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Systematic Review

Forensic Epigenetics for Casework: A Systematic Scoping Review of DNA Methylation-Centred Applications and Validation Evidence

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Abstract

Background/Objectives: Forensic epigenetics complements DNA profiling by informing donor age and trace origin, but technical promise does not establish casework validity. We mapped applications and validation evidence. **Methods:** Scopus, PubMed/MEDLINE and Web of Science were searched through 15 June 2026. Empirical reports were assigned a dominant application and an author-defined C1–C5 evidence category (not a readiness score). **Results:** Of 596 assessed full texts, 338 entered the evidence map (322 empirical studies and 16 contextual records). Among studies, 312/322 (96.9%) fell within C1–C3, 10 were C4 and none met the C5 definition of documented routine implementation in the indexed and retrievable corpus. C4 records advanced evaluation designs, which may reveal unsuccessful transfer or unresolved bias rather than acceptable casework performance. Age estimation (177 studies) and body-fluid/tissue identification (92) accounted for all C4 evidence. Of 272 identifiable methylation workflows, chemical conversion predominated; 23 studies were non-methylation, 26 reused methylation data without a new assay and one workflow remained unresolved. **Conclusions:** Published evidence is substantial but heterogeneous; internal performance cannot substitute for independent validation, inter-laboratory reproducibility, calibrated uncertainty, laboratory-specific procedures, controlled reporting and proportionate governance. Study-level counts do not quantify the proportion of distinct validated methods or establish absence of unindexed operational use.

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1. Introduction

Short tandem repeat (STR) profiling supports the comparison of DNA profiles and the evaluation of propositions concerning the contributor or contributors to a biological trace [1,2]. It does not, by itself, determine the cellular origin of the DNA, the donor's age, the biological history of the sample, or the activity through which the trace was deposited. These questions become relevant when contributor-focused profiling alone does not resolve what biological material is present or what additional biological information can legitimately be inferred. Forensic epigenetics therefore operates alongside, rather than in place of, conventional STR profiling by supplying complementary information for distinct investigative or evidential questions.

Epigenetics refers to molecular modifications that influence genome function without changing the underlying DNA sequence. DNA methylation has received the greatest practical attention in forensic research because it is measurable in DNA-based workflows and can be associated with tissue identity, age, environmental exposure, and other biological states [3–5]. Unlike inherited STR alleles, however, methylation patterns may vary across tissues, with age, and in relation to environment, disease, exposure, and sample history. This same biological variability is the source of forensic information and a major validation burden.

The literature now covers several distinct applications. DNA methylation-based age estimation has progressed from CpG discovery to targeted prediction models, external validation, and inter-laboratory exercises [6–10]. Body-fluid and tissue identification has developed around tissue-specific differentially methylated regions and targeted CpG assays [11,12]. Other areas include monozygotic-twin differentiation and exposure/lifestyle inference [13–16]. These applications do not have the same forensic role. Age estimation and most donor-characteristic inferences are predominantly investigative; body-fluid or tissue identification may have more direct evidential relevance because it concerns the nature of the biological material. Even then, identifying a body fluid does not establish how, when, or through which activity the material was deposited. Epigenetic assays likewise complement rather than displace conventional forensic biology. Visual or alternate-light examination can help locate or visualise suspected biological material before sampling; microscopy can provide morphological information about cells or other structures; and presumptive or confirmatory tests provide different levels of support for the presence or identity of particular biological materials. Epigenetic assays address a different layer by interrogating molecular marks in extracted nucleic acid to support tissue-of-origin, age, or other donor-characteristic inference. These functions are complementary, and conventional examination or testing may remain necessary even when an epigenetic assay is used. Integrated molecular studies have also combined methylation information with microbial DNA, nearby genetic variants, or multi-marker sequencing [17–19]. Such combinations were treated in this review as supporting workflows or cross-cutting features rather than evidence that the combined workflow is routinely implemented.

STR and epigenetic methods nevertheless share core validation requirements. Both require defined intended use, analytical accuracy and precision, repeatability and reproducibility, sensitivity studies, evaluation of low-template and degraded material, mixture studies, contamination control, thresholds, laboratory-specific validation, inter-laboratory

or proficiency-testing evidence, quality assurance, and controlled reporting [20–23]. These requirements are not unique to forensic science in principle; what is distinctive is the intended forensic use. Methods may be applied to limited, degraded, mixed, or otherwise compromised traces, and their results may inform investigative or judicial decisions, so validated scope, uncertainty, interpretation rules, and reporting limits must be explicit. Published developmental or collaborative evidence can support a laboratory's decision to undertake internal validation, but it cannot replace that laboratory-specific process. Practical transfer also depends on laboratory context: a specialised epigenetic research workflow may perform strongly under defined conditions, whereas a forensic laboratory considering routine use must demonstrate performance with its own personnel, instrumentation, pre-analytical procedures, thresholds, quality system, and reporting rules [20–23]. Table 1 summarises these validation parallels as a conceptual comparator only; it does not treat STR profiling or serology as a parallel classified corpus.

Table 1. Comparison between validation requirements for short tandem repeat (STR) profiling and forensic epigenetic applications.

Validation Domain	STR Profiling	Forensic Epigenetics	Implication for Forensic Use
Marker characteristics	The STR genotype is normally consistent across nucleated cells of the same individual, apart from rare biological or technical exceptions.	Methylation states may vary with tissue, age, environment, disease, exposure, and time.	Biological variability is both the source of information and a potential confounder.
Analytical validation	Accuracy, precision, sensitivity, specificity, reproducibility, contamination control, thresholds, and instrument/software performance are established.	The same core characteristics apply, with additional attention to method-specific conversion, digestion, or detection efficiency; methylation bias; CpG coverage; batch effects; and platform-specific calibration.	Strong analytical performance is necessary for both, but assay-specific artefacts differ.
Sensitivity and low-template testing	Low-template effects, allele dropout, stochastic imbalance, and analytical thresholds are evaluated.	Low DNA quantity may be compounded by pre-analytical and assay-related losses during conversion or restriction digestion, as well as stochastic methylation measurements.	Minimum input and reportable-result criteria must be established for each assay.
Degraded samples	Fragment size, locus dropout, inhibition, and partial profiles are evaluated.	Degradation may affect DNA recovery, chemical or enzymatic conversion, restriction digestion, target amplification, and tissue-associated methylation signals.	Short targets and representative aged or environmentally exposed samples are required.
Mixtures	Validation designs and interpretation protocols address multiple contributors and contributor ratios.	Mixtures may contain multiple donors, multiple tissue types, or both, altering measured methylation and classification.	Donor and tissue mixture behaviour must be tested explicitly.
Interpretation	Results generally support comparison and evaluation concerning the contributor or contributors to DNA findings.	Outputs commonly estimate age or classify tissue, fluid, exposure, or another biological characteristic.	The intended forensic question and permitted inference must be defined before validation.
Statistical reporting	Validated statistical approaches express the support for competing propositions.	Reporting may require calibrated probabilities, prediction intervals, error distributions, sensitivity/specificity, and false-classification rates.	A point estimate or strong classification metric alone is insufficient for forensic reporting.

Table 1. *Cont.*

Validation Domain	STR Profiling	Forensic Epigenetics	Implication for Forensic Use
Laboratory-specific validation	Laboratories establish procedures, thresholds, interpretation rules, and limitations before implementation.	The same requirement applies, with additional local assessment of tissue, population, platform, model, and pre-analytical workflow.	Published validation cannot replace internal laboratory validation.
Inter-laboratory and proficiency-testing evidence	Collaborative exercises, quality systems, and proficiency testing are established.	Such evidence remains limited and application-specific.	Between-laboratory reproducibility remains a major implementation gap.
Evidential role	Primarily addresses the contributor or contributors to DNA results; further propositions require separate evaluation.	Age and many donor-characteristic inferences are mainly investigative; body-fluid/tissue findings may inform propositions about the biological nature of the trace and, together with other case information, contribute to subsequent activity-level evaluation.	Body-fluid identification does not by itself establish how or when deposition occurred.
Ethical and governance issues	Concerns include privacy, database use, kinship, and interpretation.	Some applications may disclose age, health, behaviour, exposure, or life-history information.	Sensitive inferences require necessity, proportionality, data protection, and reporting limits.

Note: This conceptual comparison is based on established forensic DNA, serology validation, and reporting guidance [20–25]. It was used to frame interpretation and was not derived from the qualifying empirical corpus. STR profiling was not reviewed as a parallel corpus and was not classified using Categories 1–5.

Epigenetic applications also require evidence directed at the validity of the inference itself. Depending on the endpoint, this includes tissue specificity, population transferability, model calibration, prediction intervals or classification uncertainty, false-positive and false-negative behaviour, biological and environmental confounding, and explicit limits on reporting. The distinction between investigative use, analytical measurement, and evaluative interpretation is therefore important, particularly when probabilistic biological results may be used in legal proceedings [24,26–28].

In this review, "casework-relevant" denotes research using forensic sample types, conditions, workflows, or interpretative questions intended to inform casework; it does not indicate established use in routine forensic practice. Routine forensic implementation was treated as the highest evidential endpoint and distinguished from commercial availability, developmental validation, collaborative exercises, isolated case applications, isolated laboratory use, or informal knowledge of use [20–25]. The full Category 5 criteria are defined in Methods and Table 2.

Although the forensic epigenetics literature has expanded rapidly, biomarker discovery, internal model performance, external validation, testing of mixtures or degraded samples, inter-laboratory reproducibility, and documented forensic implementation represent different forms of evidence [9,10,29,30]. A high coefficient of determination, low mean absolute error, or high classification accuracy may demonstrate technical promise without establishing that the result can be interpreted and reported reliably for a defined forensic use.

