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RNA Detection Technologies: A Method-Centric Guide to Principles and Reproducibility

Midhun Krishnan Vasanthakrishnan[1], Marion Hogg[2] and Kif Liakath-Ali[1, 3, *]

[1]School of Biological Sciences, Highfield Campus, University of Southampton, Southampton, UK
[2]Department of Biosciences, School of Science and Technology, Nottingham Trent University, Clifton campus, Nottingham, UK
[3]Institute for Life Sciences, University of Southampton, Southampton, UK
*For correspondence: kif.liakath-ali@soton.ac.uk

Abstract

RNA detection techniques have expanded into a diverse methodological landscape spanning hybridization, amplification, imaging, and sequencing. In this review, we provide a method-centric synthesis of the major technologies that define this landscape, emphasizing how each method's core principle, practical strengths, and sources of variability shape its reproducibility. Beginning with foundational approaches, we trace the development of isothermal amplification, quantitative and digital PCR, microarrays, single-molecule imaging, multiplexed spatial methods, and amplification-free digital quantification. We then examine the transformative impact of bulk, single-cell, long-read, direct-RNA, and spatial transcriptomics, as well as CRISPR-based detection and metabolic labeling for RNA dynamics. Across these technologies, we focus on reproducibility as a defining dimension of evaluation: mature methods benefit from established standards, whereas newer approaches remain pre-standardization and require careful, experiment-specific controls. Rigorous method selection must be guided by the biological question, required resolution, sample constraints, and the maturity of each method's reproducibility framework. We conclude that RNA detection methods form interconnected methodological paths of problem-solving rather than simple replacements.

Keywords: RNA detection, Transcriptomics, Quantitative PCR, RNA sequencing, Spatial transcriptomics, Reproducibility standards

Introduction

RNA is one of the most informative molecular readouts of cellular state, capturing both gene activity and the regulatory dynamics that shape it [1]. As a result, reliable RNA detection and measurement have become essential across nearly all areas of life sciences. Determining where and when RNA molecules are expressed now represents one of the field's most routine and consequential measurements.

Over the past five decades, the available methods have expanded from a single membrane-based assay into a large and heterogeneous toolkit spanning hybridization, enzymatic amplification, sequencing, imaging, and computational prediction. This diversification has greatly broadened what can be asked of a transcriptome, from a simple presence/absence question to isoform-resolved, spatially mapped, and temporally dynamic measurements. Yet it has also introduced a practical challenge: data that should agree often do not, and the discrepancy frequently originates in the method rather than the biology.

The reproducibility of a PCR-based RNA measurement is shaped by the reverse-transcription step, amplification chemistry, library preparation workflow, and analysis or computational pipeline—sources of variability that differ qualitatively from one method to the next, complicating cross-study comparison.

Choosing an RNA detection method is, in effect, choosing a particular profile of sensitivity, throughput, spatial or temporal information, and, critically, a particular standardization framework. Some methods are governed by mature, community-agreed reporting standards and reference materials, whereas others remain pre-standardization, with reproducibility that must be justified study-by-study. Understanding how each method works and where its variability arises is the most direct route to using it rigorously and interpreting its results with confidence.

To highlight the evolution of the field as a coherent progression rather than a catalog of techniques, this review organizes the major RNA detection methods in a broadly chronological and conceptual sequence. This structure allows us to infer how each method addressed the limitations of its predecessors and opened the conceptual space for the next. In many cases, a method was developed expressly to overcome a defined constraint of an earlier one, creating methodological paths of problem-solving that extend across decades. For each technology, we provide a concise overview of its core principle and practical use, its strengths and limitations, and the reproducibility-relevant considerations that determine whether its results travel reliably between laboratories. Categorization by detection principle is treated as secondary and summarized in a comparative table (Table 1), allowing the chronological narrative to foreground how the field has expanded, diversified, and matured.

