

Liquid Biopsies and ctDNA Analysis

Molecular principles, laboratory workflow, and clinical interpretation

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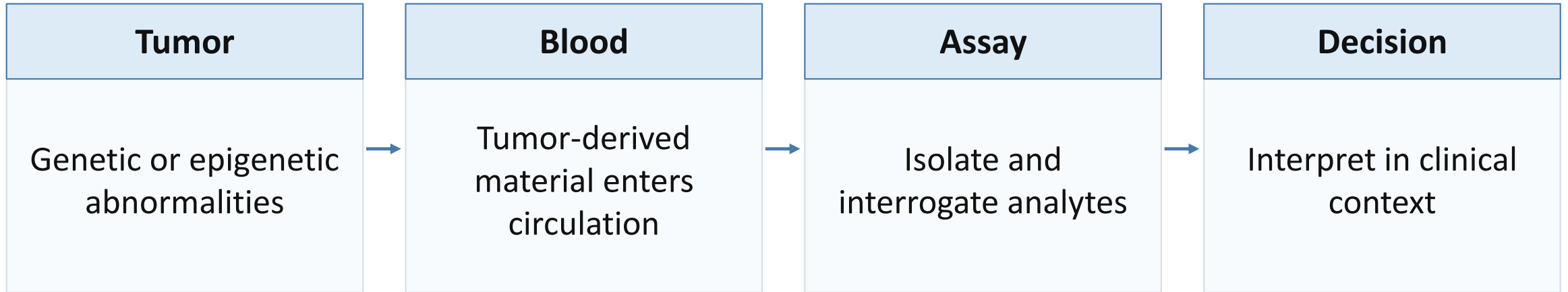
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Learning objectives

- Distinguish ctDNA from other circulating tumor-derived analytes.
- Explain why rare tumor alleles can be detected amid normal cfDNA.
- Outline preanalytic, analytic, and interpretive vulnerabilities.
- Appraise clinical indications without conflating validity and utility.

The central diagnostic idea



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- A blood draw can provide molecular information that otherwise may require tumor tissue.
 - Liquid biopsy complements—not universally replaces—histopathologic sampling.

What constitutes a liquid biopsy?

Definition

- Relatively noninvasive sampling of tumor-associated material from a body fluid.
- Plasma is the principal specimen discussed here for ctDNA.

Not equivalent to tissue

- Does not directly show tissue architecture, grade, or invasion.
- Analyte yield varies with disease state and assay design.

Tumor-derived analytes in circulation

Analyte	Biological form	Principal analytical information
ctDNA	Fragmented tumor-derived cfDNA	Sequence variants; methylation signatures
Circulating tumor cells	Intact malignant cells	Cell-associated nucleic acid / phenotype
Extracellular vesicles	Membrane-enclosed material	Molecular cargo under investigation
Trace proteins	Soluble tumor-associated products	Complementary biomarker signals

Cell-free DNA is not synonymous with ctDNA

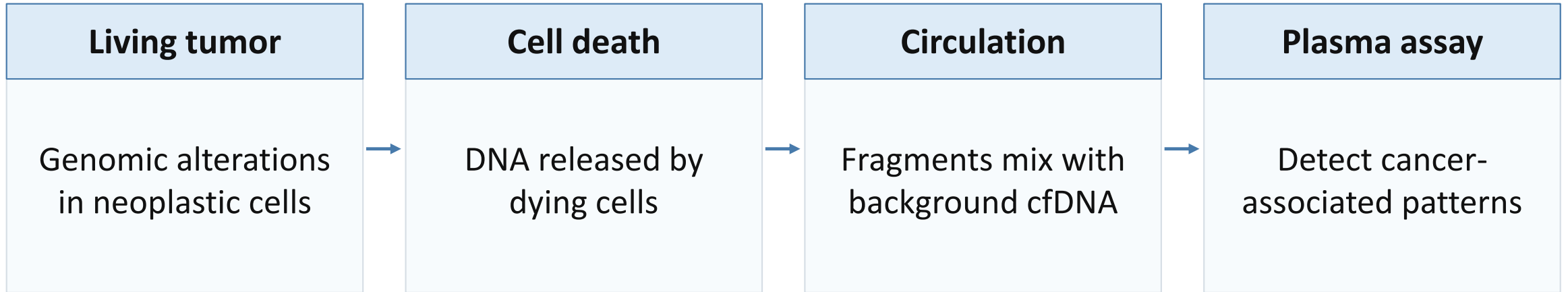
CfDNA

- DNA fragments from multiple normal and abnormal cellular sources.
- Most circulating DNA in plasma may be nontumor-derived.

CtDNA

- The tumor-derived fraction of the cfDNA pool.
- Identification depends on tumor-associated molecular features.

How tumor DNA enters blood



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- Tumor-derived DNA can also be carried in circulating tumor cells.
 - The assay sees a molecular mixture, not a pure tumor genome.

Circulating cells and free DNA are distinct

Circulating tumor cells

- Whole malignant cells detached from tumor sites.
- Potentially enable cellular and phenotypic studies.

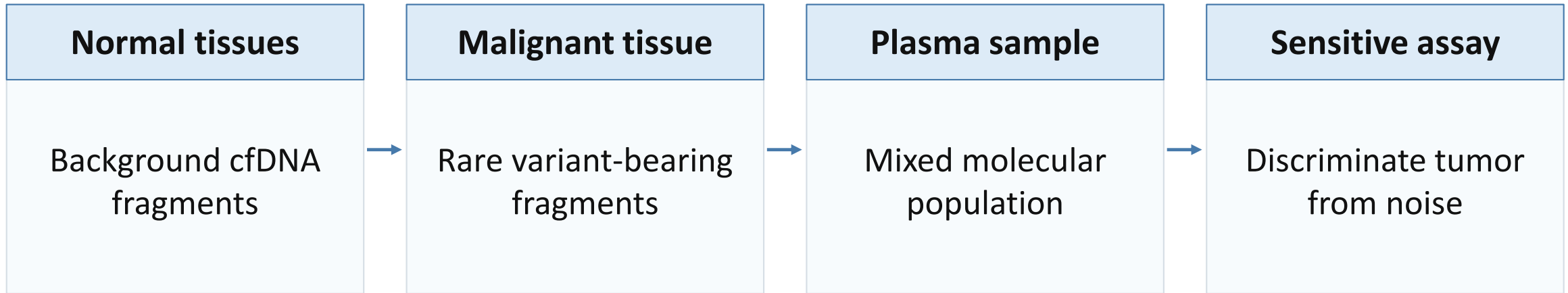
Circulating tumor DNA

- Free tumor-derived nucleic acid fragments.
- Suitable for highly sensitive sequence and methylation analyses.