Earlier syntheses described the principal applications, analytical approaches, and broader forensic-genetics or epigenomic context [3,30]. The additional synthesis in this review is not simply a larger catalogue of publications: it links each qualifying empirical report to a defined forensic endpoint and molecular stratum, maps the documented validation evidence at study/report level, separates platform capability from endpoint-specific forensic validity, and identifies where transferability, uncertainty, inter-laboratory reproducibility, and implementation documentation remain missing.

Table 2. Author-defined validation evidence categories.

Category	Working Definition	Evidence Supporting Assignment	What the Category Does Not Establish
1	Exploratory discovery	Marker discovery, association, descriptive evidence, or initial proof of concept with limited validation testing.	A validated assay, model, or forensic interpretation.
2	Initial technical evidence	Assay or model development with internal performance, training/test splitting, cross-validation, or limited technical testing, but little independent or representative forensic validation.	External validation, transferability beyond the development setting, or support for routine forensic implementation.
3	Pre-implementation validation	Independent or external validation, broader technical testing, or evaluation with relevant forensic samples, while important gaps remain in transferability, mixtures, uncertainty, reproducibility, or reporting.	Readiness for routine casework without further laboratory-specific validation and resolution of the remaining gaps.
4	Advanced validation evidence under defined conditions (evaluation, not proof of success)	For a selected assay or model, stronger external, collaborative, inter-laboratory, representative-sample, interpretative, or implementation-oriented evidence with defined limitations.	Validation of the application area as a whole, completed laboratory-specific validation, transferability beyond the tested conditions, or routine use.
5	Documented routine forensic implementation	Published, indexed, and retrievable evidence of routine forensic casework implementation following laboratory-specific validation, standard operating procedures, quality assurance, and defined interpretation/reporting practices.	Accreditation, legal admissibility, universal transferability, or routine use beyond the documented setting(s).

Note: The categories summarise the type of published evidence found in this review at the level of the individual empirical study/report. "Evidence supporting assignment" describes the evidence pattern documented in a report; it is not a checklist in which every element must be present. The breadth of an advanced evaluation does not establish a satisfactory transfer or interpretation outcome. The categories do not estimate the proportion of distinct assays, models, or method families that are validated; they do not certify legal admissibility, accreditation, proficiency-testing status, laboratory implementation, or routine use outside the indexed and retrievable corpus; and they do not replace laboratory-specific validation. Operational decision rules and boundary examples are provided in Supplementary Table S9.

Accordingly, this systematic scoping review had four linked objectives: (1) to map the principal forensic epigenetic applications and molecular strata represented in the retrieved literature; (2) to classify, at the level of the qualifying empirical study/report, the reported validation evidence using the author-defined Categories 1–5 in Table 2; (3) to characterise analytical principles and downstream platforms separately from forensic endpoints; and (4) to identify the evidential gaps separating discovery, pre-implementation validation, advanced validation under defined conditions, and documented routine forensic implementation. An additional interpretative aim was to distinguish analytical performance from the validity, transferability, uncertainty, and reportability of the forensic inference. The review was not designed as a bibliometric analysis of citation impact, publication productivity, or research networks. The categories were not designed as a numerical score, a universal quality scale, an accreditation standard, or a legal admissibility test.

2. Results

2.1. Study Selection and Composition of the Evidence Base

Within the evidence map, an empirical primary study was defined as an original investigation that directly evaluated a defined forensic endpoint or a method or model intended to support such an endpoint under the review eligibility criteria; contextual records were original relevant sources that did not meet that endpoint-focused empirical definition. The database searches identified 2212 records. After removal of 1557 duplicate

or overlapping records, 655 records remained; 44 were excluded at title/abstract screening, 611 reports were sought, 15 were not retrieved, and 596 were assessed in full. Of these, 338 records were included in the evidence map: 322 empirical primary studies and 16 contextual records. A further 143 full-text reports were retained outside the evidence map for background, comparator, or citation-tracking purposes, and 115 were excluded or non-qualifying (Figure 1). Of the 143 reports retained outside the evidence map, 81 are represented in Table S2 as 69 background/secondary records and 12 comparators. The remaining 62 are individually identified in Supplementary Table S8: 43 reviews retained for citation tracking and 19 background/context reports. Table S2 is a study-level register rather than a separate PRISMA denominator and also retains 10 records (seven excluded and three non-human) that are listed in Table S3 to document their final roles; these overlapping table entries do not represent additional PRISMA reports. None of the 143 reports retained outside the evidence map contributed to empirical application totals, Categories 1–5, or validation claims.

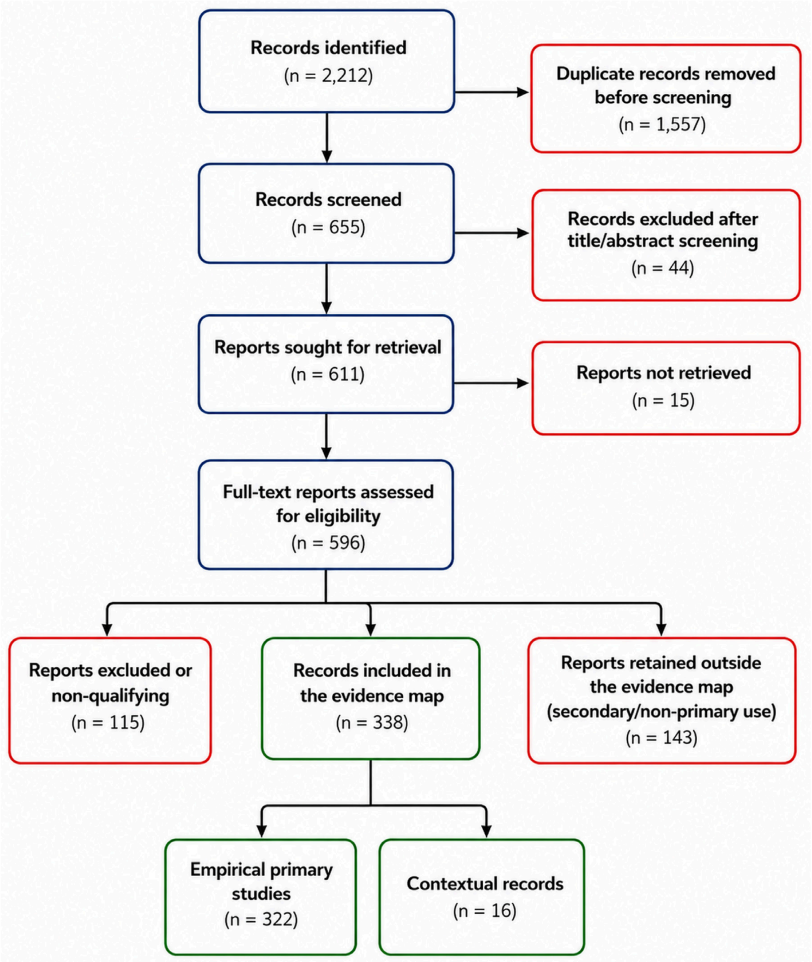


Figure 1. *Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) flow diagram [31]. Of 596 full-text reports assessed, 338 records were included in the evidence map (322 empirical primary studies and 16 contextual records), 143 were retained outside the evidence map for background, comparator, or citation-tracking purposes, and 115 were excluded or non-qualifying. Individual full-text exclusion or non-qualifying reasons are reported in Supplementary Table S3. Of the 143 retained outside the map, 81 are documented in Table S2 (69 background/secondary and 12 comparator reports), and 62 are documented separately in Table S8. Ten excluded/non-human entries are intentionally listed in both Tables S2 and S3 for traceability, not counted twice. The 15 reports not retrieved were not among the 596 assessed full texts.*

The 322 empirical primary studies were assigned to five mutually exclusive primary application areas. Age estimation accounted for 177 studies (55.0%), body-fluid/tissue identification for 92 (28.6%), monozygotic-twin differentiation for 21 (6.5%), sequencing/platform/method development for 20 (6.2%), and other donor-characteristic inference for 12 (3.7%) (Figure 2). Primary application reflected the dominant endpoint of each study; secondary application and cross-cutting tags were non-additive and did not alter these totals. These percentages describe the retrieved, methylation-centred corpus, not the relative volume of research across the whole field; search coverage differed by application and molecular stratum (Methods Section 4.2; Supplementary Table S5).

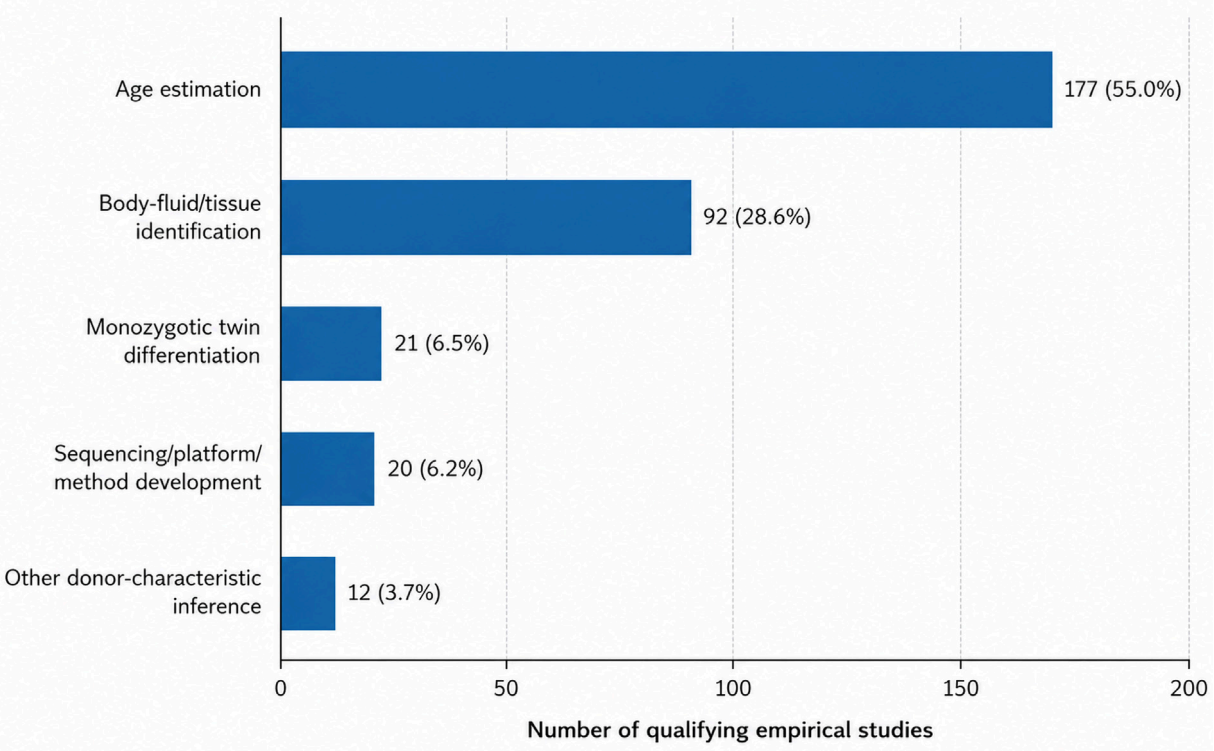


Figure 2. *Primary forensic application areas among the 322 qualifying empirical studies. The five application areas were mutually exclusive and assigned according to the dominant application of each study; application and cross-cutting tags were non-additive.*