Table 1. Categorical comparison of the major RNA detection technologies

Method (year) [Reference]	Detection principle	Throughput	Spatial information	Reproducibility standard	Practical tier
Northern blot (1977) [3]	Size-resolved hybridization	Single target	No	In-study controls	Low-cost, low-throughput
NASBA/LAMP/RPA (1991–2006) [5,6,57]	Isothermal amplification	Low-plex	No	Assay-specific	Field/point-of-care
RT-qPCR (1993–2001) [9–11]	Reverse transcription + real-time PCR	Low-plex	No	MIQE/MIQE 2.0 [10,11]	Routine, ubiquitous
ddPCR (2011) [14]	Partitioned digital PCR	Low-plex	No	dMIQE; benchmarked vs. qPCR [13]	Specialized quantification
Microarray (1995) [16,17]	Parallel probe hybridization	Transcriptome (fixed)	No	MAQC [16]	Mature, declining
smFISH (1998–2008) [19]	Single-molecule hybridization imaging	Low gene count	Subcellular	In-study controls	Specialized imaging
MERFISH/seqFISH+ (2015–2019) [21,22]	Multiplexed barcoded imaging	100s–10,000s genes	Subcellular	Pre-standardization [21,22]	Specialized imaging
NanoString nCounter (2008) [27]	Amplification-free digital barcodes	Up to ~800	No	Clinical validation [24]	Targeted, clinical
RNAscope (2012) [25]	Branched-DNA in situ amplification	Low-plex	Subcellular (FFPE)	In-study controls	Pathology mainstay
Bulk RNA-seq (2008) [29,30]	Short-read cDNA sequencing	Transcriptome	No	SEQC, ERCC, ENCODE [29–31]	Discovery default
scRNA-seq (2009–2015) [36–38]	Barcoded single-cell sequencing	Transcriptome × cells	No (cell identity)	Benchmarks; no consortium [37,38]	Widely used, complex

Long-read/direct RNA (2013–2018) [43–45]	Full-length/native sequencing	Transcriptome (isoforms)	No	Pre-standardization	Specialized
Spatial transcriptomics (2013–2022) [46–50]	Spatially barcoded/in situ sequencing	Transcriptome	Tissue–subcellular	Pre-standardization	Rapidly growing
CRISPR detection (2017–2018) [51–53]	Programmable nuclease + reporter	Low-plex	No	Pre-standardization	Point-of-care
Metabolic labeling (2008–2018) [54–56]	Nucleoside recoding + sequencing	Transcriptome (dynamics)	No (temporal)	Pre-standardization	Specialized research

1. Northern blotting

Early RNA detection methods demonstrated that specific transcripts could be identified within complex mixtures, using solution hybridization and radiolabeling approaches that laid the conceptual groundwork for later advances [2]. However, these early techniques offered limited resolution and quantification, and they could not visualize distinct RNA species within a heterogeneous sample. What the field needed was a method that could separate RNA molecules by size and then detect specific sequences within that resolved background. This need set the stage for the development of the Northern blot. Introduced by Alwine, Kemp, and Stark, the Northern blot was the first method to detect a specific RNA species against a complex background [3]. In this approach, RNA is separated by size on a denaturing gel, transferred to a membrane, and detected by hybridization with a labeled complementary probe. In practice, the method remains valued for one capability that newer technologies provide only indirectly: it reports transcript size explicitly, enabling isoforms, precursors, and degradation products to be distinguished on a single membrane. Northern blotting remains the gold standard for highly structured and modified RNA molecules, such as transfer RNAs, and the recently identified tRNA-derived fragments (tDRs) [4].

Despite its conceptual elegance, Northern blotting has notable limitations. Its sensitivity is low, typically requiring microgram-scale input RNA; its throughput is limited to a small number of targets; and the workflow is labor-intensive, often spanning several days. From a reproducibility standpoint, quantification is inherently semi-quantitative and depends on factors such as probe quality, hybridization conditions, transfer efficiency, and densitometry. No consortium-level standard governs the method and, as such, inter-laboratory comparability rests heavily on careful internal controls and transparent reporting. Nevertheless, Northern blotting established the foundational principle, sequence-specific hybridization, as a readout on which most subsequent targeted RNA detection methods were built. It remains an important historical and conceptual source for the field, illustrating how early methodological constraints shaped the development of more sensitive, scalable, and standardized technologies.