Biological shedding is heterogeneous

- Tumor presence does not guarantee enough ctDNA in a particular blood sample.
- Burden, anatomical site, biology, and treatment state affect shedding.
- Low tumor fraction produces biologically driven false-negative risk.
- A negative plasma finding is not proof that a lesion is absent.

Why the background matters



- Analytic sensitivity must be considered relative to the fraction of tumor DNA in the input.
- Sample processing can further increase the normal-DNA background.

Spatial tumor heterogeneity

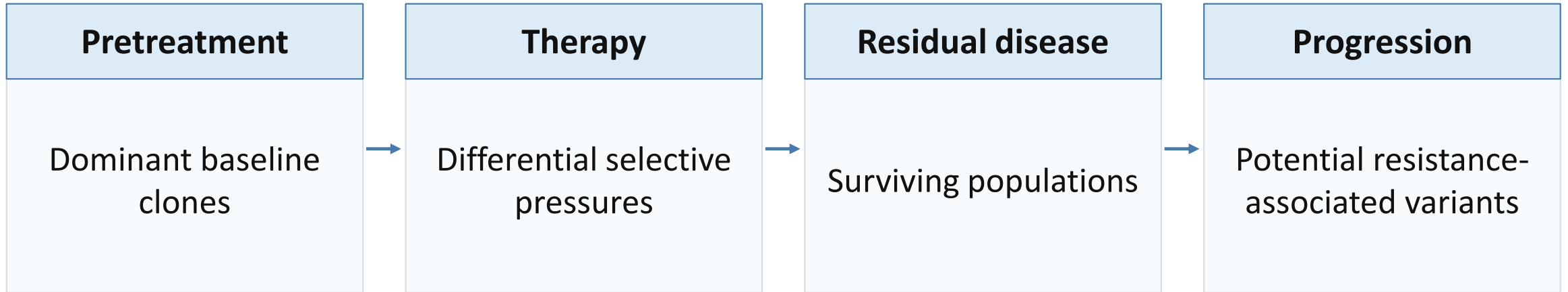
Single tissue biopsy

- Samples one lesion or region.
- May not reflect every metastatic clone.

Plasma sampling

- May contain DNA shed from more than one tumor site.
- Cannot ensure every lesion is represented.

Temporal tumor heterogeneity



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- Serial liquid biopsies can sample molecular change over time.
 - Observed changes require consideration of tumor fraction and assay variability.

Why early-stage detection is difficult

- Small or low-shedding tumors may contribute very little ctDNA.
- Tumor-associated signals can be below the assay's detection capability.
- Population screening amplifies the consequences of false positives.
- Clinical benefit must be demonstrated separately from detection alone.

Genomic signals of tumor origin

Tumor signal	Illustrative meaning	Interpretive caution
Recurrent driver mutation	Known cancer-associated alteration	Not inherently specific to one tumor or tissue
Patient-specific passenger mutations	Individualized tumor molecular signature	Requires convincing tumor linkage
Epigenetic signature	Cancer-associated methylation pattern	Cell-type origin depends on validated classifier

Driver versus passenger variants

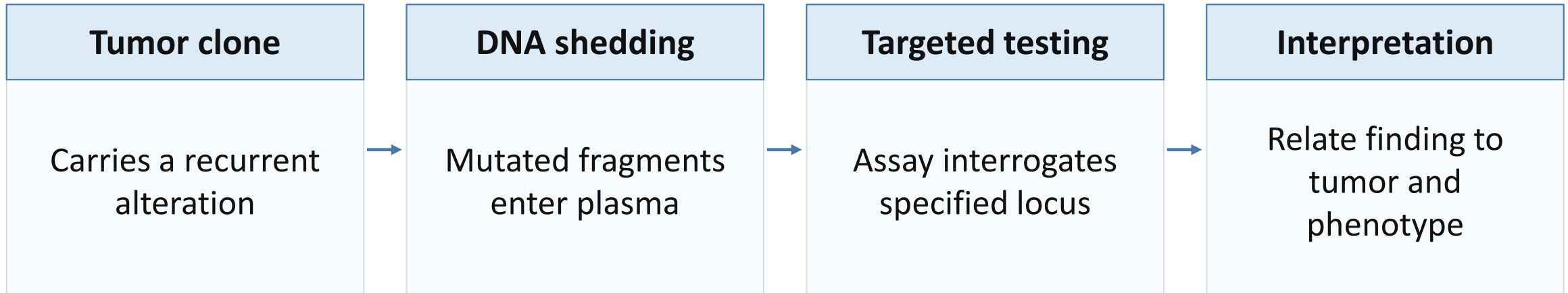
Driver alterations

- Selected because they confer a neoplastic advantage.
- May recur across independent tumors.

