REVIEWS

Translating RNA sequencing into clinical diagnostics: opportunities and challenges

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Abstract | With the emergence of RNA sequencing (RNA-seq) technologies, RNA-based biomolecules hold expanded promise for their diagnostic, prognostic and therapeutic applicability in various diseases, including cancers and infectious diseases. Detection of gene fusions and differential expression of known disease-causing transcripts by RNA-seq represent some of the most immediate opportunities. However, it is the diversity of RNA species detected through RNA-seq that holds new promise for the multi-faceted clinical applicability of RNA-based measures, including the potential of extracellular RNAs as non-invasive diagnostic indicators of disease. Ongoing efforts towards the establishment of benchmark standards, assay optimization for clinical conditions and demonstration of assay reproducibility are required to expand the clinical utility of RNA-seq.

Next-generation sequencing
(NGS). High-throughput, massively parallel sequencing technology that is used in various applications, including whole-genome sequencing, exome sequencing and RNA sequencing.

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RNA is a dynamic and diverse biomolecule with an essential role in numerous biological processes. From a molecular diagnostic standpoint, RNA-based measurements have the potential for broad application across diverse areas of human health, including disease diagnosis, prognosis and therapeutic selection. Technological advancements have continually shaped the way that RNA-based measurements are used in the clinic (BOX 1). With the evolution of next-generation sequencing (NGS) technologies, the use of RNA sequencing (RNA-seq) to investigate the vast diversity of RNA species is an obvious and exciting application, which opens up entirely new opportunities for improving diagnosis and treatment of human disease. RNA-seq provides an in-depth view of the transcriptome, detecting novel RNA transcript variation[1]. Beyond operating as an open platform technology, RNA-seq has a number of potential advantages over gene expression microarrays, including an increased dynamic range of expression, measurement of focal changes (such as single nucleotide variants (SNVs), insertions and deletions), detection of different transcript isoforms, splice variants and chimeric gene fusions (including previously unidentified genes and/or transcripts), and, fundamentally, it can be performed on any species. Although RNA-seq assays are now commercially available[2,3], these early tests belie the considerable promise for broader applicability of RNA-seq-based clinical tests.

Here we review a selection of current and potential clinical applications of RNA-seq, focusing on differential expression, rare or fusion transcript detection and allele-specific expression, before discussing the emerging areas in pathogen detection and measurement of non-coding RNA (ncRNA) species. An overview of the clinically relevant RNA species discussed within the Review is summarized within FIG. 1, focusing on those RNA species that hold the greatest promise for directly impacting current and future clinical testing, with an understanding that the full diversity of RNA species and their putative roles are covered in other reviews[4,5]. In addition, we do not review NGS assays and technologies, as this topic is well reviewed and beyond our intended scope. We finish by discussing the challenges faced in translating this technology into clinical practice, including the regulatory environment and ongoing efforts to establish reference standards and the best practices for RNA-seq as a clinical test that is capable of high reproducibility, accuracy and precision.

Opportunities enabled by RNA-seq

As in whole-genome and whole-exome sequencing, RNA-seq involves sequencing samples with billions of bases across tens to hundreds of millions of paired or unpaired short-reads. This vast amount of short-read RNA-seq data must be bioinformatically realigned and assembled to detect and measure expression of hundreds of thousands of RNA transcripts. Not only can RNA-seq detect underlying genomic alterations at single nucleotide resolution within expressed regions of the genome, it can also quantify expression levels and capture variation not detected at the genomic level, including the

How technology has shaped the evolution of RNA as a clinical biomarker

Historically, gene expression analysis within the clinic has primarily focused around single gene tests using quantitative reverse transcription PCR (qRT-PCR)[131], such as in the detection of the influenza virus[132]. This method has several advantages, including being fast, accurate, sensitive, high-throughput in terms of the number of clinical samples that can be analysed, cost-effective and requiring low sample input. For these reasons and the historic nature of this platform, qRT-PCR is generally deemed the 'gold standard' method for measuring transcript levels, particularly in the clinical space; however, there are a number of limitations, including the fact that although it is a high sample throughput technology, relatively few markers or measurements can be made in a single assay. After the initial studies describing the relatively reproducible hybridization-based methods to assess the expression of multiple gene targets using arrayed probes on solid surfaces[133], it became clear that this microarray technological revolution would lead to new opportunities for clinical assay development. Measurement of several RNA targets at one time (as 'gene expression profiles') became associated with potential diagnostic or prognostic parameters in research. It was crucial to assess the clinical validity of these technologies for multi-gene profile tests. From a gene expression standpoint, MammaPrint (Agendia) provides an excellent example of a microarray-based clinical test that simultaneously measures the expression of 70 genes in breast tumours as a profile to help predict the risk of recurrence[134]. Although powerful, microarray-based assays can have limitations in some environments, such as those related to laboratory-to-laboratory variation in sample preparation that can affect reproducibility. Moreover, for some applications, microarray signal-to-noise ratios can affect the limit of detection. Interestingly, a number of additional cancer multi-gene profile tests are clinically available, such as OncoTypeDX (Genome Health)[135] for breast cancer recurrence risk and Prolaris (Myriad)[136] for prostate cancer aggressiveness. These tests are based on qRT-PCR technologies, rather than microarrays, largely owing to the belief that qRT-PCR is more reliable, reproducible, sensitive and accurate.