DNA methylation remained the dominant molecular stratum: 288 of 322 empirical studies (89.4%) were DNA-methylation studies, 12 (3.7%) were mixed-molecular studies, and 22 (6.8%) were transcriptomic/non-coding RNA studies. The latter were retained as a distinct molecular stratum and were not described as DNA-methylation evidence; they were captured without a dedicated non-coding RNA search and are not an exhaustive RNA literature sample. Application-by-molecular-stratum counts are provided in Supplementary Table S5.

2.2. Distribution of Validation Evidence

Using the final operational rules, each of the 322 empirical study reports was assigned to the highest category supported by its documented evidence (Methods Section 4.4; Table 2; Supplementary Table S9). The final distribution was Category 1 = 19 (5.9%), Category 2 = 139 (43.2%), Category 3 = 154 (47.8%), Category 4 = 10 (3.1%), and Category 5 = 0. Accordingly, 312/322 studies (96.9%) remained exploratory, provided initial technical evidence, or were at a pre-implementation validation stage. Because classification was performed at the study-report level, this percentage describes the distribution of published study reports and should not be interpreted as the proportion of distinct assays, models, or method families that are

insufficiently validated. Within the principal DNA-methylation stratum, the corresponding distribution was Category 1 = 16, Category 2 = 124, Category 3 = 138, Category 4 = 10, and Category 5 = 0; thus, 278/288 DNA-methylation studies (96.5%) remained within Categories 1–3. The 10 Category 4 studies concerned selected age-estimation models and body-fluid/tissue assays subjected to advanced evaluation under defined conditions; Category 4 records the evaluation design and documentary breadth, including unsuccessful transfer or residual systematic bias, rather than universally satisfactory validation performance. No empirical study met the Category 5 definition of documented routine forensic implementation within the indexed and retrievable corpus.

The category distribution differed among applications (Figure 3). Age estimation contained seven Category 4 studies and body-fluid/tissue identification contained three; all other primary application groups remained within Categories 1–3. Contextual records were reported separately and were not assigned a Category 1–5 classification. Full study-level classifications and rationales are provided in Supplementary Tables S2 and S4, with application-level distributions summarised in Supplementary Table S5.

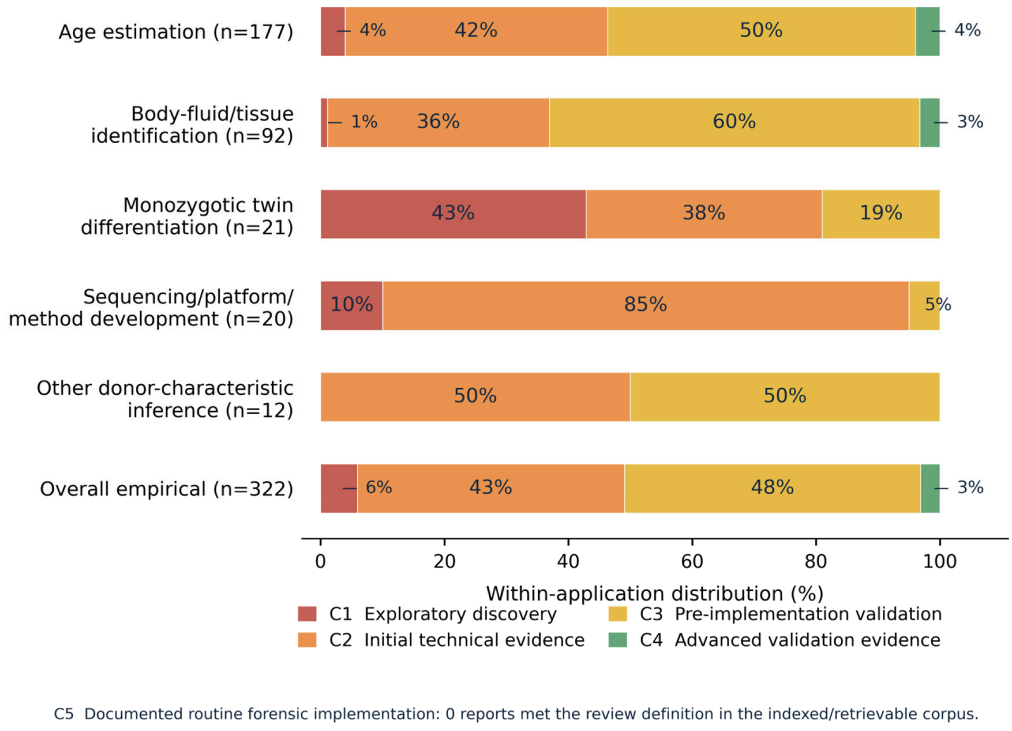


Figure 3. *Distribution of validation evidence categories within the five mutually exclusive empirical primary application areas and in the overall empirical corpus. Percentages were calculated within each row; rounded values may not sum to 100%. The legend provides abbreviated category descriptions. Category 4 ("advanced validation evidence") denotes an advanced evaluation design, not necessarily a successful transfer or validation outcome. Category 5 (documented routine forensic implementation) was not assigned to any study in the indexed and retrievable corpus.*

2.3. Age Estimation

In this review, "age estimation" denotes inference of a person's chronological age from a biological sample, including chronological age at death when postmortem material is used. It does not denote postmortem interval or time since death, sample/stain age (time since deposition), or wound age. Postmortem interval/time-since-death and time-since-deposition endpoints were treated as distinct from chronological-age estimation; as reported in Section 2.5, no qualifying empirical study had either as its dominant primary application. Wound-age estimation was outside the mapped primary endpoints of this review and was not sepa-

rately analysed. Age estimation was the largest empirical application area and contained the greatest number of studies with advanced validation evidence. Its distribution was Category 1 = 7, Category 2 = 75, Category 3 = 88, Category 4 = 7, and Category 5 = 0. Selected advanced-validation studies include receiving-laboratory and collaborative/inter-laboratory evaluations [9,10,32–35]. A further receiving-laboratory, cross-platform transportability evaluation is described in Table 3 and Supplementary Tables S10 and S11. Category 4 applied only to selected assays or models tested under defined external, collaborative, transferability, or inter-laboratory conditions; it did not indicate that age estimation as a whole was established as a routine forensic service.

The age-estimation literature included targeted models developed for defined tissues such as blood, saliva or buccal cells, with extensions to semen, bone, teeth, postmortem samples used for age-at-death estimation, and other difficult matrices [36–51]. Studies at Category 3 or 4 reported independent datasets, receiving-laboratory transfer, collaborative exercises, casework-relevant stains, or broader technical testing. These designs were heterogeneous. Cross-validation or a training/test split contributing to feature selection, choice among candidate models, fitting, or parameter tuning remained Category 2 evidence. A clearly held-out validation set not used for those development steps could contribute to Category 3 when it provided endpoint-specific validation beyond model fitting, but did not by itself establish Category 4.

Remaining constraints included population and tissue transferability, degraded or low-template traces, mixtures, calibration across laboratories and instruments, failure criteria, and transparent reporting of prediction uncertainty. Analytical accuracy was therefore necessary but insufficient for a defined forensic use. Reported error estimates in living-donor blood or buccal samples and estimates of chronological age at death from bone or teeth concern different target populations and matrices; they should not be pooled or ranked without matching endpoints, age distributions and measurement conditions.

2.4. Body-Fluid and Tissue Identification

Body-fluid and tissue identification was the second largest empirical application area, with Category 1 = 1, Category 2 = 33, Category 3 = 55, Category 4 = 3, and Category 5 = 0 [52–54]. The Category 3 block reflected substantial single-laboratory and forensic-like pre-implementation testing, while Category 4 remained restricted to selected assays evaluated through advanced external, collaborative, or inter-laboratory designs.

The evidence centred on tissue-specific DNA methylation and differentially methylated region or CpG assays for semen, saliva, blood, vaginal or menstrual material, skin or epidermal traces, and broader tissue-of-origin questions [11,55–57]. Parallel non-coding RNA strategies and overlapping platform tags—including methylation arrays, quantitative PCR (qPCR), SNaPshot, and pyrosequencing—were also represented [17,18,58–63]. Because these target and platform tags were descriptive and non-additive, their study-level frequencies are reported in Supplementary Tables S6 and S7 rather than used as application denominators.