2. Isothermal amplification using NASBA, LAMP, and RPA approaches

Parallel efforts focused on making amplification rapid, equipment-light, and deployable outside conventional laboratories. This led to the development of isothermal amplification, in which nucleic acids are amplified at a constant temperature rather than through thermal cycling. Nucleic acid sequence–based amplification (NASBA) was the first widely adopted example, using a coordinated reverse transcriptase/RNase-H/T7 polymerase reaction to amplify RNA isothermally [5]. Loop-mediated isothermal amplification (LAMP) expanded the concept by using multiple primers and a strand-displacing polymerase to generate large amounts of product with simple visual readouts [6], while recombinase polymerase amplification (RPA) pushed the temperature requirement down to near-ambient levels through recombinase-driven primer invasion [5].

These methods have become widely accessible in resource-limited settings, with RT-LAMP incorporated into World Health Organization–endorsed tuberculosis diagnostics [7]. Their strengths include speed (typically 30–60 min), minimal equipment, and tolerance of crude samples, whereas their limitations include complex primer design, nonspecific amplification, and largely qualitative or semi-quantitative outputs. Reproducibility remains assay- and target-specific rather than governed by a unifying standard, and sensitivity claims must be interpreted in context; LAMP can outperform

conventional PCR for some targets yet underperform nested PCR for others [8]. RPA has since become a preferred pre-amplification step for CRISPR-based detection, illustrating how this approach continues to support newer technologies.

3. Reverse transcription and quantitative PCR

The quantification of specific transcripts was transformed by the advent of real-time PCR. Higuchi and colleagues first demonstrated that amplification could be monitored continuously by fluorescence [9]. Heid and colleagues then formalized real-time quantitative PCR through the cycle-threshold concept [10]. The comparative 2^(−ΔΔCt) method of Livak and Schmittgen later became the standard for relative quantification [11]. Reverse-transcription quantitative PCR (RT-qPCR) remains the most widely used targeted RNA method and the routine validation benchmark for higher-throughput technologies, owing to its sensitivity (down to ~10 copies), low cost, rapid turnaround, and ubiquitous instrumentation. Its central reproducibility challenges are well-recognized: results depend on reverse-transcription efficiency, primer design, and reference-gene normalization, and inter-laboratory comparability was historically poor until formal standards were introduced. The MIQE guidelines, and their subsequent revision as MIQE 2.0, defined the minimum information required for credible qPCR reporting [12,13] and remain the strongest determinant of whether qPCR results are reproducible.

Droplet digital PCR (ddPCR) represents the digital evolution of this methodological stream. By partitioning a reaction into ~20,000 nL droplets and scoring each as positive or negative, the system by Hindson and colleagues yields absolute molecule counts by Poisson statistics, eliminating the need for a standard curve [14]. In direct comparisons, ddPCR has shown markedly improved precision for low-abundance targets, including an approximately sevenfold improvement in day-to-day reproducibility for serum/plasma microRNA measurement [15]. Its limitations include a narrower upper dynamic range and lower throughput than qPCR, making it best suited for absolute quantification, rare targets, and inhibitor-rich samples.

4. DNA microarrays

While RT-qPCR established a sensitive and standardized framework for targeted transcript quantification, it remained inherently limited to low throughput. The emergence of DNA microarrays marked the field's first leap into genome-wide expression profiling, extending hybridization-based detection to thousands of transcripts simultaneously. DNA microarrays extended sequence-specific hybridization from single targets to the whole transcriptome. They are termed DNA microarrays because the capture probes immobilized on the array surface are DNA oligonucleotides, either in the form of complementary-DNA (cDNA) spots, as in the pioneering work by Schena and colleagues [16], or as high-density synthetic oligonucleotides, as developed by Lockhart and colleagues [17]. These spatially addressed DNA probes enabled thousands of transcripts to be measured in parallel by hybridization, transforming gene expression analysis into a genome-scale assay. Microarrays powered the first generation of transcriptome-wide studies and remain embedded in validated clinical signatures where fixed, well-characterized probe sets are advantageous.