As we enter the era of next-generation sequencing (NGS) technologies, RNA sequencing (RNA-seq) can be brought to bear on clinical gene testing. RNA-seq-based tests can provide unprecedented flexibility, sensitivity and accuracy to gene expression measurements. Moreover, the diversity of RNA species opens up simultaneous measurements of rare transcripts, splice variants and non-coding RNA species. For example, the diverse reach of RNA species from RNA-seq is becoming increasingly relevant, particularly in cancer. In addition to providing direct detection of RNA from fused genes, RNA-seq detection of specific oncogenic splice variants, such as from EGFR[137] and androgen receptor[31], will probably have prognostic and therapeutic relevance. Indeed, whereas microarrays and qRT-PCR are a closed platform, with clearly defined transcript detection and measurement, RNA-seq is an open platform by nature. Likewise, the ability to identify novel transcripts may introduce clinical interpretation challenges, perhaps with analogous 'variants of unknown significance' terminology found in clinical genomic DNA sequencing. Still, and perhaps more than is often appreciated, establishment and standardization of methods for assessing reproducibility, accuracy and precision in a variety of clinically relevant conditions are needed to facilitate adoption of RNA-seq tests in the clinical laboratory setting.

differentially expressed genes, tens of thousands of differentially expressed gene isoforms and can detect mutations and germline variations for hundreds to thousands of expressed genetic variants (thus facilitating the assessment of allele-specific expression of these variants), as well as detecting chimeric gene fusions, transcript isoforms and splice variants[5,9]. In addition, RNA-seq can characterize previously unidentified transcripts and diverse types of ncRNAs, including microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs) and tRNAs[5]. Indeed, the open platform of RNA-seq for detecting and measuring temporally dynamic RNA species sets the stage for considerable challenges and even more considerable opportunities associated with RNA-seq moving into the clinical test environment.

Detecting aberrant transcription in human disease

mRNA expression profiling. Multigene mRNA signature-based assays are being increasingly incorporated into clinical management. These assays use various technology platforms to measure mRNA expression of different multigene panels and have broad clinical application (TABLE 1). For example, in breast cancer, recent clinical guidelines support the use of multigene mRNA-based prognostic assays to assist in treatment decisions in conjunction with clinicopathological factors[10,11]. Indeed, the OncotypeDx 21-gene expression assay was recently validated in a prospectively conducted study in breast cancer[12]. Clinically relevant breast cancer gene expression signatures were compared using microarrays and RNA-seq and reported strong correlation for expression of genes from the OncotypeDx and MammoPrint signatures across platforms (Spearman correlations of 0.965 and 0.97, respectively)[13]. In other work[14], systematic evaluation of RNA-seq-based and microarray-based classifiers found that RNA-seq outperformed arrays in characterizing the transcriptome of cancer and performed similarly to arrays in clinical endpoint prediction.

AlloMap is a non-invasive gene expression-based blood test that is used to manage the clinical care of heart transplant recipients, providing a quantified score for the risk of rejection based on the measurement of expression of 20 genes, a subset of which are related to immune system activation and signalling[15,16]. The potential for using RNA-seq in immune-related diseases is expanding rapidly, and the ability to quickly target and sequence the repertoire of T cell and B cell receptors from patients is beginning to mature, using techniques such as those from Adaptive Biotechnologies and ImmunoSeq. These strategies allow examination of immune-related diseases and immunotherapy response in new ways, as exemplified in a recent report in which RNA-seq and exome sequencing were used together to evaluate mutation load, expressed neoantigens and immune microenvironment expression as predictors of response to immune checkpoint inhibitor therapy in melanoma[17].

Gene fusions. Oncogenic gene fusions are well recognized for their pathogenic role in cancer. In some cases, recurrent gene fusions correlate with specific tumour subtypes, allowing gene fusion status to be used for

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Open platform
A technology platform that does not depend on genome annotation, or on predesigned species-specific or transcript-specific probes, for transcript measurement. RNA-seq technology functions as an open platform allowing for unbiased detection of both known and novel transcripts.

Single nucleotide variants
(SNVs). Single nucleotide (A, T, G or C) alterations in a DNA sequence.

expression of alternative transcripts[1,6]. Similar to serial analysis of gene expression (SAGE), a predecessor tag-based sequencing method for genome-wide expression analysis[7], RNA-seq allows quantification of transcripts without pre-defining the RNA targets of interest and provides improved detection of RNA splice events[1,6]. Unlike most historical platforms for clinical RNA measurement, such as microarrays and quantitative reverse transcription PCR (qRT-PCR), RNA-seq is fundamentally an open platform technology, allowing both quantification of known or pre-defined RNA species and the capability to detect and quantify rare and novel RNA transcript variants within a sample[1]. RNA-seq also has a greater dynamic range for quantifying transcript expression compared to microarray technology[8], providing the potential for increased detection of variation within a sample. Overall, RNA-seq can identify thousands of

diagnostic purposes. According to the 2008 WHO (World Health Organization) classification, diagnosis of acute myeloid leukaemia (AML) can be made regardless of blast count based on detection of recurrent genetic abnormalities, such as the t(8;21)(q22;q22) translocation, *RUNX1–RUNX1T1* fusion (involving isoforms of runt-related transcription factor 1 (*RUNX1*); this fusion is also known as *AML1–ETO*)[18]. Gene fusions have also been associated with prognosis and have been suggested as biomarkers for screening and assessment of cancer risk, as exemplified by the *TMPRSS2–ERG* fusion (involving transmembrane protease serine 2 gene (*TMPRSS2*) and v-ets avian erythroblastosis virus E26 oncogene homologue (*ERG*)) in prostate cancer[19].