Reported validation evidence included collaborative exercises, independent or inter-laboratory testing, developmental validation, casework-oriented samples, and targeted marker validation [12,52–54,64–66]. The main unresolved requirements were reproducible interpretation thresholds, sensitivity and specificity estimates, limits of detection, tissue-source mixtures versus mixtures of individual donors, low-template and degraded stains, environmental challenges, compatibility with downstream STR profiling, and broader inter-laboratory or proficiency-testing evidence. Experiments containing two biological fluids from one person cannot automatically establish performance on mixtures from two individuals.

Actual casework material was nevertheless represented in both major application areas. Examples included a semen-identification assay evaluated on 33 police casework samples [65], a semen-age model applied to an actual rape case [67], and age-estimation studies using actual crime-scene blood or casework skeletal samples [68,69]. Body-fluid/tissue studies also included material from 18 forensic cases [70] and two actual casework samples examined with combined STR and epigenetic approaches [71]. These reports demonstrate direct contact with case material, but an assay yielding a measurement on a controlled reference specimen, an interpretable result on a mock trace, and a reportable conclusion from an actual submitted forensic item represent different evidential circumstances. A single case application or a small case series was kept distinct from collaborative validation, laboratory implementation, and documented routine use; no separate corpus-wide casework prevalence is therefore reported.

2.5. Other Forensic Applications

No qualifying empirical study had postmortem interval or time-since-deposition inference as its dominant primary application. Related work remained represented in the wider evidence base through secondary application tags, background/secondary records, and non-human evidence [72–77]. For example, Bilinsky (2025) [73] was classified by its dominant body-fluid/tissue-identification endpoint despite a descriptive time-interval tag; Hashiyada (2019) [72] was retained as background/secondary, and Sjöholm (2018) [76] concerned non-human rat-brain material rather than eligible human forensic PMI inference. These examples reflect the eligibility and dominant-endpoint rules rather than an absence of related research. The individual study dispositions can be traced in Supplementary Tables S2 and S3. These sources also show why age at death, time since death, and time since deposition should not be treated as interchangeable endpoints: decomposition, temperature, tissue specificity, agonal state, environmental exposure, and casework sampling can alter both the measured signal and its interpretation.

Monozygotic-twin differentiation comprised 21 empirical studies: Category 1 = 9, Category 2 = 8, Category 3 = 4, Category 4 = 0, and Category 5 = 0 [13,14,78–86]. The evidence supported biological plausibility and increasing technical sophistication across DNA-methylation and several non-coding RNA approaches; one retained study also had an analytically unresolved sequencing workflow. Important constraints remained tissue dependence, age-related and stochastic divergence, longitudinal stability, cohort size, and reproducibility under forensic sample conditions. Discrimination among known within-pair reference materials should not be conflated with validated inference from unknown trace material across tissues or over time.

Other donor-characteristic inference comprised 12 empirical studies: Category 1 = 0, Category 2 = 6, Category 3 = 6, Category 4 = 0, and Category 5 = 0. The empirical evidence included targeted smoking- and alcohol-related studies [15,16,87,88]. Philibert et al. [89] was retained as a contextual record because the reported toolkit was evaluated for insurance and treatment monitoring rather than a defined forensic inference. Additional qualifying work addressed multi-trait or smoking-related models [90–92]. Wang et al. [93] had height as the dominant endpoint, while multi-trait work also assessed height alongside other characteristics [90]. Primary-application counts therefore reflect dominant endpoint assignment and should not be read as counts of every trait examined. Individual-level inference in this broader domain remained vulnerable to confounding, population transferability, tissue effects, uncertainty, privacy concerns, and overinterpretation.

Sixteen contextual records provided ethical, legal, governance, field-development, or directly relevant biological and technical context. Because they fell outside the qualifying

empirical endpoint framework, they were not assigned a Category 1–5 classification and did not contribute to Categories 1–5.

2.6. Molecular Strata, Detection Principles, and Analytical Platforms

Sequencing, platform, and method-development studies accounted for 20 primary empirical records: Category 1 = 2, Category 2 = 17, Category 3 = 1, Category 4 = 0, and Category 5 = 0 [19,94–99]. Platform capability was treated as supporting an endpoint rather than as a forensic endpoint in itself.

An identifiable methylation-discrimination workflow was reported for 272 empirical studies. Chemical conversion, predominantly bisulfite, was the primary principle in 243 studies (89.3% of identifiable workflows). Restriction-digestion approaches accounted for 18, hybrid/comparative multiple principles for 5, direct native-DNA methylation detection for 3, and enzymatic conversion, affinity/enrichment, and native-DNA fragmentomics for one study each. MSRE and MDRE were classified as restriction-digestion approaches and were not treated as enzymatic conversion. Outside this denominator, 23 empirical studies were non-methylation studies, 26 reused existing methylation data without performing or reporting a new molecular assay, and one study, Zeng et al. [86], retained an unresolved analytical workflow and was not assigned a definitive nanopore or PCR-amplified workflow.

Methodological and readout/platform tags were recorded separately and were non-additive. Chemical conversion appeared as a methodological tag in 248 studies, MSRE in 19, MDRE in 8, enzymatic conversion in 3, direct native-DNA detection in 3, and affinity/enrichment and fragmentomics in one study each. Readout tags included computational/public-data modelling (104), capillary electrophoresis or fragment analysis (98), pyrosequencing (80), and quantitative PCR, droplet digital PCR, methylation-specific PCR, or high-resolution melting (qPCR/ddPCR/MSP/HRM; 66). Additional tags comprised massively parallel or next-generation sequencing, including targeted sequencing (MPS/NGS; 61), SNaPshot or single-base extension (SBE) with capillary electrophoresis (59), methylation microarrays (44), other expression/readout or not reported (36), MassARRAY/Agena EpiTYPER (14), nanopore/direct single-molecule detection (3), and lateral-flow/dipstick readout (1). These frequencies overlap and must not be added to the empirical denominator. Quantification-only qPCR, methods merely cited, and reused arrays or sequencing datasets were not counted as generated platforms; reused/public-data analysis was coded only in its dedicated tag. Detailed full-text-based study-level coding is reported in Supplementary Tables S6 and S7, with Zeng 2026 [86] retained as analytically unresolved.

Table 3 summarises the empirical application-level findings and reports the contextual records separately. It presents the complete category distribution because the small number of Category 4 studies applies only to selected assays or models and does not define the status of an entire application area. Table 3 presents the 10 Category 4 studies individually; Supplementary Table S10 provides the corresponding detailed sample, laboratory, performance, forensic-condition, and limitation fields. Supplementary Table S11 further records descriptive relationships among the ten reports and the difference between unique biological contributors, shared specimens and repeated technical measurements where source-identifiable; it does not provide an additional independent-method denominator.

Table 3. Category 4 studies: advanced evaluations under defined conditions.

Study/Intended Use	Samples and forensic Conditions	Validation Design/Laboratories	Key Findings and Remaining Limitations
Pfeifer (2020) [9]—age estimation; transfer of published blood/buccal pyrosequencing models.	151 postmortem blood samples (1 day–96 y) and 149 buccal swabs (13 days–95 y) were collected; 100 buccal samples were used for the initial local model evaluation and 49 additional buccal samples were later used as a test set. Twenty-one fresh-blood samples were analysed for fresh-versus-stored/postmortem comparison.	One receiving laboratory first tested the published Bekaert blood and buccal models directly, then retrained local models. The retrained blood model was evaluated on the 206 online Bekaert blood records (external to the local retraining set); the retrained buccal model was evaluated on 49 additional local buccal samples. The publication does not report how the 100/49 buccal split was selected.	Direct transfer performed poorly (MAD 9.84 y blood). For buccal samples, the source contains an internal numerical discrepancy: the abstract reports an initial MAD of 8.32 y, whereas the Results section reports 8.68 y for the 100-sample direct application. Apparent fit after local retraining improved to MAD 5.55 y (blood) and 4.65 y (buccal), whereas post-retraining evaluation yielded MAD 6.49 y on the 206 Bekaert blood records and 4.47 y on the 49 buccal test samples. The study therefore documents informative transfer failure and local adaptation, but not multi-laboratory implementation or routine use.
Heidegger (2022) [10]—semen age estimation; VISAGE ET-13 targeted bisulfite-MPS assay.	99 assay-validation preparations across methylation standards and semen materials; concordance DNA and FTA-card stains from two semen donors (33 and 42 y). Sensitivity inputs were 50, 20, 10 and 1 ng into bisulfite conversion.	Five VISAGE laboratories performed one collaborative ET-13 validation run each, assessing reproducibility, concordance, species specificity and a low-input series. The sensitivity experiment used methylated DNA standards rather than a broad donor-level semen series.	Most markers/samples met the 1000-read threshold down to 10 ng, with a small number of 10-ng dropouts; every 1-ng sample had at least two marker dropouts (36 dropouts in total). Methylation variability increased below 50 ng. Mock-stain age deviations were 5.4 y and 2.0 y for the two donors. Thus, 1 ng was tested but did not demonstrate acceptable assay sensitivity; broader donor/casework validation remains required.
Kampmann (2024) [32]—blood age estimation; five-CpG bisulfite-pyrosequencing protocol on PyroMark Q48.	49 blood donors aged 18–64 y plus methylation controls; samples analysed in triplicate. No aged/degraded casework stains were tested.	Five forensic genetic laboratories used a common protocol and existing age model in a collaborative exercise.	Overall MAD 3.62 y. Demonstrated inter-laboratory feasibility and exposed platform/QC constraints, but trace robustness, broader population transferability and routine implementation remained unshown.
Heidegger (2025) [33]—blood/buccal age estimation; VISAGE Enhanced Tool and associated models.	Technical testing used methylation controls plus blood/saliva; model evaluation used 160 blood and 100 buccal samples independent of the original VISAGE testing sets. Sensitivity maintained to 5 ng.	Six laboratories completed technical phases; three governmental laboratories evaluated the age-prediction models.	MAE 3.95 y (blood) and 4.41 y (buccal). One laboratory showed systematic blood-age bias; excluding it reduced blood MAE to 3.1 y. Supports inter-lab robustness but reinforces laboratory-specific validation.
Onofri (2025) [34]—blood-age estimation; Ge.F.I. five-locus bisulfite–SBE/SNaPshot assay.	Blood and dried mock-stain specimens from 22 Italian volunteers. Six laboratories contributed 528 technical replicate records (four per subject/laboratory), not 528 independent donors.	Ten laboratories participated across three phases; six supplied final quantitative data. Replicates were averaged to subject level before laboratory-specific leave-one-out cross-validation (LOO-CV). Phase II/III alignment used age-informed regressions.	Mean laboratory MAD increased from 3.07 y to 4.41 y with subject-level LOO-CV; laboratory effects explained 33.8% of residual variance. The 5.24-y mean width of a 95% CI around the cross-laboratory mean is not a single-case prediction interval. Preprocessing may introduce optimism if age-based alignment was not refitted inside CV; the source does not establish this. Only 22 donors were studied; see S10 for complete methodological details.