Their limitations are equally well-defined: a closed design that restricts measurement to pre-selected probes, cross-hybridization between related sequences, and a compressed dynamic range. Reproducibility became a central focus of the landmark MicroArray Quality Control (MAQC) project, which demonstrated that both intra- and inter-platform measurements could be made reproducible with appropriate standardization [18]. This effort established the template for later sequencing–reproducibility consortia. Although progressively displaced by RNA sequencing for discovery applications, DNA microarrays retain a practical role wherever a stable, validated content panel is sufficient and where regulatory-grade reproducibility is required.

5. Single-molecule and multiplexed RNA imaging

As hybridization-based profiling expanded to the transcriptome through DNA microarrays, a parallel effort sought to recover the spatial information that array-based methods inherently lack. In situ hybridization achieves this by hybridizing RNA molecules in their native place within cells and tissues, preserving spatial context that solution-based methods cannot capture. Early implementations demonstrated that RNA could be visualized directly, but true single-molecule resolution emerged when Femino and colleagues first imaged individual transcripts in fixed cells [19]. Raj and colleagues then made single-molecule fluorescence in situ hybridization (smFISH) robust and quantitative by tiling each target with ~30 singly

labeled oligonucleotide probes, so that only their coincidence produced a detectable fluorescent spot [20]. smFISH provides absolute transcript counts per cell with subcellular resolution and very low false-positive rates, though at the cost of low gene throughput. However, this technique can only be used on unique sequences, limiting its usefulness for analyzing mature miRNA levels vs. pre-miRNA levels or tDRs vs. mature tRNAs. This requirement for long, probe-tillable regions also makes smFISH poorly suited for short mRNAs and many long noncoding RNAs that lack a sufficiently unique sequence for robust probe design.

Scaling this principle to the transcriptome required multiplexing. MERFISH (multiplexed error-robust fluorescence in situ hybridization) achieves this through error-robust combinatorial barcoding across sequential imaging rounds, enabling hundreds to thousands of genes to be profiled at single-molecule resolution [21], while seqFISH+ reaches a comparable scale using pseudocolor barcoding [22]. MERFISH reports detection efficiencies of ~80% with misidentification rates near 4% at the ~10,000-gene scale [23], and its measurements show strong concordance with bulk and single-cell RNA sequencing [24]. The dominant reproducibility challenge for multiplexed imaging lies in computation: image segmentation, spot calling, and barcode decoding lack consensus pipelines, so quantitative claims require in-study replicates and explicit reporting of detection efficiency and misidentification rates. In addition to these computational sources of variability, multiplexed imaging also faces intrinsic physical limitations, most notably optical crowding, where high local transcript density prevents individual molecules from being resolved even with perfect analysis.

6. RNAscope in situ hybridization

As single-molecule imaging matured, a major practical advance came from the commercialization of robust in situ hybridization chemistry. RNAscope, developed by Wang and colleagues and marketed by Advanced Cell Diagnostics (now part of Bio-Techne), addressed the long-standing problem of high background in tissue hybridization through a paired-probe design in which only correctly adjacent probes nucleate a branched amplification tree [25]. Because the target RNA is not enzymatically copied, the method achieves near-single-molecule sensitivity while preserving stoichiometry, and it performs reliably in formalin-fixed, paraffin-embedded tissue—an essential requirement for pathology. RNAscope has become a widely used method for tissue-level transcript localization in both research and diagnostic settings.

