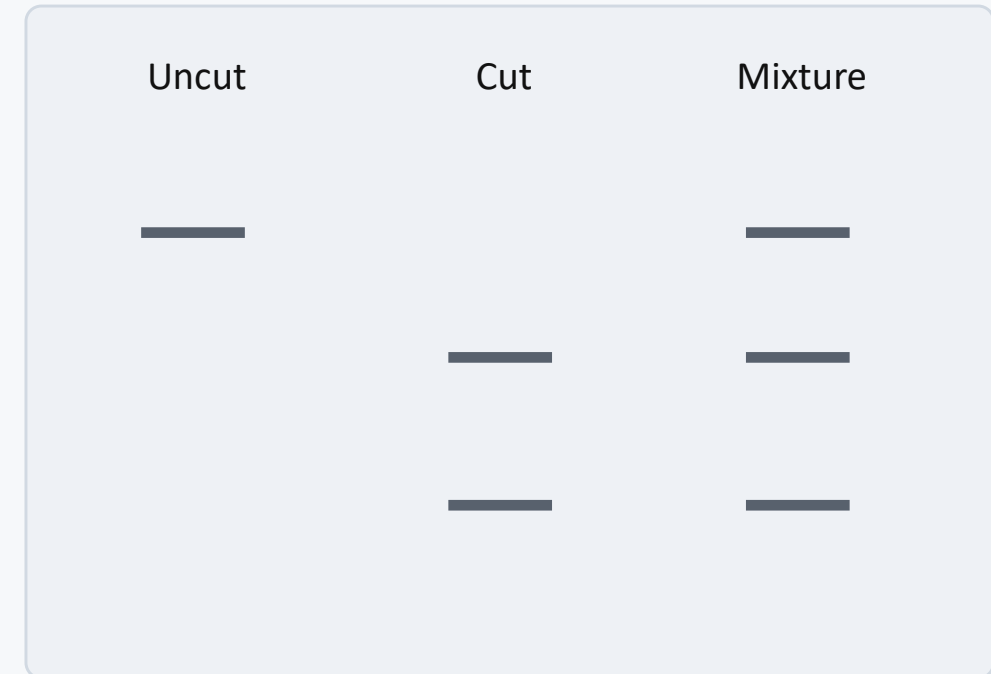
Cut site altered

Different fragment pattern

Best for a known, recurrent variant

Case answer: Use restriction fragment analysis

- PCR amplifies a defined interval containing the altered recognition site.
- Restriction digestion produces the expected allele-dependent fragment pattern.
- Electrophoresis distinguishes cut and uncut classes.
- The approach is appropriate because the causal variant and informative site are known.



Conceptual gel bands; Not measured fragment lengths

Schematic — not patient or laboratory data

Case: Same-sized DNA products, different bases

- Two samples produce amplicons of equal length.
- A single-base substitution is suspected within the amplified locus.
- Amplicon sizing cannot establish the sequence difference.
- Which method allows the base order to be compared with a reference?