Passenger alterations

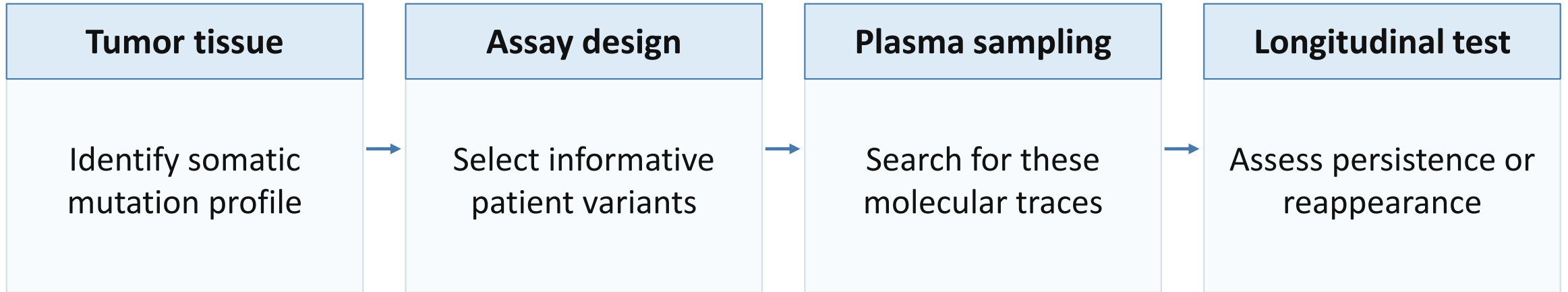
- Acquired during clonal evolution without necessarily driving malignancy.
- Collectively can identify one patient's tumor lineage.

Recurrent point mutations



- A recurrent alteration can be analytically recognizable in a background of wild-type sequences.
- Tumor specificity is not automatically assured by the variant name alone.

Patient-specific mutation signatures

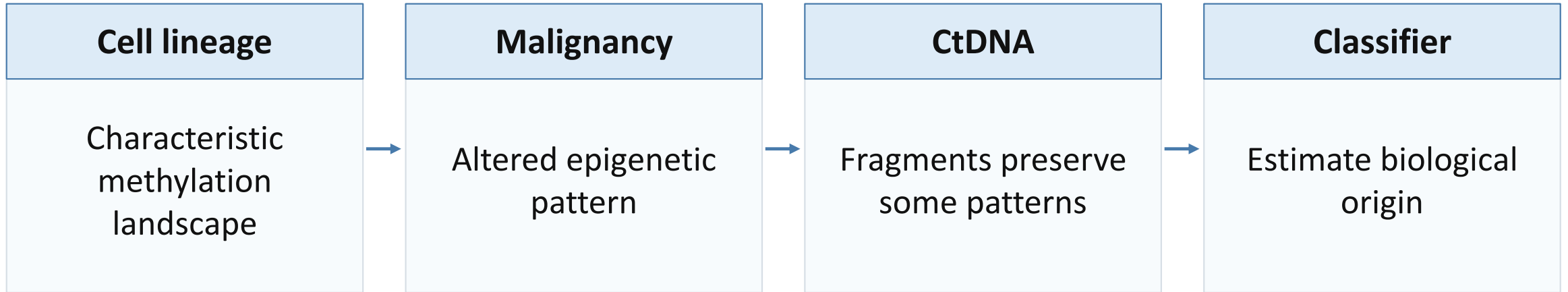


- A bespoke signature can be more specific to the known tumor lineage.
- A tumor-informed assay depends on suitable tissue and reliable baseline profiling.

Epigenetic alterations as biomarkers

- DNA methylation can change without altering nucleotide sequence.
- Cancer-associated methylation patterns may serve as molecular signatures.
- Cell-type dependence creates a possible tissue-of-origin signal.
- A validated classifier is needed before inferring primary tumor site.

Methylation and tissue-of-origin inference



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- This is an inference from molecular patterns, not direct morphologic identification.
 - Sensitivity and specificity depend on assay development and validation.

Complementary molecular analytes

Proteins and vesicles

- Tumor-associated proteins and extracellular vesicles can enter blood.
- May capture biology not represented by DNA alone.

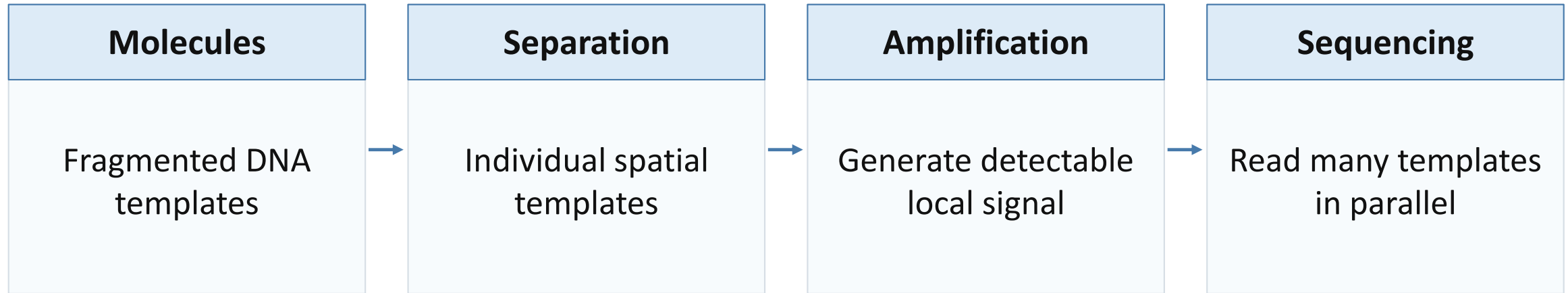
CtDNA strengths

- Directly interrogates mutation or methylation signatures.
- Can support patient-specific molecular tracking.

Why NGS changed the field

- Massively parallel sequencing reads many DNA molecules independently.
- Mixtures of normal and abnormal molecules can be analyzed.
- High depth increases the opportunity to detect rare variant alleles.
- Input and error characteristics still determine real sensitivity.

Core NGS mechanism



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- The output comprises reads that must be assigned to genomic locations.
 - Bioinformatics distinguishes candidate variants from noise.

Targeted panels concentrate sequencing effort

Targeted gene panel

- Enriches selected genomic intervals.
- Provides deep coverage of chosen targets.

Broad genomic approach

- Covers more regions but distributes resources.
- May be less efficient for extremely rare alleles without specialized design.

A sensitive assay begins with a clear question

Clinical question	Appropriate target concept	Key limitation
Identify actionable variant	Relevant mutation panel	Alteration must be covered and detectable
Track a known tumor	Patient-specific variants	Requires prior tumor profile
Infer tumor origin	Epigenetic patterns	Needs validated origin classifier
Explore heterogeneous disease	Broader genomic profiling	Lower shed and complex alterations remain difficult

Variant allele frequency (VAF)

- VAF expresses variant-bearing reads relative to total reads covering a locus.
- Low VAF may reflect a small tumor-derived contribution to cfDNA.
- VAF does not equal the percentage of malignant cells in the patient.
- Copy number, zygosity, clonality, and biology complicate translation.