The platform's proprietary nature brings both strengths and constraints. Probe design, amplification chemistry, and detection reagents are centrally controlled, contributing to consistent wet-lab performance but limiting customization and multiplexing relative to methods such as MERFISH. BaseScope, a related assay within the same commercial suite, extends the approach to splice junction–specific and short-target detection, enabling discrimination of closely related isoforms and even single-exon or single-nucleotide differences [26]. As with other imaging-based methods, reproducibility ultimately depends on image quantification and segmentation, spot calling, and thresholding, which remains less standardized than the underlying chemistry. Transparent reporting of analysis parameters and controls is therefore essential for credible quantitative claims.

7. NanoString nCounter

While RNAscope provided a robust, commercial solution for spatial transcript detection in tissue, the need for equally standardized, amplification-free quantification in bulk samples led to the development of the NanoString nCounter platform. Developed by Geiss and colleagues and commercialized as a turnkey system, NanoString nCounter introduced amplification-free, digital, multiplexed RNA counting. Each transcript is captured by a probe pair and tagged with a color-coded molecular barcode that is imaged and counted directly, eliminating reverse transcription and enzymatic amplification from the workflow [27]. This design makes the assay notably robust to degraded and formalin-fixed material, which has become its principal practical strength, and the platform's commercial standardization ensures highly consistent wet-lab performance across laboratories.

Its constraints are equally characteristic: a fixed panel architecture (up to ~800 targets), no discovery capability, and reliance on a dedicated instrument. However, reproducibility is high in the sense most relevant to clinical deployment. The PAM50-based Prosigna assay, built on the nCounter platform, was analytically validated for formalin-fixed, paraffin-embedded breast tumor specimens and received regulatory clearance [28], making nCounter one of the few RNA detection technologies with formal, documented clinical-grade reproducibility.

8. Bulk RNA sequencing

While platforms such as NanoString provided standardized, amplification-free quantification for fixed panels, the need for open, discovery-scale measurements drove the field toward sequencing-based approaches. RNA sequencing replaced hybridization with direct, hypothesis-free counting of cDNA fragments. Foundational studies in mammalian cells by Mortazavi and colleagues [29] and in yeast by Nagalakshmi and colleagues [30] established that short-read sequencing could quantify transcripts genome-wide with a wide dynamic range while also detecting novel transcripts and splice junctions, capabilities summarized in early reviews [31]. Bulk RNA-seq rapidly became the default discovery method for transcriptome-wide expression profiling. Its limitations include adapter ligation, amplification and library preparation biases, batch effects, and the need for computational analysis, although user-friendly web-based pipelines have reduced the barrier to entry. Beyond these technical considerations, bulk measurements also average across all cells in a sample, obscuring cellular heterogeneity, failing to detect rare populations, and providing no spatial information.

Reproducibility has been examined more extensively for bulk RNA-seq than for any other RNA detection method. The SEQC/MAQC-III consortium demonstrated that relative expression measurements are robust across laboratories and platforms when appropriate filtering is applied, while noting that absolute and transcript-level quantification remain more variable [32]. External RNA spike-in controls provide an amplification-independent calibration anchor [33], and ENCODE established widely used data standards for sequencing depth and replicate concordance [34] [59]. Complementing these efforts, the Recount3 initiative has reprocessed tens of thousands of publicly available RNA-seq datasets through a uniform computational pipeline, providing harmonized gene- and exon-level quantifications that remove a major source of between-study variability [35]. The practical consequence is that bulk RNA-seq delivers reproducible differential-expression results when spike-ins, adequate depth, and standardized analysis pipelines—whether local or community-curated resources such as Recount3—are used.

9. Single-cell RNA sequencing

Although bulk RNA-seq transformed transcriptome-wide measurement, it necessarily averages across all cells in a sample, prompting the development of methods capable of resolving the cellular heterogeneity that bulk profiles obscure. Tang and colleagues reported the first single-cell transcriptome [36], and droplet-based methods—Drop-seq [37] and inDrop [38]—subsequently scaled the approach to tens of thousands of cells by barcoding individual cells before pooling. A key reproducibility innovation was the unique molecular identifier (UMI), which tags individual molecules prior to amplification so that PCR duplicates can be removed and counts corrected [39,40]; UMIs materially reduce amplification noise and have become foundational to modern single-cell protocols. In practice, platform choice is consequential, and it is best guided by published benchmarks rather than vendor claims: comparative studies have systematically evaluated single-cell methods for sensitivity, precision, and cost [41, 42].