Several US Food and Drug Administration (FDA)-approved targeted agents have clinical biomarkers amenable to RNA-seq, including agents with activity against known oncogenic fusions. The prototypical example is the marked efficacy of kinase inhibitors (for example, imatinib) in *BCR–ABL1*-positive (involving breakpoint cluster region (*BCR*) and tyrosine-protein kinase ABL1 (*ABL1*)) chronic myeloid leukaemia (CML)[20]. While karyotyping is typically used for diagnosis of CML, qRT-PCR measurement of *BCR–ABL1* transcripts is recommended to monitor the molecular response during kinase inhibitor treatment[21]. Continued advances in long-read RNA-seq technology promise to further expand the utility of fusion transcript measurements, allowing detection of mutation phasing by measuring across the full fusion transcript. Recently, single-molecule long-read RNA-seq was applied to longitudinal samples from patients with *BCR–ABL1*-positive CML with poor treatment response[22]. The results provided a clonal view of the range of resistance mutations, distinguishing between compound mutations in the same RNA molecule and independent alterations present on different molecules of the *BCR–ABL1* fusion transcript. The authors reported sensitive detection that resulted in the identification of several mutations that escaped detection by routine clinical analysis and, for one of the proof-of-concept cases, detected a known drug-resistant mutation in a longitudinal patient sample four months earlier than detected by Sanger sequencing[22].

Aside from monitoring *BCR–ABL1* fusion transcript levels during treatment, current clinical guidelines rarely recommend RNA-based detection of gene fusions. The *EML4–ALK* fusion (involving echinoderm microtubule associated protein like 4 (*EML4*) and anaplastic lymphoma receptor tyrosine kinase (*ALK*)) was originally reported in a subset of non-small-cell lung cancers (NSCLC) in 2007, and the ALK inhibitors crizotinib and ceritinib gained FDA approval in ALK-rearrangement-positive NSCLC in 2011 and 2015, respectively. *EML4–ALK* is typically detected by fluorescence *in situ* hybridization (FISH) using commercial break-apart probes that flank a highly conserved translocation breakpoint in the *ALK* genomic locus[23], with the emerging use of immunohistochemistry-based strategies to detect overexpression of ALK protein[24]. Recent clinical guidelines recommend against using qRT-PCR-based *ALK* fusion detection for treatment selection in lung

cancer owing to the challenges of RNA sample quality in routine formalin-fixed paraffin-embedded (FFPE) pathology samples, as well as the risk of false negatives resulting from limitations in detecting fusions involving novel ALK fusion partners[25].

In recent years, clinical detection of gene fusions has advanced beyond assays to detect individual fusion events to the introduction of RNA-seq assays, which allow a more comprehensive evaluation of potential gene fusions. For example, the FoundationOne Heme assay uses RNA-seq with genomic sequencing to detect common gene fusions in haematological cancers and sarcomas[2,3]. Reports are emerging of clinical responses by patients receiving treatment on the basis of gene fusion detection with this assay[2,3]. RNA-seq promises to expand the repertoire of detectable gene fusions, not only by capturing more subtle intrachromosomal rearrangements but also allowing detection of fusion products with uncharacterized fusion partners. Efforts are underway to catalogue gene fusions detected across various tumour types using RNA-seq data[26], although additional studies are needed to define the clinical value for identified fusions.

Alternative transcripts. Alternative transcript variants, arising from splicing alterations or structural variants, have been identified and implicated in a range of human diseases, including developmental disorders[27], neurodegenerative disorders[28,29] and cancers[30]. There is also growing evidence that the presence of alternative transcripts can have therapeutic implications. For example, expression of the alternatively spliced androgen receptor variant 7 (*AR-V7*) has been detected in the circulating tumour cells of ~30% of men with castration-resistant prostate cancer and is associated with reduced response to androgen receptor-directed therapies[31,32]. Similarly, expression of the tumour-specific epidermal growth factor receptor (*EGFR*) variant III *(EGFRvIII)* transcript is well described in glioblastoma, arising from an in-frame deletion encompassing exons 2–7 (REF. 33); clinical trials targeting *EGFRvIII* are underway[34]. In some cases, the mechanisms contributing to the generation of alternative transcripts may be missed by exome sequencing. Alternative breast cancer 1 (*BRCA1*) transcripts have been identified in a subset of patients with breast cancer who have a family history of breast and/or ovarian cancer[35]. Notably, these patients had previously tested negative for pathogenic *BRCA1* or *BRCA2* mutations by conventional genomic analysis[35]. It is anticipated that RNA-seq data will provide a more complete view of altered splicing and disease-specific transcripts, and that the growing body of transcriptome data will be a rich resource for discovery of disease-specific isoform transcripts as potential diagnostic markers and therapeutic targets.