Depth is not the same as sensitivity

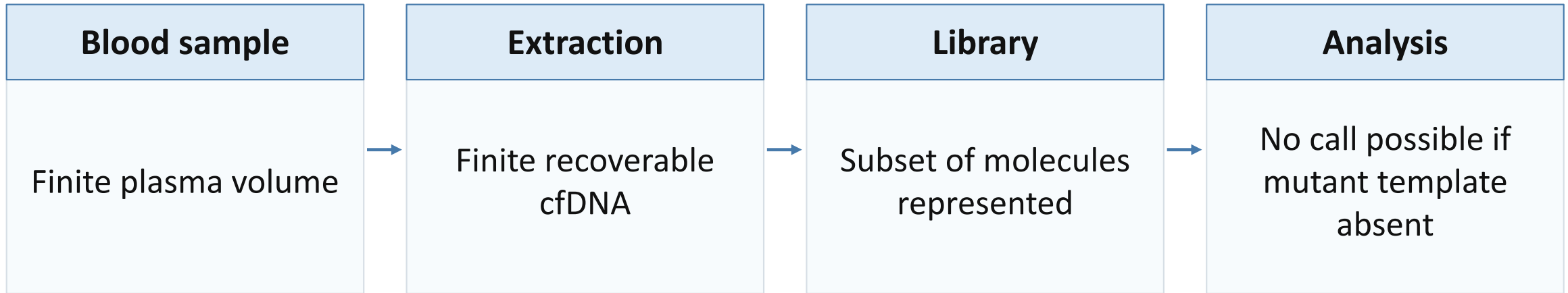
Sequencing depth

- Number of reads covering a locus.
- More reads can improve sampling of rare alleles.

Actual detection capability

- Also depends on molecules, errors, assay design, and background.
- High nominal depth cannot recover absent tumor fragments.

Input molecules create a hard limit

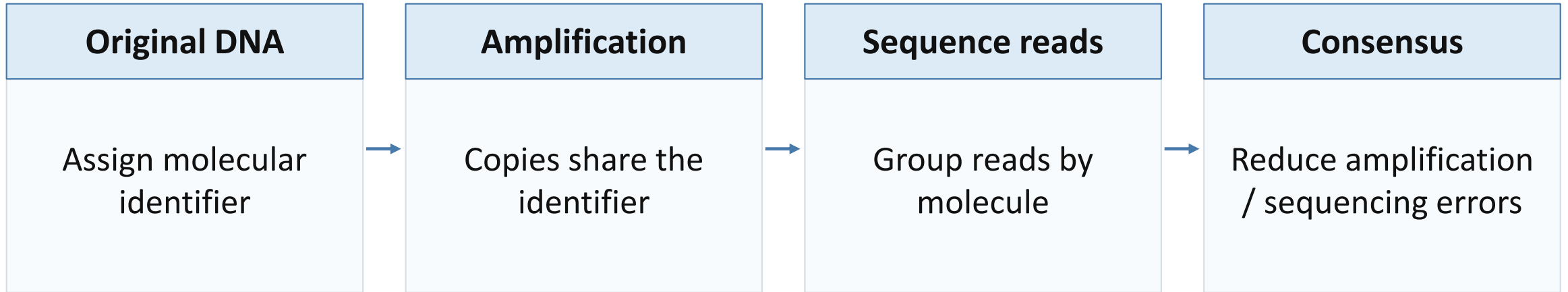


- Extra PCR copies are not new independent biological molecules.
- Sampling variability becomes important near the detection limit.

Sequencing artifacts can mimic rare variants

- Base-calling, DNA damage, and amplification introduce technical noise.
- Rare true events must be separated from systematic artifacts.
- Error suppression is especially important for low-frequency ctDNA.
- Every platform needs a validated detection threshold and quality controls.

Molecular barcodes and error suppression



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- This is a conceptual explanation of a common error-suppression approach.
 - Barcoding does not correct biological confounding such as clonal hematopoiesis.

Digital PCR and NGS answer different questions

Feature	Targeted digital PCR	Targeted NGS
Core strategy	Interrogate defined alterations	Sequence multiple selected regions
Best-fit question	Is this known variant present?	What variants are represented in the panel?
Tradeoff	Limited multiplexed discovery	More complex informatics and error modeling
Interpretation	Known target coverage	Report only within validated assay scope

Tumor-informed versus tumor-naïve designs

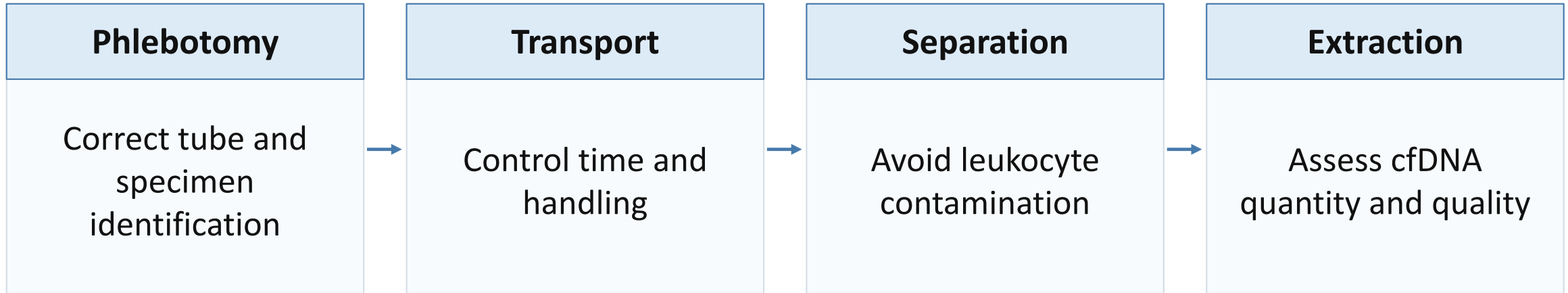
Tumor-informed

- Initial tumor sequencing identifies personalized variants.
- Useful concept for highly specific longitudinal tracking.