The limitations of single-cell RNA-seq are well recognized: pronounced dropout of low-abundance transcripts, complex analysis workflows with no single canonical pipeline, and loss of spatial context. Unlike bulk RNA-seq, no MIQE- or SEQC-equivalent consortium standard governs single-cell measurement, so reproducibility relies on transparent reporting, technical replicates, and benchmark-guided platform selection. Despite these challenges, single-cell RNA-seq has become a central tool for dissecting cellular diversity, developmental trajectories, and regulatory states at a resolution unattainable by bulk methods. Despite these challenges, single-cell RNA-seq has become a central tool for dissecting cellular diversity, developmental trajectories, and regulatory states at a resolution unattainable by bulk methods. Its importance is reflected in the large number of specialized protocols that have diverged from the original implementations, optimized for high throughput, full-length coverage, perturbation screens, multi-omics integration, or ultra-low-input samples, underscoring how deeply embedded single-cell profiling has become across biological contexts.

10. Long-read and direct RNA sequencing

Both bulk and single-cell RNA-seq rely on short reads that reconstruct isoform structure only by inference. Long-read sequencing emerged to measure full-length transcripts directly. Early work using PacBio's single-molecule real-time (SMRT) sequencing demonstrated that circular consensus sequencing could generate high-fidelity, full-length cDNA reads, enabling accurate reconstruction of complete isoforms, and revealing transcript structures that short reads cannot unambiguously assemble. Sharon and colleagues provided one of the first demonstrations of single-molecule long-read sequencing of the

human transcriptome, resolving complete isoforms at scale [43]. Nanopore direct RNA sequencing, introduced by Garalde and colleagues, extended this principle by sequencing native RNA molecules without reverse transcription or amplification, thereby preserving isoform structure, poly(A)-tail length, and base modification–sensitive signal information within a single read [44]. This capability was applied at the transcriptome scale to the human poly(A) RNA population by Workman and colleagues [45].

Long-read and direct RNA sequencing provide the most straightforward route to address isoform-resolved and epitranscriptomic questions. Their limitations are well-recognized: lower per-base accuracy and throughput than short-read sequencing and, most importantly for reproducibility, a strong dependence on basecalling and modification-calling software versions. As these computational steps materially affect read identity, isoform assignment, and modification detection, software versions and model parameters should be explicitly recorded. As the field continues to mature, transparent reporting and version-controlled analysis remain essential for credible, reproducible long-read and direct RNA measurements.

11. Spatial transcriptomics and in situ sequencing

Although long-read and direct RNA sequencing resolved transcript structure at the molecule level, they did so at the cost of spatial context, motivating a complementary lineage of methods that restored positional information to sequencing. In situ sequencing, described by Ke and colleagues, read short transcript barcodes directly within fixed tissue by rolling-circle amplification of padlock probes [46]; STARmap extended this principle to three-dimensional tissue volumes through hydrogel embedding and in-tissue barcode sequencing [47]. Capture-based spatial transcriptomics took the opposite approach: instead of sequencing in place, transcripts were captured onto a spatially barcoded surface for ex situ sequencing. The original method by Ståhl and colleagues [48] was refined to near-cellular resolution by Slide-seq [49] and to sub-micron resolution by Stereo-seq [50], enabling progressively finer spatial mapping of transcriptomes.

In practice, capture-based methods provide unbiased, transcriptome-wide spatial discovery, whereas image-based methods offer targeted, single-molecule confirmation. The shared limitation is a fundamental trade-off between spatial resolution and capture efficiency or gene-panel size. The dominant reproducibility concern is computational: deconvolution of multi-cell capture spots and assignment of transcripts to individual cells rely on competing algorithms that do not yet converge on a consensus solution. As a result, spatial transcriptomics remains in the pre-standardization category, where transparent reporting of analysis parameters, benchmarking across tools, and in-study controls are essential for credible quantitative interpretation.