Reference

ATG C T A A G

Sample

ATG T T A A G

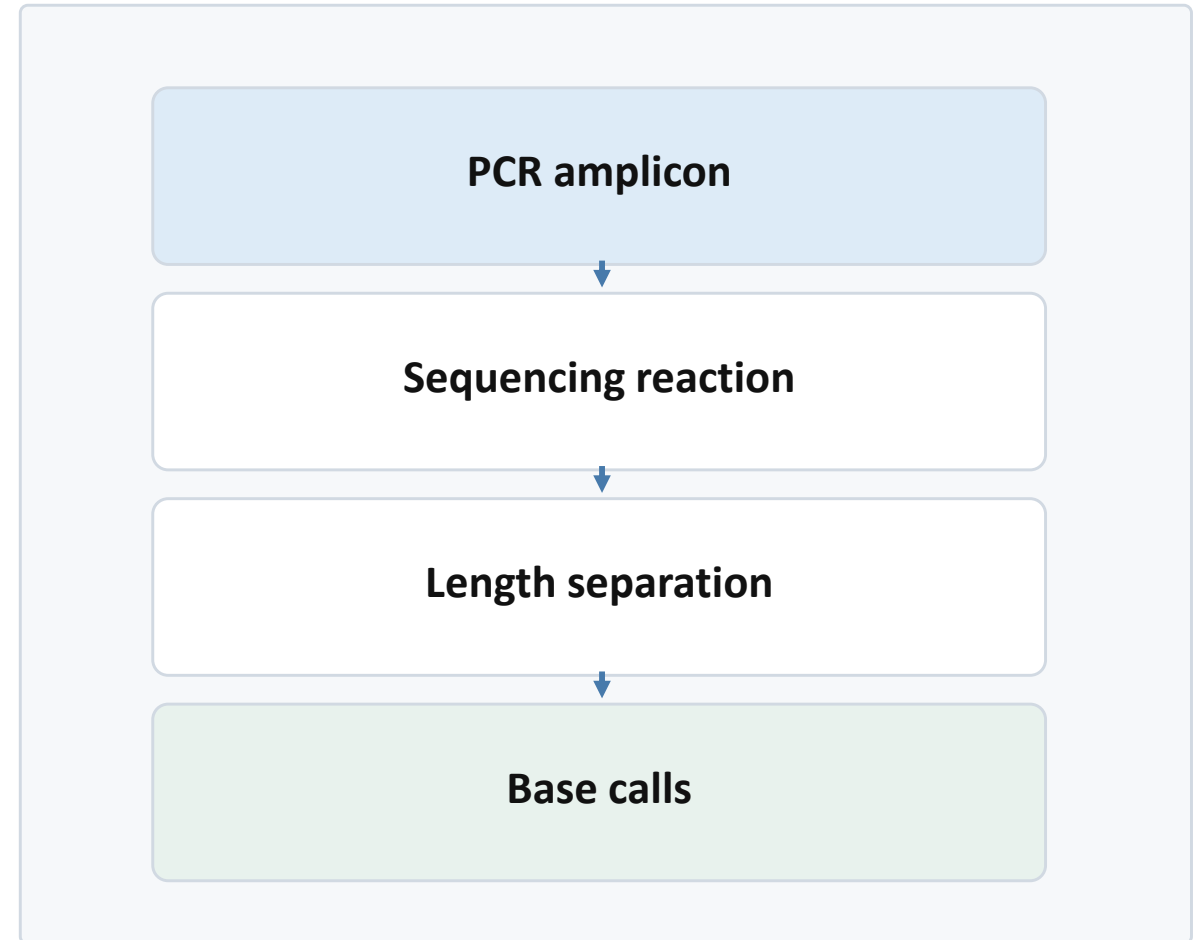
C → T

Hypothetical substitution; No patient sequence implied

Schematic — not patient or laboratory data

Case answer: Use Sanger sequencing

- Sequence a defined PCR product with terminator-labeled extension reactions.
- Size-separate the terminated products to recover nucleotide order.
- Compare the sample sequence against the relevant reference.
- The length match does not rule out a nucleotide substitution.



Case: Possible large FMR1 expansion

- Clinical suspicion of fragile X syndrome remains high.
- Conventional PCR detects no clearly expanded product.
- The missing band cannot be interpreted as definitive absence of an expansion.
- Which complementary assay does Robbins identify for very large expansions?