Allele-specific expression. Allele-specific expression (ASE) can arise through multiple mechanisms, including genetic imprinting, X-chromosome inactivation and allele-specific transcription[36]; in some cases, ASE has been associated with predisposition to disease[37,38]. One

Reference standards
Highly characterized and standardized control materials that are used to ensure accuracy and comparability of assays.

Spearman correlation
Statistical measure of the strength of association between two rank-ordered variables.
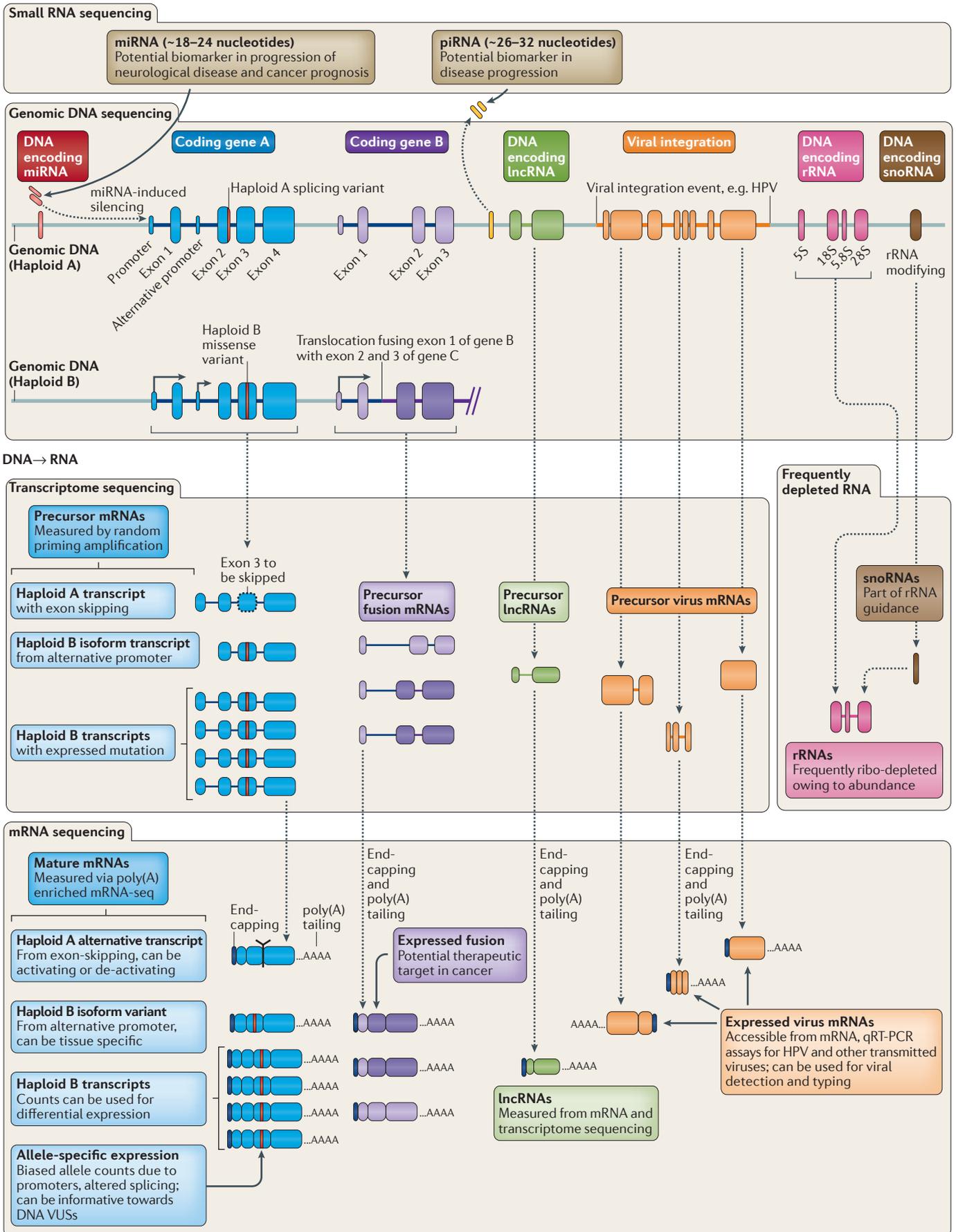
Phasing
Evaluation of closely situated mutations to determine whether they reside on the same or different alleles.

Break-apart probes
A DNA probe system used to detect rearrangements involving specific loci. Probes for a region 5′ of the designated breakpoint are labelled with one colour and probes for a region 3′ of the breakpoint are labelled with another colour. An overlapping signal (such as yellow for red and green probes) indicates a normal pattern, whereas distinct signals (that is, red and green) indicate the presence of a rearrangement.

Structural variants
Genomic variants, other than single-nucleotide variants, involving large regions of DNA, including insertions, deletions, inversions and duplications.