12. CRISPR-based RNA detection

Where spatial transcriptomics restored positional information to sequencing, a parallel development introduced an entirely new recognition chemistry for nucleic acid detection. Programmable CRISPR nucleases enabled sequence-specific detection through target-activated collateral cleavage. SHERLOCK, developed by Gootenberg and colleagues, coupled RPA pre-amplification to Cas13, whose activation triggers collateral cleavage of a quenched reporter to yield a fluorescent or lateral-flow signal with single-base specificity [51]; a multiplexed, quantitative version followed shortly thereafter [52]. DETECTR, by Chen and colleagues, applied the analogous collateral activity of Cas12a to DNA targets [53]. These assays are oriented toward rapid, low-equipment, point-of-care detection, with single-base discrimination particularly valuable for variant and allele identification.

Their limitations are characteristic of early-stage diagnostic platforms: dependence on pre-amplification for maximal sensitivity, which introduces contamination risk; largely qualitative or semi-quantitative output; and a still-developing evidence base for reproducibility. Validation has so far come from a limited number of laboratories, and no MIQE- or SEQC-equivalent consortium standard yet governs CRISPR-based detection. As a result, these methods are best treated as application-specific assays that require empirical validation, explicit reporting of amplification steps, and interpretation as qualitative or semi-quantitative readouts rather than absolute measurements.

13. Metabolic labeling to visualize nascent RNA

All steady-state RNA measurements share a fundamental limitation: they cannot distinguish synthesis from turnover. Metabolic labeling resolves this temporal dimension. Building on 4-thiouridine pulse-labeling and biochemical separation

of newly synthesized RNA [54], SLAM-seq introduces a nucleoside-recoding chemistry that converts incorporated 4-thiouridines into a characteristic T→C signature, allowing new and pre-existing transcripts to be distinguished directly from sequencing data at single-nucleotide resolution [55]. TimeLapse-seq achieved the same conceptual separation through an independent recording chemistry that marks newly synthesized RNA without requiring physical enrichment [56]. These approaches quantify synthesis and degradation rates and distinguish transcriptional from post-transcriptional regulation.
Their limitations are primarily practical: incompatibility with most primary tissues and the need to optimize labeling time and dose against cytotoxicity and metabolic perturbation. Reproducibility depends critically on transparent reporting of these labeling parameters, as they determine the effective temporal resolution and the fraction of labeled transcripts. As with several emerging methods in this review, metabolic-labeling approaches are not yet covered by a formal consortium standard, placing them in the pre-standardization category where careful optimization and explicit documentation are essential for credible interpretation.

Choosing a method: aligning technology with the biological question

The single most important determinant of a successful RNA measurement is not the method but the question it is meant to answer; the biological question should dictate the method, never the reverse. Before selecting a technology, several criteria must be made explicit. First, the nature of the target: whether the aim is to quantify a small number of known transcripts or to survey the transcriptome without prior hypotheses, a distinction that separates targeted assays from discovery platforms. Second, the type of quantification required, relative vs. absolute, since applications such as rare-variant or low-abundance detection benefit from the standard-curve-free absolute counting of droplet digital PCR, which has demonstrated superior precision over real-time PCR for such targets [15]. Third, the dimensions of information needed: bulk abundance, single-cell resolution, spatial location, or temporal dynamics, each of which sharply constrains the candidate methods. Fourth, the characteristics of the sample, including input amount and whether material is fresh, frozen, or formalin-fixed, as degraded or archival specimens favor amplification-free counting approaches validated for such material [28].
Fifth, and often overlooked, is the maturity of the method's reproducibility framework. Technologies governed by consortium standards such as MIQE for quantitative PCR [12] or the SEQC/MAQC-III assessment for RNA sequencing [32] offer measurable inter-laboratory comparability, whereas newer methods require study-specific controls. An additional molecular constraint is the epitranscriptomic modification status of the RNA itself, since heavily modified species such as tRNAs are often refractory to reverse transcription–based approaches and can therefore be systematically under-detected in amplification-dependent workflows. Finally, practical constraints such as cost, turnaround time, equipment availability, and analytical expertise should be used to narrow the final choice. Where platform choice is consequential, as in single-cell sequencing, selection should be guided by published head-to-head benchmarks rather than vendor claims [41,42]. The most robust experimental designs frequently combine methods, pairing a discovery platform with an orthogonal validation assay so that the strengths of one offset the limitations of another. This often requires collaboration between groups with complementary expertise, which is itself a strength: it brings together distinct methodological expertise and ensures that validation is performed independently of discovery. Defining these criteria in advance remains the most reliable route to a reproducible and interpretable result.