Tumor-naïve

- Plasma assay does not require bespoke tumor sequencing.
- May interrogate recurrent variants or epigenetic patterns.

Preanalytical quality starts before sequencing



- Leukocyte lysis adds non-tumor DNA and can dilute the ctDNA fraction.
- Preanalytical parameters must follow a validated laboratory protocol.

Plasma is generally preferred to serum

Plasma

- Obtained from anticoagulated blood.
- Reduces contamination from DNA released during clotting.

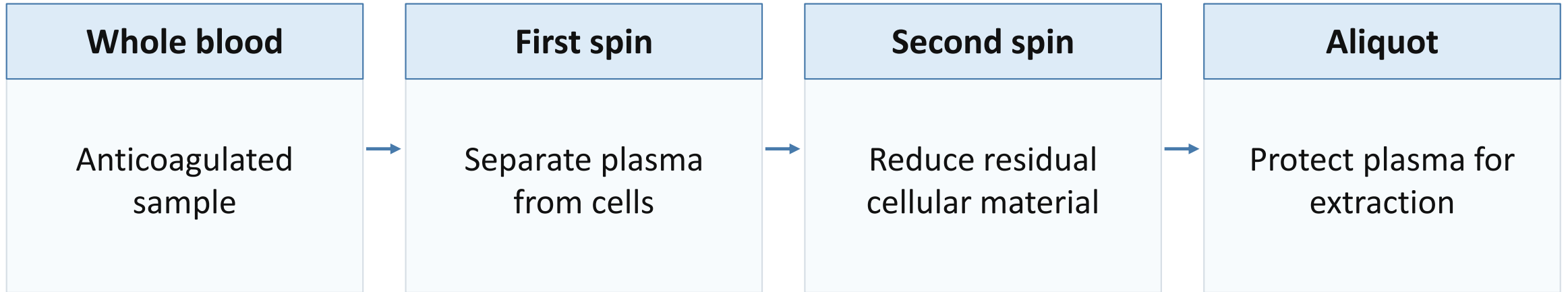
Serum

- Clotting can release leukocyte-derived DNA.
- Additional background reduces relative tumor fraction.

Tube choice and processing delay

- Routine EDTA tubes require prompt plasma separation.
- Stabilizing tubes can support delayed processing within validated conditions.
- Delay and agitation can elevate normal cfDNA through cellular breakdown.
- Do not substitute generic transport assumptions for assay instructions.

Plasma separation is a quality-critical step

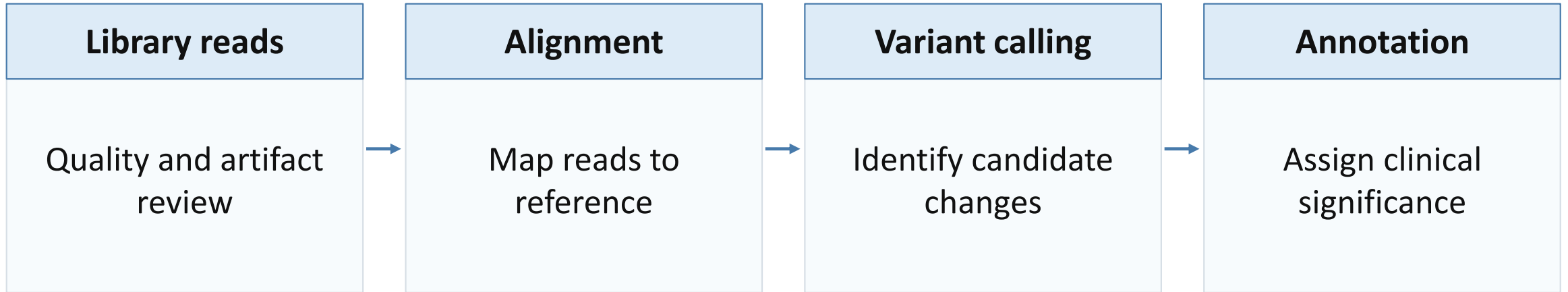


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- Avoid carrying over the buffy coat during pipetting.
 - Specific centrifugal conditions must be laboratory-validated.

Preanalytic failure pathways

Failure	Likely effect	Preventive control
Leukocyte lysis	High normal DNA background	Timely separation / stabilizing tube
Buffy-coat carryover	Cellular DNA contamination	Validated two-step separation
Insufficient plasma	Low unique molecule count	Volume sufficiency checks
Mislabeled time point	Incorrect kinetic interpretation	Accurate collection documentation

Sequence analysis is a pipeline



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- Each step has separate quality thresholds and opportunities for errors.
 - Clinical reporting follows laboratory review of annotated calls.

Alignment establishes genomic context

- Short reads must be mapped to a reference genome.
- Mismapping or repetitive regions can create spurious variant calls.
- Coverage uniformity matters as much as global read count.
- Alignment quality is a prerequisite for reliable interpretation.

Variant calling requires calibrated thresholds

- Candidate alleles are compared with reference sequence.
- Read support, base quality, strand pattern, and artifact profile matter.
- Low-frequency calls must satisfy assay-specific validation rules.
- A reportable variant is not automatically a clinically actionable variant.