Table 3. *Cont.*

Study/Intended Use	Samples and forensic Conditions	Validation Design/Laboratories	Key Findings and Remaining Limitations
Lee (2022) [35]—age estimation (primary) with body-fluid typing as an enabling secondary endpoint; SNaPshot multiplexes.	Each of 12 laboratories received 24 standardised test samples derived from blood, saliva and semen; four progressively complex tasks covered the complete workflow, including undisclosed sample characteristics.	Twelve forensic laboratories participated in a full-workflow collaborative exercise.	Mean absolute age-prediction error across participant data: 2.7 y (semen), 5.0 y (blood), 3.8 y (saliva); body-fluid results were mostly conclusive (98%). Analyzer, buffer, input and local workflow effects remained; local validation/SOPs required.
Liu (2022) [52]—spermatozoa identification; 3-plex HhaI MSRE-PCR assay.	Nine blinded volunteer-derived mock samples: two semen, two saliva, one blood, and four semen-containing two-fluid mixtures at defined ratios.	Six of eight invited Taiwanese forensic laboratories completed a collaborative test, applying their local presumptive testing, DNA extraction and quantification before the shared MSRE-PCR assay.	All six laboratories reported concordant expected sperm-identification results on prepared samples. Local analytical thresholds, broader proficiency testing, actual casework samples and routine reporting/QA remain unestablished; see S10.
Kim (2024) [53]—organ/tissue identification; EPIC marker discovery followed by methylation-SNaPshot multiplex.	Korean/Asian autopsy material for development and internal validation; multiplex tested on 88 autopsy samples. Independent German comparison: 80 samples from 10 individuals.	Development laboratory plus independent German inter-laboratory comparison; sensitivity and mixture experiments were also reported.	Internal detection was 100% across nine tissue types; independent comparison correctly identified 74/80 samples (92.5%). Lung/kidney and threshold/dropout effects remained; no routine implementation.
Jung (2016) [54]—body-fluid identification; seven-CpG methylation SNaPshot multiplex.	30 prepared specimens covering blood, saliva, semen and vaginal fluid; staged tasks included known/unknown sources and mixtures, progressing to extraction and bisulfite conversion.	Seven laboratories completed a staged collaborative exercise spanning capillary analysis through the full analytical workflow.	Six laboratories generated consistent results through complex tasks and all laboratories concurred on final interpretations. Sensitivity/error-rate thresholds and formal interpretive guidelines were not established.
Poussard (2023) [100]—saliva/buccal age estimation; cross-platform transfer of published Jung age-prediction models from SNaPshot to pyrosequencing.	Saliva and buccal swabs from 115 French individuals aged 0–88 y. Analytical adaptation used methylation standards for trueness, sensitivity and repeatability; DNA-input testing covered 300, 150, 75 and 37.5 ng, with 75 ng selected as the practical compromise.	A receiving forensic laboratory adapted primers/PCR conditions and the analytical platform before evaluating the published tissue-specific and multitissue models on the 115-donor cohort. The donor cohort was used for performance evaluation rather than for setting the analytical adaptation conditions.	MAE 3.5 y (saliva), 3.9 y (buccal) and 3.2 y (multitissue); approximate 95% prediction intervals were ±9 y for saliva and ±10 y for the other models. The study explicitly tested cross-platform transportability and documented age-range and DNA-input limitations, supporting C4 under defined conditions; it remained single-laboratory and did not test mixtures, degraded traces, inter-laboratory reproducibility or routine implementation.

Note: Category 4 denotes evidence from advanced evaluation of the defined assay or model under the conditions tested, not necessarily successful validation. It does not indicate validation of an entire application area, completion of each laboratory's internal validation, universal transferability, routine forensic implementation, accreditation, or legal admissibility. Performance values are reported only to characterise the source-specific validation evidence and were not pooled across studies. An unsuccessful receiving-laboratory transfer or systematic between-laboratory bias may be informative for advanced evaluation without demonstrating acceptable casework performance.

3. Discussion

The findings provide a structured account of the published evidence rather than a verdict on the ultimate value of any method. The main result is that most qualifying publications document discovery, initial technical evidence or pre-implementation validation, whereas advanced evaluations and documented implementation remain uncommon. These report-level counts must not be interpreted as estimates of the number of distinct assays or

models that are insufficiently validated. The more consequential gap is the limited accumulation of evidence on transferability, interpretative uncertainty, laboratory implementation and controlled reporting beyond the development setting. Category 4 is not a common performance grade: an independent transportability experiment that exposes bias and a multi-laboratory collaborative exercise may both document advanced evaluation while yielding different conclusions about the method itself.

The absence of Category 5 evidence is therefore a finding about documentation within the published corpus, not a declaration about all operational practice. Unpublished local validation or laboratory experience may exist, but it cannot be counted as documented routine implementation in a systematic map of indexed and retrievable literature [20–25].

The comparison with STR practice clarifies why this distinction matters. Established forensic DNA validation separates developmental evidence from laboratory-specific validation and requires procedures, limitations, interpretation rules and quality parameters to be supported by validation data [20–23]. The comparison in this review is conceptual rather than a parallel categorisation of STR methods. Epigenetic applications add an inferential requirement: validation must address not only whether the molecular signal can be measured reproducibly, but whether the resulting biological estimate or classification remains valid, transferable and reportable for the intended forensic use [9,10,26,32,33]. Analytical robustness is therefore necessary but not sufficient; biological and interpretative validity must also be demonstrated under the tissues, populations, sample states and reporting conditions for which the conclusion is intended.

Confidence in the molecular synthesis is greatest for DNA-methylation-centred applications. Mixed-molecular and transcriptomic/non-coding RNA studies were kept separate because a shared forensic endpoint does not make molecular evidence interchangeable, and validation claims must remain tied to the molecular stratum examined.

Age estimation has the largest evidence base and the highest number of studies documenting advanced evaluation designs, including independent validations and inter-laboratory exercises [9,10,32–35]. Recent model development also illustrates that technically promising internal performance may still precede external or inter-laboratory validation [101]. Its principal role remains investigative: it may narrow a pool of unknown persons or provide contextual intelligence when no matching profile is available, but it is not an identification method and remains complementary to STR confirmation [1,30]. At a high level, this lead-generating role is analogous to investigative genetic genealogy in that both can support investigative prioritisation without replacing evidential profile comparison [102]. The main unmet requirements are no longer marker discovery alone. They include robust transfer across tissues, age ranges and populations; low-template, degraded and mixed samples; platform and laboratory effects; explicit failure criteria; model-version control; calibration; and prediction uncertainty that can be communicated in a forensic report.

Body-fluid and tissue identification addresses a more trace-centred question and may bear more directly on evidential interpretation. Targeted methylation assays for semen, saliva, blood, vaginal or menstrual material, skin and other tissues can complement conventional forensic biology [12,52–54,64–66]. The remaining gap is the transition from strong assay performance to reproducible interpretation across mixtures, degraded or low-template stains, environmental challenges and downstream STR workflows, with predefined thresholds and error characteristics. Analytical classification of a fluid or tissue must also remain separate from any later inference about the activity through which the material was deposited [24]; a body-fluid result does not by itself establish mechanism, timing or activity context.

Less developed applications expose related but distinct gaps. Postmortem and difficult-matrix studies rely on different biological assumptions for age at death, time since death and time since deposition, with decomposition, temperature, tissue, agonal state, environment and sampling affecting the signal [72–77]. Monozygotic-twin differentiation still requires stronger evidence beyond comparison of known within-pair reference materials, particularly for unknown traces, cross-tissue performance, temporal stability, cohort size and reproducibility under realistic sample conditions [13,14,78–86]. Other donor-characteristic inference—including exposure/lifestyle-related traits and body-size phenotypes such as height—remains particularly vulnerable to confounding, population and tissue transferability, privacy concerns and individual-level uncertainty [15,16,87–93]. Wang et al. [93] focused specifically on height, whereas multi-trait work also assessed height alongside other characteristics [90]. These domains remain further from reportable individual-level inference than age estimation or body-fluid/tissue identification.

Analytical platforms should be judged through the forensic endpoint they are intended to support. Massively parallel sequencing, arrays, nanopore or other direct methylation methods, restriction-digestion assays, chemical or enzymatic conversion, and droplet digital PCR can differ in sensitivity, throughput, accessibility and multiplexing [19,94–99], but none is a forensic endpoint in itself. The technology synthesis therefore separates the primary methylation-discrimination principle from methodological and readout tags. This distinction prevents technical novelty or platform capability from being mistaken for endpoint-specific forensic validation.

Computational models are directly relevant to the review objective of distinguishing technical performance from transferable forensic validity. Machine-learning methods can improve prediction or classification [41,43,90,95,103,104], but strong internal performance may reflect the structure of the development dataset rather than performance under new populations, laboratories, platforms or sample conditions. Validation of these models therefore requires external testing, assessment of dataset shift, calibration, version control, explicit failure criteria and uncertainty that can be communicated in a report; interpretability may also be important where model behaviour affects the limits of the forensic conclusion.