Small RNA sequencing

miRNA (~18–24 nucleotides)
Potential biomarker in progression of neurological disease and cancer prognosis

piRNA (~26–32 nucleotides)
Potential biomarker in disease progression

Genomic DNA sequencing

DNA encoding miRNA

Coding gene A

Coding gene B

DNA encoding lncRNA

Viral integration

DNA encoding rRNA

DNA encoding snoRNA

miRNA-induced silencing

Haploid A splicing variant

Viral integration event, e.g. HPV

Genomic DNA (Haploid A)

Promoter
Exon 1
Alternative promoter
Exon 2
Exon 3
Exon 4
Exon 1
Exon 2
Exon 3
5S
18S
5.8S
28S
rRNA modifying

Haploid B missense variant

Translocation fusing exon 1 of gene B with exon 2 and 3 of gene C

Genomic DNA (Haploid B)

DNA→ RNA

Transcriptome sequencing

Precursor mRNAs
Measured by random priming amplification

Exon 3 to be skipped

Precursor fusion mRNAs

Precursor lncRNAs

Precursor virus mRNAs

Frequently depleted RNA

snoRNAs
Part of rRNA guidance

Haploid A transcript
with exon skipping

Haploid B isoform transcript
from alternative promoter

Haploid B transcripts
with expressed mutation

rRNAs
Frequently ribo-depleted owing to abundance

mRNA sequencing

Mature mRNAs
Measured via poly(A) enriched mRNA-seq

End-capping and poly(A) tailing

End-capping and poly(A) tailing

End-capping and poly(A) tailing

End-capping
poly(A) tailing

Expressed fusion
Potential therapeutic target in cancer

Haploid A alternative transcript
From exon-skipping, can be activating or de-activating

...AAAA

...AAAA

...AAAA

...AAAA

Haploid B isoform variant
From alternative promoter, can be tissue specific

...AAAA

...AAAA

Expressed virus mRNAs
Accessible from mRNA, qRT-PCR assays for HPV and other transmitted viruses; can be used for viral detection and typing

Haploid B transcripts
Counts can be used for differential expression

...AAAA
...AAAA
...AAAA

...AAAA
...AAAA
...AAAA

AAAA...

Allele-specific expression
Biased allele counts due to promoters, altered splicing; can be informative towards DNA VUSs

lncRNAs
Measured from mRNA and transcriptome sequencing

of the most common mechanisms for ASE is genomic imprinting, whereby one allele is silenced through DNA methylation and histone modifications, leaving biased expression of the transcribed single nucleotide polymorphisms (SNPs) in a parent-of-origin specific manner. Imprinted gene clusters are frequently associated with human disease, as disease syndromes can arise from alterations on the single non-silenced, parental allele. For example, Angelman syndrome, a neurogenetic disorder associated with intellectual disability, speech impairment and a risk of seizures, is a well-studied imprinting disorder caused by deficient maternal allele expression of ubiquitin protein ligase E3A (UBE3A) in the brain[39]. In addition to epigenetic and transcriptional regulation, post-transcriptional mechanisms such as alternative splicing and protein-truncating mutations can also contribute to ASE[40]. For example, variants affecting splicing can cause exons to be skipped leading to ASE of variants contained within the exon[41]; likewise, a premature stop codon can lead to nonsense-mediated decay of one allele, resulting in ASE of the other[42,43].

Evaluating ASE within RNA-seq data can inform our understanding of regulatory variation and aid in the functional interpretation of genetic variants[44]. Initial applications of ASE-RNA-seq focused on genomic regions that contribute to variation in transcript expression levels, termed expression quantitative trait loci, looking at Nigerian individuals from the International HapMap project. RNA-seq of lymphoblastoid cell lines derived from these individuals, coupled with the corresponding genotypes from the HapMap project, resulted in the identification of over 1,000 genes for which genetic variation influenced transcript levels or splicing and showed high concordance between polymorphisms located near genes and ASE[45]. More recently, as part of the Genotype–Tissue Expression (GTEx) Program, RNA-seq is being carried out on samples from a broad range of tissues from hundreds of post-mortem donors to, in part, examine the influence of genetic variation on gene expression. By analysing ASE in the pilot GTEx data, the effects of truncating mutations on nonsense-mediated transcript decay were characterized, demonstrating the utility of using RNA-seq and ASE analysis to aid in the functional interpretation of genetic variants at the DNA level[43].

Given that some events may be difficult to detect or predict, ASE can be an important correlative biomarker towards identifying a pathogenically relevant genetic variant. Overexpression of a mutant allele may indicate

the presence of an otherwise unidentified promoter mutation or intronic variant impacting splicing. For example, ASE analysis of transforming growth factor beta receptor 1 (TGFBR1) has been observed and associated with an increased risk of colon cancer, even though the mechanism for ASE has not been identified[37]. Within our own work, we used RNA-seq and ASE analysis to characterize both the chromosomal parent-of-origin and the extent of X-inactivation in a female child with mild cognitive impairment[46]. By performing family-trio (child and both parents) whole-exome sequencing and RNA-seq, we defined a de novo heterozygous deletion encompassing 1.6 kb on chromosome X, which contained several genes associated with neurological dysfunction; using SNPs as phasing markers, we demonstrated that the focal deletion was present on the paternal allele. The RNA-seq data further provided the ability to use ASE analysis to estimate skewed X-chromosome inactivation, demonstrating favoured expression of the cytogenetically normal maternal allele, which we suggested contributes to the observed modest phenotype. Notably, RNA-seq provided a unique advantage over the traditional Humara assay, which measures DNA methylation of the androgen receptor gene on chromosome X, providing both a parent-of-origin and chromosome-wide view of X-inactivation[46].