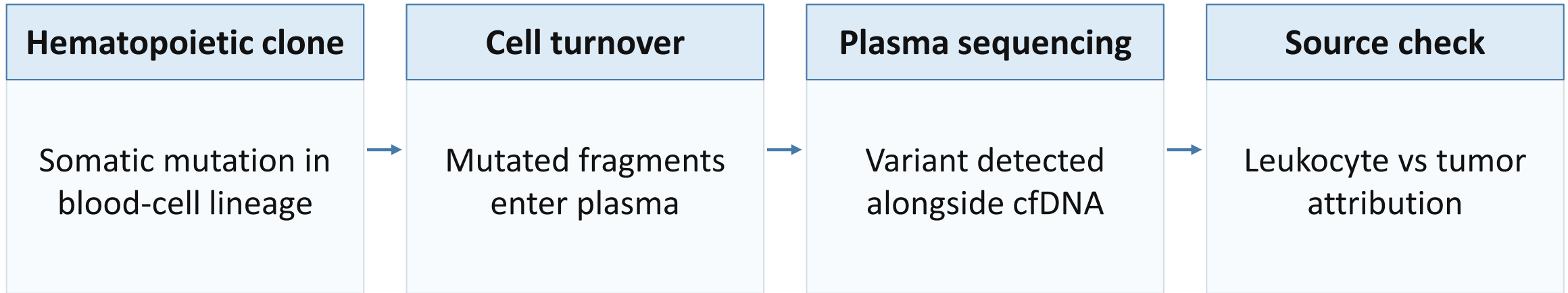
Variant annotation has multiple layers

Layer	Question answered	Example of caution
Identity	What change and gene?	Correct transcript and variant nomenclature
Pathobiology	Tumor driver or incidental finding?	Tumor source not proven by plasma alone
Clinical evidence	Diagnostic / prognostic / predictive?	Cancer type and therapy context differ
Actionability	Does the finding alter management?	Requires current indication / evidence review

Clonal hematopoiesis is a major confounder

- Nonmalignant hematopoietic clones can carry somatic mutations.
- DNA shed from these clones contributes to plasma cfDNA.
- A biologically real plasma mutation may be unrelated to the solid tumor.
- Matched leukocyte testing can distinguish blood-cell and tumor origin.

Why clonal hematopoiesis mimics cancer



- Analytic detection can be correct while tumor attribution is wrong.
- This is a clinical false positive for tumor genotyping, not necessarily a sequencing error.

Clonal hematopoiesis versus true ctDNA

Characteristic	Clonal hematopoiesis	Tumor-derived ctDNA
Source	Nonmalignant hematopoietic clone	Malignant neoplastic clone
Plasma variant	Often technically real	Technically real when detectable
Matched leukocyte DNA	Can show concordant variant	Not expected for a strictly tumor-specific somatic variant
Clinical use	Do not misattribute to solid tumor	Interpret against tumor type and context

Germline variants are a separate issue

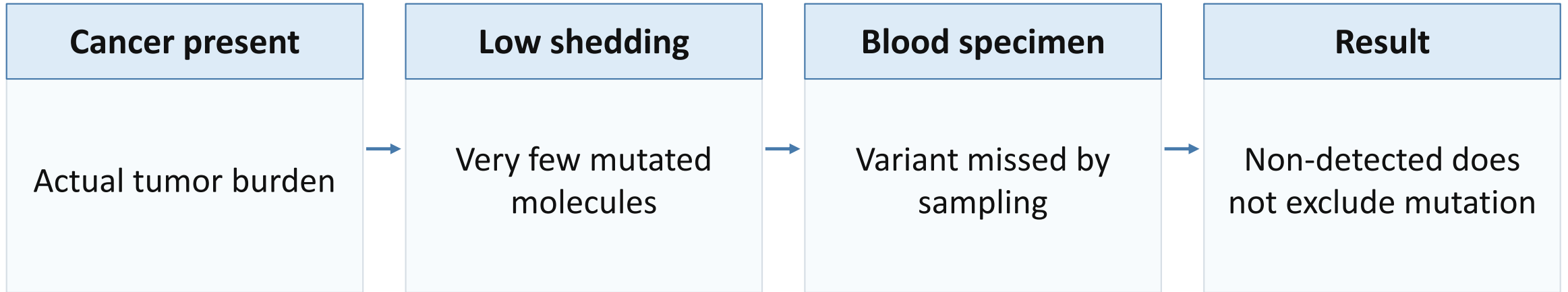
Somatic tumor alteration

- Acquired by malignant clone.
- May be useful for tumor genotyping or monitoring.

Inherited germline variation

- Constitutional variant across normal tissues.
- Requires appropriate confirmatory germline testing when suspected.

Why a negative plasma test can mislead



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- The relevant alteration may be present in tissue despite a negative plasma result.
 - Consider reflex tissue testing when clinically indicated.

Copy-number and fusion detection is harder

- Performance varies across molecular alteration classes.
- Plasma assays may be less sensitive for fusions and copy-number changes.
- Tissue-based testing may still be preferred for many cancer settings.
- Interpret “no alteration detected” only within the assay’s technical scope.

Sensitivity, specificity, and clinical value

Term	Central question	Why it matters
Analytical validity	Does assay detect what it claims?	LOD, accuracy, precision, interference
Clinical validity	Is the result associated with disease or outcome?	Prognostic or diagnostic relationship
Clinical utility	Does result-guided care improve decisions/outcomes?	Evidence beyond association

A result requires a two-axis interpretation

Tumor biology axis

- Expected shedding / stage / treatment.
- Chance that a real tumor alteration reached plasma.

Assay axis

- Coverage / detection limit / quality.
- Chance that a present tumor fragment would be called.

Interpreting plasma-positive findings

- Confirm the variant is within validated assay coverage and QC.
- Distinguish tumor-derived from hematopoietic or germline origins.
- Evaluate alteration-specific clinical evidence for this cancer.
- Document whether the finding is diagnostic, prognostic, or predictive.

Interpreting plasma-negative findings

- Do not equate non-detection with absence of malignancy.
- Review tumor type, disease burden, collection timing, and sample adequacy.
- Determine whether the target and variant class are reliably assessed.
- Pursue tissue testing when a negative plasma result is not informative.