Tables 3 and 4 illustrate the central distinction between analytical performance and forensic validity. A study may show a sensitive assay or accurate model while still leaving unresolved questions about transferability, mixtures, degradation, uncertainty, reproducibility, laboratory procedures and reporting. The category framework may support training and quality-assurance discussions by making these evidence types explicit, but it is not itself a quality score or implementation standard. Progress beyond pre-implementation evidence requires representative sample testing, defined limits of detection and interpretation, reproducible protocols, laboratory-specific validation, inter-laboratory evidence and controlled reporting [9,10,20–26,32,33].

In U.S. federal practice, Daubert and Federal Rule of Evidence 702 provide a useful legal analogue because they focus on methodological reliability, error, standards and reliable application to the case [105,106]. Validation can therefore inform legal scrutiny of reliability, but it is not equivalent to admissibility, which remains a case- and jurisdiction-specific legal determination.

Table 4. Application-level summary of validation evidence.

Application Area/Evidence Role	n	Category Distribution (C1/C2/C3/C4/C5)	Main Forensic Purpose	Most Advanced Evaluation Reported	Main Limitations Before Forensic Use
Age estimation	177	7/75/88/7/0	Estimate chronological age or age at death from a defined tissue.	Repeated targeted modelling; independent, collaborative, and inter-laboratory evaluations for selected models.	Tissue/population transferability; low-template, degraded, and mixed traces; local calibration; prediction uncertainty.
Body-fluid/tissue identification	92	1/33/55/3/0	Classify biological material or tissue of origin.	Targeted CpG/tissue-specific differentially methylated region (tDMR) assays with developmental, collaborative, independent, and inter-laboratory studies for selected assays.	Thresholds; sensitivity and specificity; donor and tissue mixtures; environmental challenges; STR compatibility; proficiency-testing evidence.
Monozygotic-twin differentiation	21	9/8/4/0/0	Differentiate genetically indistinguishable twins through epigenetic or non-coding RNA divergence.	Genome-wide and targeted studies with selected additional-sample and forensic-scenario testing.	Cohort size; tissue/age effects; longitudinal stability; low-template traces; reproducibility.
Sequencing/platform/method development	20	2/17/1/0/0	Develop analytical methods that support one or more forensic applications.	Multiplex bisulfite-MPS, direct detection, conversion alternatives, low-input and combined workflows.	Endpoint-specific validation; reproducibility; workflow compatibility; interpretation and reporting.
Other donor-characteristic inference	12	0/6/6/0/0	Infer selected donor characteristics beyond age, including exposure/lifestyle-related traits and body-size phenotypes such as height.	Repeated associations and selected targeted or multi-trait models with limited transferability testing; one height-focused methylation model.	Confounding; individual-level uncertainty; population/tissue transferability; phenotype-specific validation; privacy; proportionality.
Contextual records	16	—	Provide ethical, legal, governance and field-level context, and retain relevant biological or technical studies that did not evaluate a qualifying forensic endpoint.	Structured contextual analyses and original biological or technical findings relevant to forensic epigenetics, but not designed as validation studies for a specific forensic use.	Reported separately; no Category 1–5 classification was assigned, and these records were not used to raise any application's validation category.

Note: C = validation evidence category. Categories 1–5 apply only to empirical primary studies. The five empirical application rows are mutually exclusive and sum to 322; the 16 contextual records are reported separately and were not assigned a Category 1–5 classification.

Ethical and legal concerns differ across applications. Age estimation and body-fluid/tissue identification are relatively circumscribed, but both can be overinterpreted if uncertainty and limits are not explicit. Sensitive epigenetic inferences concerning exposure or lifestyle may reveal personal information and invite conclusions beyond the underlying data [4,5,27–29,88–92]. Trauma- or stress-related associations raise similar governance con-

cerns, but they were not represented as a dominant qualifying empirical forensic endpoint in this evidence map. For such applications, necessity, proportionality, data protection, population bias, reporting boundaries and the risk of transferring group-level associations to individual-level conclusions are part of defining what can responsibly be reported.

Across the field, the principal missing evidence is validation depth rather than additional marker discovery alone. For age estimation, priorities include harmonised reporting of tissue, age range, population composition, calibration, prediction intervals, platform effects, failure criteria, degraded and mixed samples, and inter-laboratory reproducibility. For body-fluid/tissue identification, priorities include standardised thresholds, sensitivity and specificity, limits of detection, donor and tissue mixtures, environmental challenges, low-template samples, STR compatibility and proficiency-testing exercises [52,54,66]. Across the selected Category 4 evaluations, documented limitations recur in different ways rather than forming a ranked score: age-prediction studies leave tissue/population transfer, local model calibration, prediction-interval coverage, casework low input or observed laboratory bias unresolved in at least some tested workflows; body-fluid/tissue studies often remain concentrated on shared prepared/mock specimens, with local thresholds, open-set/tissue-confusion behaviour, mixture interpretation and casework reporting limits incompletely addressed. These are qualitative source-specific patterns, not prevalence estimates for distinct assays or all 322 studies (Table 4, Tables S10 and S11). More generally, reporting should identify the sample and tissue, assay, model and version, validation population, calibration and uncertainty. Independent validation, inter-laboratory networks, external quality assessment, laboratory-specific procedures and transparent expert interpretation are the steps most clearly missing between the current evidence base and documented routine implementation.

Figure 4 brings together the primary application counts, validation-category distribution, and principal gaps and priorities for future research.

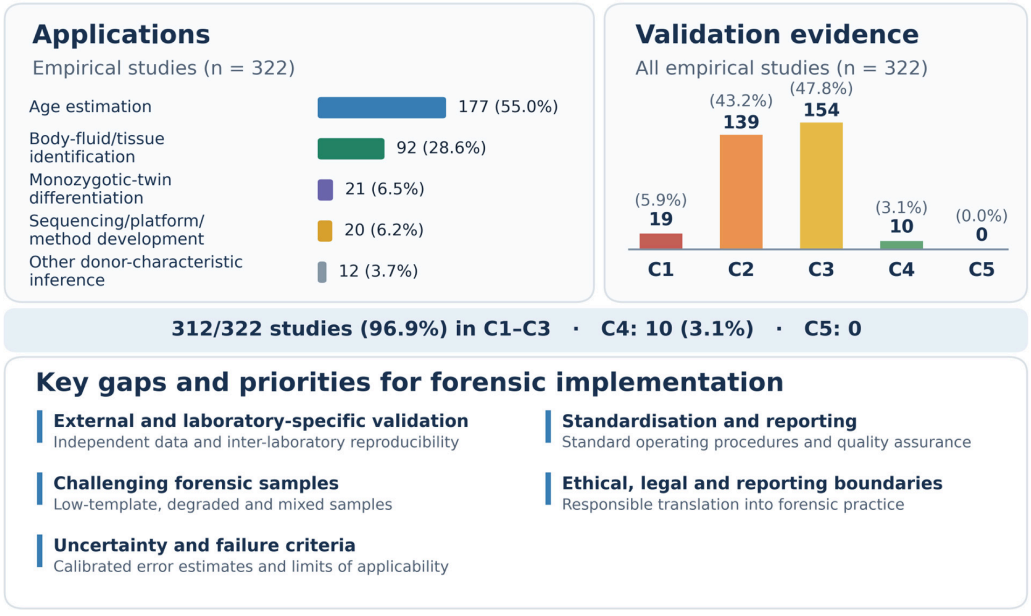


Figure 4. *Empirical distributions (n = 322) by application and Categories 1–5 (**upper**) panels. The (**lower**) panel is a qualitative synthesis of implementation priorities, not a frequency ranking of deficiencies assessed across all studies. C5 = 0 means no indexed, retrievable report met the review's routine-implementation criteria; operational use elsewhere is not excluded.*

4. Materials and Methods

4.1. Review Design and Reporting

This study was designed as a systematic scoping review. Its purpose was to map a heterogeneous evidence base rather than pool diagnostic accuracy or evaluate an intervention. A scoping design was appropriate because forensic epigenetics spans different applications, biological materials, analytical platforms, statistical models, validation designs, and translational endpoints. Reporting followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses extension for Scoping Reviews (PRISMA-ScR) [31]. No review protocol was registered or prepared as a separate written document. An initial structured charting framework and provisional validation-evidence categories were progressively refined during full-text assessment and charting. Their final definitions were consolidated before synthesis and consistently applied across the empirical corpus, as detailed below and in the Supplementary Materials.

The author-defined validation evidence categories were used only to organise the reported evidence. They were not intended to certify legal admissibility, accreditation, proficiency-testing status, or routine implementation, and they do not replace jurisdiction-specific review or laboratory-specific validation.

4.2. Search Strategy, Eligibility Criteria and Study Selection

Searches were conducted in Scopus, PubMed/MEDLINE, and Web of Science Core Collection, with a final search date of 15 June 2026. The searches were limited to English-language records published from 1 January 2010 to the final search date. The 2010 date was applied consistently as the lower temporal eligibility limit. No separate biological, historical, or milestone-based rationale for selecting exactly 2010 was documented; it is therefore reported as a methodological temporal restriction, and relevant earlier literature may have been missed. Search strategies differed in scope and syntax across databases. Scopus used one broad cross-application search plus five modular searches that expanded application-specific vocabulary for age estimation, body-fluid/tissue identification, post-mortem interval/time since deposition, monozygotic twins, and sequencing/platforms. PubMed/MEDLINE and Web of Science used single combined cross-application queries and did not reproduce those five modules separately. The databases were therefore complementary rather than exact replications, and topic-specific retrieval sensitivity could differ across them. Across the database-specific strategies, combinations of forensic terms were used with epigenetic, DNA methylation, methylome, age-estimation, body-fluid/tissue, postmortem, time-since-deposition, monozygotic-twin, exposure, trauma, stress, validation, casework, and platform-related terms, as appropriate to each database syntax and search component. The strategy was centred on DNA methylation and did not use a separate non-coding RNA search block. Complete database-specific strategies, interface-limit notes, and raw retrieval counts are provided in Supplementary Table S1.