Degner et al.[47] provided one of the first reports to ascertain RNA-seq read-mapping allele specificity, demonstrating a mapping bias for alleles with SNPs represented in the reference sequence, compared to that of the alternative allele, thus producing reference-biased ASE[45,47]. Filtering to remove biased sites resulted in an enrichment of SNPs with ASE in genes with previously reported cis-regulatory mechanisms or gene imprinting[47]. Measurement of ASE can also be confounded by difficulty aligning RNA split reads that harbour neighbouring SNPs and small indels, which can also lead to reference-biased ASE[42]. Recent reports provide recommendations for bioinformatic analysis and data processing to address these and other challenges and introduce tools for improved detection of ASE from RNA-seq data[42,48].

Extracellular RNAs. The investigation of extracellular RNAs (exRNAs) in biofluids to monitor disease is a rapidly growing area of diagnostic research. exRNAs are released from all cells in the body and are protected from degradation by carriage within extracellular vesicles or association with RNA-binding proteins (RBPs) and lipoproteins[49–52,160]. Measurement of exRNA is appealing as a non-invasive method for monitoring disease; as biofluids are more readily accessible than tissues, more frequent longitudinal sampling can occur. Transcripts from many tissue types, including neurons from the brain, have been shown to be accessible and detectable in plasma samples[53]. One obvious drawback is a lack of tissue specificity, as biofluids contain exRNAs and RBPs released from many tissue types. However, the size of the organ or tissue and proximity to the biofluid can influence the RNAs detected. For example, plasma samples have high levels of transcripts released from the

◀ Figure 1 | **Diversity of RNA species detection enabled by RNA sequencing applications.** Various RNA sequencing (RNA-seq) methodologies can be used to measure diverse, clinically relevant RNA species. Small RNA deep sequencing uses size selection to sequence various small non-coding RNAs, including microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs). Precursor RNAs can be measured using random primer amplification and oligo(dT) primers can be used to select polyadenylated transcripts. RNA-seq also allows for detection and measurement of alternative transcripts, chimeric gene fusion transcripts and viral RNA transcripts, as well as evaluation for allele-specific expression. HPV, human papillomavirus; lncRNA, long non-coding RNA; poly(A), polyadenylation; qRT-PCR, quantitative reverse transcription PCR; rRNA, ribosomal RNA; snoRNA, small nucleolar RNA; VUSs, variants of undetermined significance.

Table 1 | **Selected examples of current RNA-based clinical tests**

RNA biomolecule	Method	Examples	Use
Viral RNA	qRT-PCR	• Influenza virus[68] • Dengue virus[69] • HIV[70] • Ebola virus[71]	Viral detection and typing
mRNA	qRT-PCR	• AlloMap (CareDx; heart transplant)[15,16] • Cancer Type ID (BioTheranostics)[143]	Diagnosis
	Microarray	Afirma Thyroid Nodule Assessment (Veracyte)[116]	Diagnosis
	qRT-PCR	• OncotypeDx (Genome Health; breast, prostate and colon cancer)[144–147] • Breast Cancer Index (BioTheranostics)[148] • Prolaris (Myriad; prostate cancer)[136]	Prognosis
	Digital barcoded mRNA analysis	Prosigna Breast Cancer Prognostic Gene Signature (Nanostring)[149]	Prognosis
	Microarray	• MammaPrint (Agendia; breast cancer)[134] • ColoPrint (Agendia; colon cancer)[150] • Decipher (Genome Dx; prostate cancer)[151]	Prognosis
miRNA	Microarray	Cancer Origin (Rosetta Genomics)[152]	Diagnosis
Fusion transcript	qRT-PCR	AML (RUNX1–RUNX1T1)[18]	Diagnosis
	qRT-PCR	BCR–ABL1 (REF. 21)	Monitoring molecular response during therapy
	qRT-PCR (exosomal RNA)	ExoDx Lung (ALK) (Exosome Dx)[161]	Fusion detection
	RNA-seq	FoundationOne Heme[2,3]	Fusion detection

AML, acute myeloid leukaemia; BCR, breakpoint cluster region; miRNA, microRNA; qRT-PCR, quantitative reverse transcription PCR; RNA-seq, RNA sequencing; RUNX1, runt-related transcription factor 1; RUNX1T1, runt-related transcription factor 1 translocated to 1 (cyclin D related).

Clinical Laboratory Improvement Amendments (CLIA). All laboratory testing on humans in the United States is regulated by The Centers for Medicare and Medicaid Services through CLIA. The purpose is to ensure quality and uniformity of laboratory tests.

liver and heart, while saliva has abundant transcripts from salivary glands and the oesophagus (K.R.V.K-J., unpublished observations). More recently, researchers are addressing this challenge by testing methods to selectively pull down extracellular vesicles derived from specific tissues, such as by immunoprecipitation for specific membrane proteins (such as L1 cell adhesion molecule (L1CAM) for neuronally derived vesicles)[54,55]. Use of exRNAs for cancer detection has parallels to extracellular DNA in that mutations can be detected and measured in RNA transcripts, provided that the mutations are transcribed. Potential advantages of exRNAs are that there are many more copies of the RNA than the DNA (making assessment potentially more sensitive) and differences in expression level can indicate that an organ or tissue is injured or diseased, in a way that cannot be described by DNA measurements. The catalogue of exRNA contains a large number of mRNAs and a range of regulatory RNAs that can be thoroughly evaluated by RNA-seq. There is growing interest in using this non-invasive analysis of exRNAs to

monitor disease, using changes in exRNAs as readouts for key disease pathways and indicators of therapeutic efficacy. Companies such as Exosome Diagnostics are developing exRNA-based Clinical Laboratory Improvement Amendments (CLIA) diagnostic tests to monitor key gene fusions (EML4–ALK) and mutations (EGFR T790M) from plasma samples[56]. ExoDx Lung(ALK) is the first such test, measuring EML4–ALK transcripts isolated from exosomes in plasma from patients with NSCLC. Notably, several groups have also used circulating RNA information to provide feedback about fetal health[57].