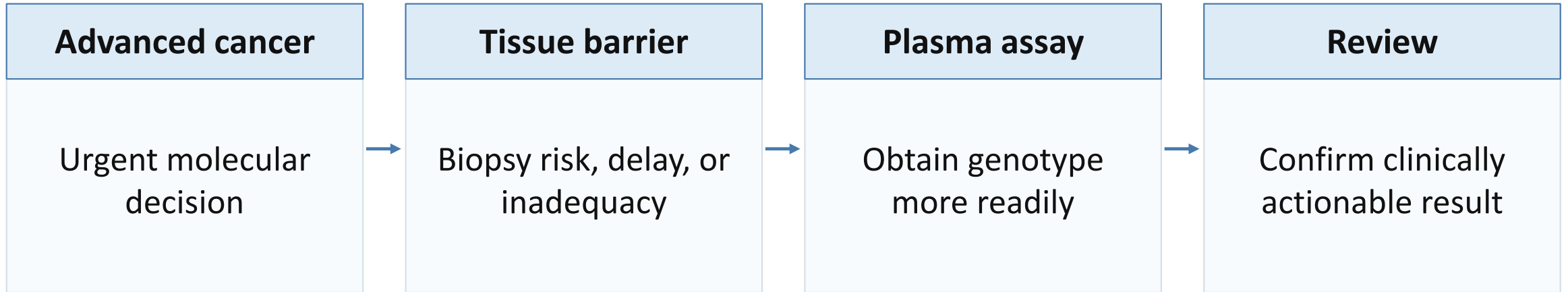
Plasma and tissue are complementary

Dimension	Tissue analysis	Plasma ctDNA analysis
Architecture	Histology, grade, invasion	No tissue architecture
Sampling	Local tumor region	Circulating, variable lesion representation
Repeat testing	May require invasive procedure	Relatively noninvasive serial sampling
Key failure	Limited tissue or spatial sampling	Low ctDNA or nontumor DNA confounding

Advanced cancer genotyping: Supported role

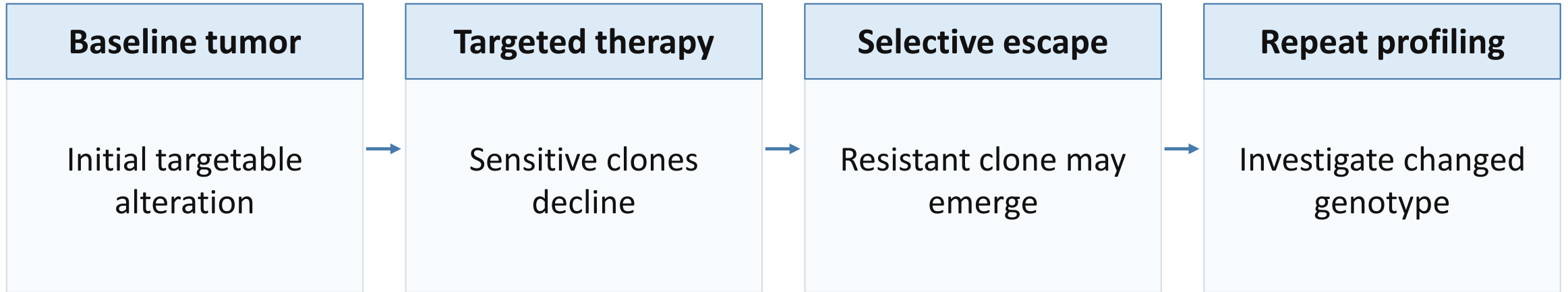
- Validated, sensitive plasma assays can identify actionable variants.
- May be especially useful when tissue is inaccessible or speed matters.
- Clinical interpretation must account for assay coverage and sensitivity.
- ESMO 2022: Tissue testing may be needed after a noninformative plasma test.

Why speed and accessibility can matter



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- This workflow is scenario dependent, not universal “liquid-first” policy.
 - Histologic classification and other essential pathology may still require tissue.

Molecular resistance at progression



- A newly detected variant may reflect resistance or subclonal selection.
- Clinical action requires validated variant–drug evidence.

Monitoring therapeutic response

Potential benefit

- Serial ctDNA changes may reflect tumor molecular burden.
- Can sometimes precede conventional detection of progression.

Why caution persists

- Levels fluctuate with shedding, timing, and assay conditions.
- Early ctDNA-guided regimen changes require clinical utility evidence.

Molecular residual disease (MRD)

- MRD refers to residual malignant material after curative-intent therapy.
- CtDNA detection may indicate persistent occult disease.
- A positive association with future relapse is clinical validity.
- Changing therapy on the result needs separate prospective utility evidence.

MRD and radiologic assessment differ

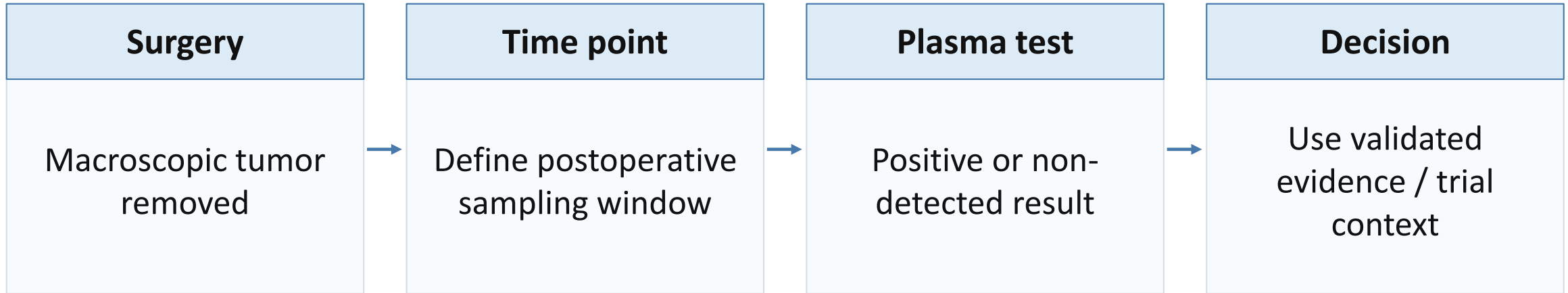
Radiologic assessment

- Identifies disease above imaging detectability.
- Cannot reliably exclude microscopic persistence.

Molecular assessment

- Searches for tumor-specific material in plasma.
- Cannot ensure a negative sample is biologically disease-free.

A postoperative ctDNA result is contextual



- A result must be tied to a defined collection time and intended use.
- FDA 2024 addresses ctDNA biomarkers in early-stage trials; It does not establish universal care algorithms.