Very large repeat expansion

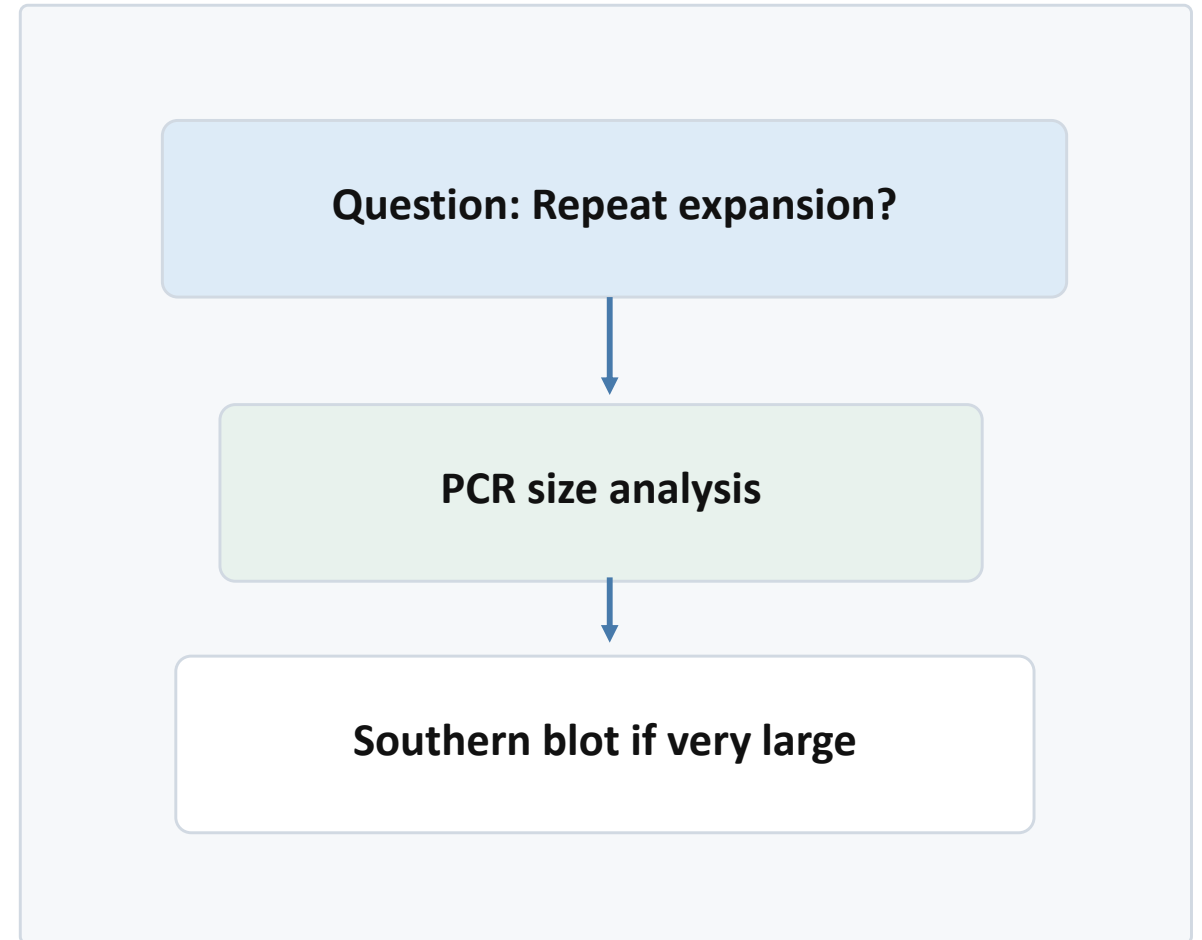


Conventional PCR may fail

Absence of product is not proof of absence of expansion

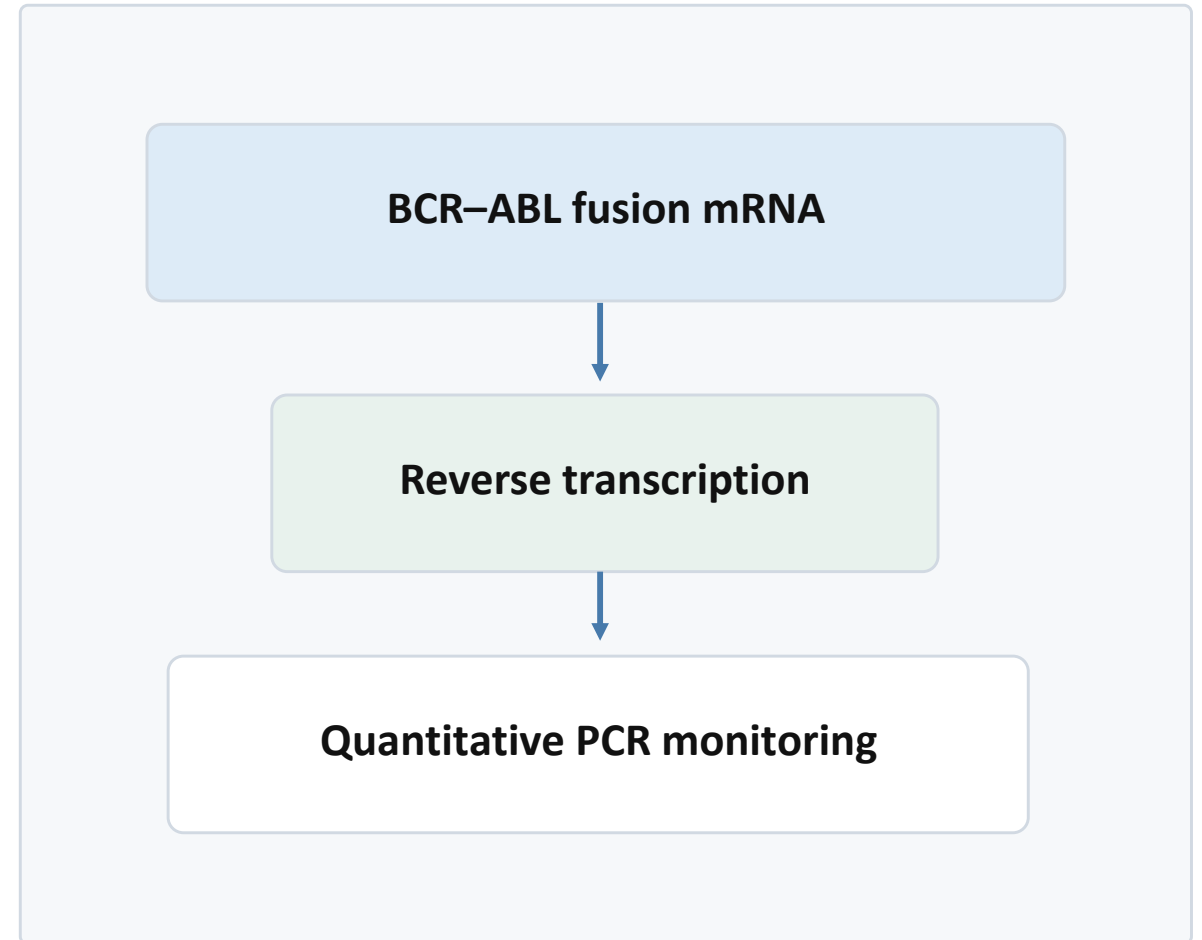
Case answer: Consider Southern blot

- The largest trinucleotide expansions may exceed conventional PCR amplification capability.
- Southern blotting of genomic DNA may therefore be required.
- The choice addresses an assay limitation rather than negating PCR utility.
- Test reporting should state the detection limits relevant to the suspected lesion.



Case: Monitoring molecular disease in CML

- A patient with CML undergoes serial assessment for a BCR–ABL fusion transcript.
- The target being measured is RNA rather than genomic DNA.
- Reverse transcription prepares the template for amplification.
- Quantitative PCR provides a molecular burden readout for follow-up.



Five concepts that govern PCR interpretation

- Primers determine which DNA interval is interrogated.
- The downstream analytical readout determines what constitutes a result.
- RNA is converted into cDNA before PCR.
- Large repeats and rearrangements create predictable detection gaps.
- Clinical interpretation requires the correct specimen and a disease-specific question.

Question: Identity / length / quantity?

Sequence → Sanger

Cut site → restriction

Length → sizing

Abundance → qPCR

References and source boundaries

- Robbins, Cotran & Kumar: Pathologic Basis of Disease. 11th ed. (2026). Chapter 5: Genes and Human Diseases.
- Primary section: “PCR Detection of DNA and RNA Sequence Alterations,” printed p. 161.
- Parent section: “Molecular Genetic Diagnosis,” printed pp. 159–161.
- The molecular schemes and sequence examples are newly constructed educational illustrations.
- Original textbook Figure 5.33 and chapter PDF were unavailable for direct comparison; no figure is reproduced.