Eligible empirical primary evidence comprised original research, validation studies, method-development studies, and casework-relevant technical studies involving epigenetic markers, analytical platforms, or interpretation. For this review, a qualifying empirical primary study was an original investigation that directly evaluated a defined forensic endpoint or a method or model intended to support such an endpoint under the eligibility criteria. DNA methylation-centred studies were the principal focus. Mixed-molecular and transcriptomic/non-coding RNA studies were retained only when they directly addressed a forensic application and were analysed separately from DNA-methylation evidence. Accordingly, the non-coding RNA stratum represents relevant records captured by the methylation-centred search and is not necessarily exhaustive. Contextual records comprised original ethical, legal, governance, or field-development records with direct implementation

relevance, together with directly relevant original biological or technical studies captured by the search that did not evaluate a defined qualifying forensic endpoint or inference. Background/secondary papers, comparators, non-human studies, and excluded records did not contribute to empirical application or Category 1–5 totals.

Duplicate records were removed before title/abstract screening. In total, 1557 duplicate or overlapping records were removed from the 2212 retrieved records, leaving 655 records for screening. Bibliographic deduplication and evidence-unit linkage were handled as separate steps. At the full-text stage, suspected linked reports were compared for title and authorship, publication type, assay/model identity, sample or dataset provenance, and whether each report contributed new empirical validation information. Correction notices, conference abstracts, preprints, and later journal reports were adjudicated case by case rather than automatically merged or counted separately. When multiple representations described the same underlying empirical work, one controlling evidence unit was retained and the relationship documented; the linked Gopalan abstract/preprint is one example. Reuse of public or previously published datasets within a distinct new analysis did not by itself make the later report a duplicate if it contributed separate validation information, whereas reports without new empirical evidence were not treated as independent evidence units. Related publications on the same assay or model remained separate study-level records when they contained distinct empirical evidence. This linkage was performed for identified overlaps during source-identity and duplicate-evidence-unit checks and was not a corpus-wide reconstruction of method families. Study selection then followed staged title/abstract screening and full-text eligibility assessment. Fifteen reports sought for full-text assessment could not be retrieved because the full texts were not available through the institutional subscriptions and access routes of the authors' universities; they were therefore not assessed for full-text eligibility. Title/abstract screening and full-text eligibility assessment were undertaken separately by two reviewers, with occasional consultation over uncertain cases. Disagreements concerning eligibility were discussed with additional co-authors, aiming for a shared evidence-based decision. Full-text records were assigned evidence roles after checks of source identity, duplicate evidence units, and eligibility. Reference lists of relevant publications were examined for potentially eligible studies. Reviews retained for citation tracking also supported contextual reading and source verification. No qualifying empirical study entered the evidence map solely through a separate citation-tracking route: all 429 records in the study-level register were traceable to one or more of the three database searches, so citation tracking did not create an additional PRISMA identification stream. The sortable Supplementary Workbook provides the database retrieval source(s) for the 429 study-level register records. The complete numerical flow is reported in Results and Figure 1. Supplementary Table S2 reports the reference-linked 429-record study-level register, including primary application, molecular stratum, evidence role, and secondary application/cross-cutting tags, and Supplementary Table S8 lists the 62 secondary/non-primary reports retained outside the evidence map that are not represented in Table S2.

4.3. Data Charting and Application Classification

The same two reviewers manually charted data from full texts, tables, figures, and available Supplementary Materials through separate assessments, with occasional consultation on uncertain evidence and discussion with additional co-authors when disagreements arose. An initial structured extraction framework was refined during full-text assessment and charting; following consolidation of the final fields, all mapped empirical studies were revisited to ensure consistent extraction. The Supplementary study-level tables report reference-linked study identities, evidence role, primary application, molecular stratum, validation category and rationale, and technology coding. Additional source notes on

biological material, molecular targets, reported performance, validation design, forensic sample conditions, limitations, and implementation requirements supported consistency checks and were not treated as a separate quantitative dataset. Missing information was coded as not reported. For validation classification, a feature that was not reported was not treated as proof that the underlying work had not been performed; rather, the study was not credited with evidence that could not be documented from the retrievable report. Exact source identity, preprints, correction notices, duplicate evidence units, and reused datasets were checked separately to prevent double-counting.

Actual casework material was defined as samples originating from real forensic investigations or casework reports. Mock or simulated samples, blinded validation materials, collaborative exercises, and proficiency-style exercises were distinguished from actual casework and were not treated as evidence of routine implementation. Because actual casework use was not coded as a dedicated, exhaustive charting variable and the application/cross-cutting tags were descriptive and not necessarily exhaustive, a corpus-wide prevalence estimate of actual casework use was not calculated retrospectively.

Each empirical primary study was assigned one dominant primary application to prevent double-counting in application totals. Application and cross-cutting tags were descriptive, non-additive, and not necessarily exhaustive. Molecular strata were coded separately as DNA methylation, mixed molecular, or transcriptomic/non-coding RNA. For the technology synthesis, each empirical record was coded along three distinct dimensions: a mutually exclusive primary methylation-discrimination principle, non-additive methodological tags, and non-additive downstream readout/platform tags. Study-performed principles and readouts were distinguished from discovery platforms, reused public or previous data, comparators or confirmation, quantification and quality-control tools, methods merely cited, and non-epigenetic components. Quantification-only quantitative PCR (qPCR) and reused arrays or sequencing datasets were not counted as generated molecular readouts. Methylation-sensitive or methylation-dependent restriction enzymes (MSRE/MDRE) were classified as restriction-digestion approaches, not enzymatic conversion.

4.4. Classification of Validation Evidence

Each qualifying empirical primary study was assigned to one of five author-defined validation evidence categories. The category framework began with provisional definitions that were refined during full-text assessment and charting. After the definitive rules were established, all 322 empirical studies were reassessed to ensure consistent application, including records classified earlier in the review. The two reviewers made separate category assessments: one completed the assignments independently, while the second occasionally consulted on uncertain cases. The resulting assignments were compared; disagreements were discussed with additional co-authors with the aim of reaching a shared, evidence-based decision. The classification was categorical rather than numerical and was applied conservatively. A report received the highest category supported by its documented evidence, considering analytical and interpretative limitations together; an isolated strong performance statistic was insufficient. Different types of evidence could independently support the same category; they were not added together as points. At C4, an advanced external transfer or collaborative design can yield an adverse result and still be described as advanced evaluation; this does not remove the separately documented limitations of that result. The assessment considered analytical performance, independent or external validation, testing of relevant samples and conditions, interpretative limits and uncertainty, and practical laboratory implementation. In brief, Category 1 denoted exploratory discovery, Category 2 initial technical evidence, Category 3 pre-implementation validation, Category 4 advanced validation evidence under defined conditions, and Category 5 documented

routine forensic implementation (Table 2). High analytical performance alone was not sufficient for Category 4 or Category 5. When evidence necessary for a higher category was not documented or remained ambiguous in the report, the study was assigned to the highest category supported by the documented evidence. This conservative rule reflects reporting uncertainty as well as validation scope and should not be read as proof that unreported work had not been performed.

Supplementary Table S9 provides operational criteria, alternative qualifying evidence patterns, and worked examples at the Category 2–3 and Category 3–4 boundaries. The examples illustrate how missing or ambiguous information is handled without reducing the categories to a numerical score. Chen et al. (2026; long non-coding RNA age-estimation study), for example, was assigned to Category 3 on the basis of evaluation in independently sourced external cohorts, although ComBat batch correction incorporated known chronological age as a biological covariate. The authors described the resulting external accuracy as an idealised scenario because age would be unknown in forensic use; this was not an independently deployable prediction pipeline. By contrast, Lee (2025) [101] remained Category 2 because test-set performance contributed to the choice of preferred models, without a subsequent untouched validation of those selected models (Supplementary Table S9B). Chen's Category 3 designation reflects an external-cohort evaluation design, not stronger or prospectively credible prediction for an unknown-age forensic sample. Poussard et al. (2023) [100] was assigned to Category 4 because a receiving laboratory evaluated cross-platform transportability of published models, documenting analytical adaptation separately from performance in a new donor cohort, as well as sensitivity, repeatability and prediction limits. These examples illustrate the documented validation evidence and its limits, rather than establishing routine casework implementation.

For this review, analytical evidence referred to the performance and reproducibility of the assay, marker panel, or model. Interpretative evidence concerned the validity, uncertainty, transferability, and limits of the forensic conclusion. Implementation evidence concerned performance under representative forensic conditions together with laboratory procedures, quality assurance, and reporting requirements. Category assignment considered these domains jointly rather than treating any single performance metric as sufficient.

The unit of classification was the individual empirical study/report, not the assay, model, workflow, laboratory, or method family. Distinct publications concerning the same or related assay/model could contribute separate study-level evidence when they contained distinct empirical validation information, whereas linked representations of the same underlying empirical work were consolidated into one evidence unit. Reuse of a dataset was documented but did not by itself determine whether two reports were duplicates. For the ten Category 4 reports, a supplementary descriptive relation register (S11) identifies documented shared assay families, originating models, receiving laboratories, technical repetitions and biological sample units, while expressly marking unestablished links. It does not reconstruct every method-development chain, and no independence-adjusted sensitivity percentage was calculated. The review did not construct a method-level denominator; category percentages therefore describe the distribution of published studies in the evidence map, not the proportion of distinct methods that are validated.