Early detection and population screening

- Screening requires careful assessment of low-prevalence populations.
- False-positive workups and missed early disease can cause harm.
- Tissue-of-origin classifiers need independent clinical validation.
- ESMO 2022 regarded asymptomatic screening as investigational.

Current evidence must be use-case specific

Use case	Evidence interpretation	Important caveat
Advanced cancer genotyping	Established role for validated assays	Noninformative result may require tissue
MRD / molecular relapse	Strong prognostic associations in many settings	Treatment benefit is indication-specific, not automatic
Response-adaptive therapy	Promising molecular dynamics	Prospective clinical utility needed
Asymptomatic screening	Investigational in ESMO 2022 framework	False-positive and false-negative consequences

What an adequate report should communicate

- Specimen, sampling time, platform, and genes or targets assayed.
- Reported variants with nomenclature and interpretive evidence.
- Limits of detection, relevant quality constraints, and non-detection caveats.
- Whether results are suitable for specific clinical decisions.

Case: Inadequate tissue in advanced cancer

CLINICAL VIGNETTE

A patient with advanced carcinoma needs molecular profiling, but the available tissue is exhausted.

What can a plasma assay contribute, and what if it is negative?

- A validated ctDNA assay may identify actionable tumor-associated alterations.
- A noninformative plasma result should not terminate tissue-based testing when feasible.
- Tissue remains necessary when morphology or molecular coverage is unresolved.

Case: Negative plasma in early malignancy

CLINICAL VIGNETTE

A localized tumor is confirmed histologically. The liquid biopsy reports no detectable tumor variant.

Which interpretation is scientifically defensible?

- Low tumor DNA shedding or finite sampling may explain non-detection.
- A negative plasma result does not overrule tissue-based diagnosis.
- Consider the analytical targets, assay limit, and collection context.

Case: A plasma mutation from blood cells

CLINICAL VIGNETTE

A plasma assay detects an oncogenic variant; The same variant is found in matched peripheral blood leukocytes.

What source should enter the differential?

- Clonal hematopoiesis may explain the plasma finding.
- A confirmed sequence change need not be derived from the solid tumor.
- Avoid assigning tumor therapy based only on an unverified plasma source.

Case: Detectable ctDNA after surgery

CLINICAL VIGNETTE

After curative-intent resection, a tumor-informed plasma assay detects a patient-specific mutation signature.

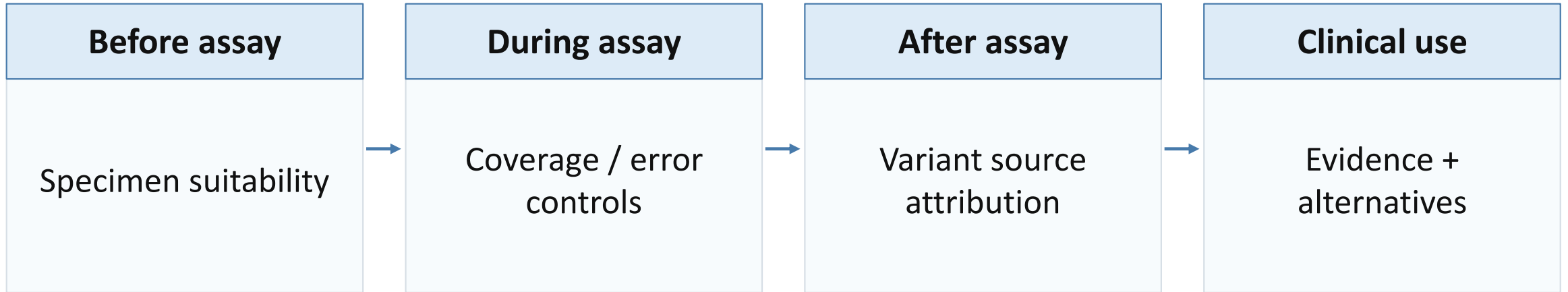
What can and cannot be concluded?

- This can be molecular evidence of residual tumor-derived material.
- Risk association is not identical to proof that changing treatment improves outcomes.
- Interpret using disease-specific trials, timing, and current clinical protocols.

Interpretation exercise: Four statements

Statement	Assessment	Reason
“ctDNA = all plasma cfDNA”	Incorrect	cfDNA includes substantial nontumor material
“Plasma-negative excludes mutation”	Incorrect	Low shedding and finite sampling
“CH can mimic tumor variants”	Correct	Hematopoietic clones shed mutant DNA
“MRD positivity proves benefit of adjuvant change”	Incorrect	Utility must be demonstrated

Analytic safeguards across the test pathway



- No single quality-control step solves all sources of uncertainty.
- Every molecular conclusion depends on a linked preanalytic-to-clinical chain.

Integrated take-home principles

- CtDNA is a tumor-derived subset of circulating cell-free DNA.
- Point mutations, patient-specific signatures, and methylation are complementary readouts.
- NGS allows rare-allele detection, but biology and assay limits remain decisive.
- A positive or negative result must be interpreted with tissue and clinical context.

Clinical interpretation: Final synthesis

- Separate analytically correct findings from true tumor attribution.
- Distinguish predictive targets, prognostic MRD associations, and screening hypotheses.
- Do not overrule tissue pathology with an uninformative plasma result.
- Demand evidence of patient benefit before changing therapy from ctDNA alone.

Primary and supporting references

Primary textbook

Robbins, Cotran & Kumar. Pathologic Basis of Disease. 11th ed. 2026; Ch. 5, pp. 164–166.

Clinical consensus

Pascual J, et al. Ann Oncol. 2022;33:750–768. Doi:10.1016/j.annonc.2022.05.520

Preanalytical guidance

Clinical Practice Guideline for Blood-based ctDNA Assays. 2024. PMCID: PMC10813828.

Additional literature and regulatory context

Biological confounding

Razavi P, et al. Nat Med. 2019;25:1928–1937. doi:10.1038/s41591-019-0652-7

Regulatory methodology

FDA. Use of ctDNA for Early-Stage Solid Tumor Drug Development. Final Guidance, Nov 2024.

Implementation update

ESMO commentary on ctDNA standardization and implementation, 19 Jun 2026.