Conclusion

Three take-home messages emerge from a chronological, method-centric assessment of RNA detection technologies. First, RNA detection technologies form progressive trajectories of problem-solving rather than a succession of replacements. Real-time PCR added quantification to amplification, and digital PCR added absolute precision; microarrays generalized hybridization, and RNA-seq removed its closed-content limitation; smFISH made hybridization single-molecule, and MERFISH made it transcriptome-wide; bulk sequencing was refined into single-cell, then spatial, then native and time-resolved variants. Viewed in this way, RNA detection methods constitute a connected toolkit, with each method occupying a distinct position defined by the biological question it is designed to answer.
Second, reproducibility across this toolkit depends strongly on the availability of established standards, benchmarks, and reporting frameworks. Mature methods like RT-qPCR, ddPCR, microarrays, and bulk RNA-seq benefit from widely used guidelines and consortium-level assessments, including MIQE, dMIQE, MAQC, SEQC, ERCC spike-ins, and ENCODE standards. Newer methods, including single-cell, spatial, long-read, direct-RNA, CRISPR-based, and metabolic-labeling

approaches, are rapidly developing their own best practices but still require careful experiment-specific controls, transparent reporting, and benchmark-aware interpretation.

Third, the field has expanded from measuring whether RNA is present or abundant toward measuring where it is located, when it is produced, how it is processed, and how confidently it can be quantified. This shift has made computational analysis an increasingly central component of RNA detection. For many modern methods, reproducibility now depends not only on sample preparation and molecular chemistry but also on choices in alignment, segmentation, deconvolution, basecalling, transcript assignment, and statistical modeling. RNA detection is therefore no longer a single-purpose measurement problem but a multidimensional methodological landscape in which method choice must be matched to the biological question, resolution required, and available validation framework.

Future methodological directions are visible in the present trajectory. The most valuable near-term advances are unlikely to be entirely new detection chemistries. Instead, they are likely to come from the standardization of methods that currently lack it, consortium benchmarking for single-cell and spatial measurement, and consensus pipelines for image-based and native RNA analysis. A parallel priority is the integration of capabilities that are currently separate, such as spatially resolved measurement of RNA dynamics. Equally important will be the disciplined validation of frontier tools, including genetically encoded recording and computational prediction, against established experimental standards. The history of RNA detection technologies suggests that the methods that endure are those whose variability is best understood and best controlled. This is where methodological effort most meaningfully strengthens the reproducibility and robustness of a technique.

Usage of AI

The authors disclose that AI-assisted tools were used during the preparation of this review. Specifically, Claude Sonnet (claude-sonnet-4-6) and Claude Opus (claude-opus-4-8), developed by Anthropic, were used to assist with textual content generation, rephrasing, and the identification of potentially relevant literature. The structural framework, conceptual direction, interpretation, reasoning, selection of content, and development of the manuscript's arguments were determined by the authors. All AI-assisted outputs were rigorously reviewed, verified for accuracy, edited, and approved by the authors, who take full responsibility for the accuracy, reliability, and credibility of the final manuscript.

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