The US National Center for Advancing Translational Sciences (NCATS) has recently launched the Extracellular RNA Communication Consortium[58] to develop the use of exRNA as a diagnostic tool[59]. They have funded several groups to help develop a catalogue of exRNAs in healthy individuals and in a number of diseases[60]. With increasing support for exRNA research, there should be substantial gains in understanding how to best examine these biomolecules and overcome variability in detection.

Non-coding RNA species. Beyond mRNA quantification and detection of alternative transcripts, RNA-seq opens up possibilities to measure a considerable diversity of RNA species including long non-coding RNAs (lncRNAs) and various short RNA species including miRNAs and piRNAs (TABLE 2). Owing to their stability and regulatory role in health and disease, miRNAs have been extensively examined as potential diagnostic markers of disease. Currently, small RNA-seq of miRNAs and other targeted miRNA-array platforms have fallen short for reliable cross-platform accuracy[61,62]. A considerable obstacle to using small RNA-seq is the low level of validation observed across PCR and sequencing platforms. The US National Institute of Standards and Technology (NIST) has begun development of small RNA controls; external RNA controls to support the validation of assay results and improve platforms are a necessary next step for the utility of small RNA-seq and are discussed further in later sections.

Of the small regulatory RNAs, miRNAs are the best studied and have an updated, well-curated repository for sequence information: miRBase[63]. With increasing accessibility and popularity of small RNA-seq, there is growing interest in using this technology in other categories of regulatory RNA. However, correct alignment and categorization is hampered by the state of the small RNA databases. Some small RNA databases are well-maintained and curated (such as the Genomic tRNA Database[64]), whereas other databases maintain sequences that are predicted but not experimentally validated. There is also substantial sequence overlap between categories of RNA, such as piRNA, tRNA and rRNA, making downstream data analysis challenging.

There are new types of regulatory RNAs for which the diagnostic potential is unknown. Circular RNAs (circRNAs) were recently rediscovered in RNA-seq experiments searching for chromosomal rearrangements in cancer[65]. Although many groups are identifying new circRNAs and their potential functional roles

in the cell, there are few reports of function in disease pathogenesis. However, circRNAs have been found in high abundance in biofluids and tissues and have been found to be more stable than mRNAs, increasing their potential for diagnostic purposes[66]. Other regulatory RNAs have had new roles identified. For example, a role for tRNAs has been reported in cancer, whereby cleavage of tRNAs produced fragments that could displace the RNA-binding protein Y-box binding protein 1 (YBX1) from oncogenic transcripts, altering stability and suppressing breast cancer growth, invasion and metastasis[67]. A current challenge is associating new RNA discoveries, newly identified sequence information and the emerging roles for regulatory RNAs that challenge dogma with disease and diagnosis.

RNA-seq for infectious disease diagnosis

RNA-based pathogen diagnostics. Given the large number of clinically important RNA viruses (HIV, the Ebola, West Nile, dengue, hepatitis A, hepatitis D, hepatitis E, coxsackie and influenza viruses, and the severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) coronaviruses), qRT-PCR assays have been developed and are commonly used in the clinic for viral detection and typing[68–71]. It is likely that many of these targets will be translated into RNA-based sequencing assays in the near future. For example, unbiased non-targeted metagenomic RNA-seq has recently been used to directly detect influenza virus RNA in respiratory fluids, with additional viral pathogens detected in a subset of cases[72]. In a public health context, RNA-seq was used to track the origin and transmission patterns of the Ebola virus during the 2014 outbreak in West Africa[73]. RNA-based amplicon sequencing is also being explored for viral quasi-species (that is, mixed allele population) assessment for hepatitis C virus (HCV) and HIV; such analyses are necessary in the clinic to determine the presence and relative quantity of drug-resistance mutations for patient therapy, which can occur as minor components in a larger viral population[74,75]. However, clinical application of RNA-based diagnostics for infectious disease is still rare beyond the qRT-PCR assays for viral pathogens.

Microbial exogenous small RNA. A tremendous diversity of exogenous RNAs from non-human sources has been seen in human plasma, which indicates there is a relationship between the host and the microbiome, food sources and/or the environment[76,77]. The sources and importance of these microbial exogenous RNAs — which may or may not be encapsulated in outer-membrane vesicles[78] — are still being explored, particularly in the context of infection[159]. However, they hold a great deal of promise for new diagnostic targets. Extensive analysis