The 16 contextual records comprised ethics, governance, legal interpretation, field development, and original biological or technical evidence that did not test a qualifying forensic endpoint. They were reported separately and were not assigned a Category 1–5 classification; they were not used to raise the validation category of any biological application. Background/secondary, comparator, non-human, and excluded records were likewise outside Categories 1–5.

In this review, Category 5 was reserved for published, indexed, and retrievable evidence that a defined method had moved beyond validation studies into routine forensic casework implementation, with laboratory-specific validation, standard operating procedures, quality-assurance conditions, defined interpretation and reporting rules, and documented limits of use. Commercial availability, developmental validation, collaborative exercises, or informal knowledge that a method may be used in some laboratories were not sufficient for Category 5 assignment [20–23,25]. Category 5 therefore did not imply universal admissibility, universal transferability, accreditation, or proof of routine use outside the published corpus. Clearly linked indexed and retrievable reports concerning the same defined method and laboratory workflow could, in principle, be considered together if they collectively documented all required implementation elements; such linked evidence would be treated as one implementation evidence package for the Category 5 assessment. This package-level implementation assessment did not alter the report-level unit used for the descriptive Category 1–5 frequency counts. Potential report relationships were recognised only where they became identifiable during source-identity checks, full-text charting or reference-list examination. Candidate links were assessed through explicitly reported assay/model identity, laboratory context and workflow; reports without a sufficiently established shared method and laboratory workflow were not combined for a possible Category 5 assessment. This targeted treatment of identifiable links was not a corpus-wide reconstruction of method families or an exhaustive search for all possible implementation packages. No linked evidence package meeting the Category 5 definition was identified.

4.5. Synthesis and Consistency Checks

The synthesis was narrative and application-based. Meta-analysis was not attempted because endpoints, tissues, sample types, platforms, models, validation designs, and performance metrics were too heterogeneous for meaningful pooling. Results therefore present the PRISMA flow, composition of the evidence base, primary application distribution, molecular strata, validation evidence categories, application-specific findings, and the separate synthesis of methylation-discrimination principles, methodological tags, and readout/platform patterns. The additional descriptive linkage of Category 4 reports in Table S11 was not used to recalculate the study/report denominators or classify distinct assays as validated or unvalidated.

Manual consistency checks confirmed that each empirical study contributed once to the appropriate evidence role, primary application and, where applicable, Category 1–5 totals; that contextual records were reported separately; and that background/secondary, comparator, non-human, and excluded records did not contribute to empirical denominators. Record identifiers, evidence roles, applications, molecular strata, categories, technology counts, manuscript statements, figures, and Supplementary Tables S2–S8 were cross-checked.

No formal risk-of-bias or source-quality appraisal was performed; the five categories mapped the type and extent of reported validation evidence and were not intended as study-quality ratings.

5. Strengths and Limitations

This review is based on a systematic multi-database search through 15 June 2026 and a final evidence map comprising 322 empirical primary studies and 16 contextual records. It separates empirical evidence from contextual, background/secondary, comparator, non-human, and excluded records; assigns each empirical study to one dominant application for non-duplicative totals; separates molecular strata; and applies source-identity and duplicate-evidence-unit checks to reduce double-counting. Distinguishing analytical, inter-

pretative, and implementation evidence alongside technology coding makes the reported validation gaps auditable. External reproducibility of the bespoke classification framework has not been established.

Several limitations should be considered. The review was restricted to English-language records published from 1 January 2010 to 15 June 2026. The exact 2010 cutoff had no separate documented milestone-based justification and should be understood as a methodological temporal restriction rather than an empirically derived boundary in the development of the field; relevant older literature may therefore be absent. Search coverage was also not identical across databases. Scopus combined a broad search with five modular application-specific searches using additional topic-specific vocabulary, whereas PubMed/MEDLINE and Web of Science used single combined cross-application queries. These strategies were complementary rather than exact replications, so topic-specific retrieval sensitivity may have differed across databases. Together with the language and date restrictions, this may have affected coverage across application areas. No dedicated search of grey or non-indexed operational literature was performed, and selective publication or indexing may therefore have influenced the retrievable evidence base; no formal publication-bias assessment was undertaken. Because the search strategy was centred on DNA methylation and did not include a dedicated non-coding RNA search block, the transcriptomic/non-coding RNA stratum should not be interpreted as exhaustive. The heterogeneity described in Methods precluded meta-analysis. The five-category classification was author-defined and has not itself been externally validated. Category assignment depended on the evidence documented in each retrievable report. Absence of reporting was treated as absence of documented evidence for classification, not proof that the underlying work had not been performed. Because the unit of classification was the study/report, multiple publications may concern the same or related assay or model; technical repeats and participating laboratories must likewise not be treated as independent biological samples; the reported category percentages therefore should not be interpreted as proportions of distinct methods or method families. Screening, charting and category assessment involved separate assessments by two reviewers, with occasional consultation and co-author discussion of disagreements. The assessments were not fully independent throughout, and pre-adjudication inter-reviewer agreement was not formally quantified. Although the charting form and category framework evolved during the review, all 322 empirical studies were reassessed under the final operational definitions.

Non-indexed or locally documented operational practice may be underrepresented, so the finding that no study met the Category 5 definition within the indexed and retrievable corpus does not prove that no laboratory has used any epigenetic method operationally. The 16 contextual records were retained separately without a Category 1–5 classification and should not be read as endpoint-specific validation evidence. Finally, no validation evidence category constitutes a jurisdiction-specific admissibility assessment, accreditation decision, or substitute for laboratory-specific validation.

6. Conclusions

Forensic epigenetics, particularly DNA methylation analysis, has developed a substantial empirical evidence base with clear forensic potential. The final evidence map comprises 322 empirical primary studies and 16 contextual records. Age estimation and body-fluid/tissue identification are the only application areas with Category 4 advanced evaluation designs in the mapped corpus, but they serve different purposes: age estimation is principally investigative, whereas body-fluid or tissue identification may contribute more directly to evidential interpretation while remaining distinct from conclusions about the activity that produced a trace.

The empirical category distribution was Category 1 = 19, Category 2 = 139, Category 3 = 154, Category 4 = 10, and Category 5 = 0. Thus, most qualifying publications documented exploratory, initial technical, or pre-implementation validation evidence. The 10 Category 4 studies concerned selected assays or models documenting advanced evaluation designs under defined conditions; these designs did not necessarily establish successful transfer or routine use. No study met Category 5 within the indexed and retrievable corpus. The framework may support training and quality-assurance discussions by making explicit why publication or strong internal performance does not, by itself, establish a validated forensic use.

The main translational gap is demonstrating that a defined forensic inference remains valid when transferred beyond its development setting and reported under real laboratory constraints. Priority areas are representative low-template, degraded, mixed and environmentally challenged samples; calibrated uncertainty and failure criteria; laboratory-specific and external validation; inter-laboratory reproducibility; controlled reporting; and clear ethical and legal boundaries. Closing these gaps would provide a stronger basis for moving selected epigenetic assays or models from promising research toward documented routine forensic implementation.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/epigenomes10040070/s1. Supplementary Table S1. Search strategies and retrieval audit. S1A. Database search strategies. S1B. Deduplication procedure. S1C. Reports sought but not retrieved (n = 15). Supplementary Table S2. Study-level record register and evidence roles (n = 429). Supplementary Table S3. Full-text reports excluded or classified as non-qualifying (n = 115). Supplementary Table S4. Validation evidence and Category assignment for empirical primary studies (n = 322). Supplementary Table S5. Evidence distributions and report accounting. S5A. Validation Category distribution by primary application (n = 322). S5B. Molecular-stratum distribution among empirical primary studies (n = 322). S5C. Study-level evidence roles (n = 429). S5D. Cross-cutting descriptive tags among empirical primary studies (non-additive). S5E. Report-level accounting. S5F. Empirical primary application by molecular stratum (n = 322). Supplementary Table S6. Primary discrimination principles, methodological tags, and readout/platform frequencies. S6A. Primary discrimination principle among identifiable methylation workflows (n = 272). S6B. Analytical-workflow status among empirical primary studies (n = 322). S6C. Non-additive methylation-method tags (n = 322). S6D. Non-additive readout/platform tags (n = 322). Supplementary Table S7. Study-level technology coding for empirical primary studies (n = 322). Supplementary Table S8. Secondary/non-primary reports retained outside the evidence map and not represented in the study-level register (n = 62). Supplementary Table S9. Operational classification decision guide and worked boundary examples. S9A. Operational decision guide. S9B. Worked boundary examples. Supplementary Table S10. Detailed characterization and qualifying evaluation routes of the 10 Category 4 studies. S10A. Detailed advanced-evaluation characteristics. S10B. Qualifying advanced-evaluation route for each C4 report (n = 10). Supplementary Table S11. Category 4 evaluation-chain relationships and non-independence considerations (n = 10). Table S2 provides the complete 429-record study-level register, with sources identified by their manuscript reference numbers; the main reference list contains the sources cited in the narrative. A sortable XLSX companion provides an integrated register of the 322 empirical studies and database-retrieval sources for the 429 study-level records. In accordance with MDPI's Instructions for Authors, citations and references used in Supplementary files are also represented in the main text and in the article reference list. For bibliographic completeness, the reference list therefore also includes all unique sources represented in Supplementary Tables S2, S3 and S8; the 500 sources not already represented among References [1–106] are cited collectively here [59,107–603].

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Abbreviations

The following abbreviations are used in this manuscript:

CpG	Cytosine–phosphate–guanine
ddPCR	Droplet digital polymerase chain reaction
DNA	Deoxyribonucleic acid
EQA	External quality assessment
HRM	High-resolution melting
MDRE	Methylation-dependent restriction enzyme
MPS	Massively parallel sequencing
MSP	Methylation-specific polymerase chain reaction
MSRE	Methylation-sensitive restriction enzyme
NGS	Next-generation sequencing
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
SBE	Single-base extension
SOP	Standard operating procedure
STR	Short tandem repeat

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