Table 2 | Regulatory non-coding RNA species

RNA species	Description	Potential clinical application
miRNA	miRNAs are ~18–24 nucleotides in length and represent the most extensively characterized group of small ncRNAs having activity in gene repression.	miRNAs are being pursued as potential biomarkers in a broad spectrum of diseases, from cancer to Alzheimer disease to cardiovascular disease. A microarray-based miRNA test is currently available for use in characterizing cancer origin[152].
piRNA	piRNAs are ~26–32 nucleotides in length, with functions in transposon repression and maintenance of germline genome integrity.	piRNAs have been implicated in cancer, with an initial study demonstrating an association between increased expression of piRNA and poor prognosis in soft-tissue sarcomas[153].
snRNA	snRNAs are ~100–300 nucleotides in length, localized to the nucleus, with functions in RNA processing and splicing.	Circulating levels of U2 snRNA fragments (RNU2-1f) have been proposed as potential diagnostic biomarkers in various tumour types, including pancreatic cancer and colorectal cancer[154].
snoRNA	snoRNAs have two main classes, box C/D snoRNAs, ~60–90 nucleotides in length, and box H/ACA snoRNAs, ~120–140 nucleotides. snoRNAs play a key role in ribosome biogenesis and rRNA modifications.	Levels of snoRNA and/or their functional fragments have been proposed as potential clinical diagnostic measures, with applications being pursued in fields such as cancer and neurodegenerative disorders. Two snoRNAs were recently identified in sputum samples and shown to have potential use as diagnostic biomarkers in lung cancer[155].
lncRNA	lncRNAs represent the category of ncRNAs that are greater than 200 nucleotides in length and function to regulate gene expression.	lncRNAs have been associated with cancer prognosis, with potential utility as biomarkers in cancer. Tests such as ExoIntelliScore Prostate include lncRNA as a biomarker[156].
circRNA	circRNAs are lncRNAs that contain a covalent bond between the 5′ and 3′ end, resulting in a continuous circular loop. circRNAs can act as miRNA sponges and regulators of splicing and transcription.	Although little is known about the association of circRNAs with disease, initial studies are exploring circRNA levels as potential biomarkers in cancer; a recent study showed an association between reduced levels of a specific circRNA (hsa_circ_002059) in gastric tumours compared to adjacent non-tumour tissue[157].
tRNA	tRNAs help with translation of mRNA to protein. tRNAs are highly structured and have many modifications to bases, making them difficult to sequence through.	Recent evidence suggests that tRNA fragments are cleaved in the presence of hypoxic or other stressful conditions. They can, in some cases, act as decoys for RNA binding proteins, causing destabilization of other transcripts[158].

circRNA, circular RNA; lncRNA; long non-coding RNA; miRNA, microRNA; piRNA, PIWI-interacting RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; tRNA, transfer RNA.

(*in vitro*, *ex vivo* and *in vivo*) and cataloguing of small RNAs produced by pathogens has been under way for several years and will provide a comprehensive reference database of exogenous RNA signals that may be useful for future clinical infection studies[79–81]. For example, *ex vivo* studies on *Neisseria meningitidis* infections in human blood have yielded dozens of small RNAs that seem to be associated with bacteraemic infections[82]. Similarly, multiple studies of the *Mycobacterium tuberculosis* microRNAome have yielded numerous biomarkers that are currently being explored for diagnostic purposes[83] and even for phenotype–genotype predictive diagnostics (such as for identifying the presence of multi-drug resistance)[84].

Pathogen mRNA. Measurement of microbial mRNA may be a useful marker of infection, as expression may improve detection in cases of low-level infections (for example, bacteraemia and cerebrospinal fluid infections) and could act as a better predictor of disease compared to direct genomic detection. For example, the simple detection of human papilloma virus (HPV) DNA is not sufficient to diagnose HPV-related squamous cell carcinoma (HPV DNA is detectable in ~14% of healthy control women[85]); thus, RNA-based diagnostics to detect HPV have been developed. HPV early oncoprotein *E6/E7* mRNA detection, as a surrogate for active infection, may provide a better predictive value for cervical cancer.

Host RNA. Host response, in the form of mRNA signatures, is also likely to become useful for monitoring specific infections. For example, upregulation of specific host immune factors (interferon beta 1 (*IFNB1*) interferon lambda 2 (*IFNL2*) and interferon lambda 3 (*IFNL3*)) was recently demonstrated for genotype 3 HCV infection, which is associated with accelerated liver fibrosis and is an independent risk factor for hepatocellular carcinoma, compared to non-genotype 3 HCV infection[86,87]. Host microRNA–gene interactions during the infection response are also proving to be a fruitful source of potential diagnostic biomarkers for specific infections, as well as distinguishing between active and latent infections; for example, the use of such markers to discriminate between latent infection and active disease with *M. tuberculosis*[88]. As with the other diagnostic uses of small RNA biomarkers described in this Review, a number of hurdles exist for the development and validation of infection assays, including sensitivity, specificity and, perhaps more difficult to overcome, the normalization of small RNA in clinical samples, which will vary between conditions, tissues and individual hosts and is an active area of study[89]. However, the application of RNA-seq provides a useful orthogonal approach to genomics-based diagnostics for clinical microbiology.

Challenges moving RNA-seq to the clinic

Translating an assay to the clinic. Translation and broader adoption of a laboratory test into the clinic involves evaluation and demonstration of analytical validity, clinical validity and, eventually, clinical utility[90] (FIG. 2). Analytical validity generally refers to the ability of the test to measure the intended biomolecules within clinically relevant conditions. Establishment of an analytically valid test can have different meanings depending on the regulatory framework that the test falls under, as discussed below. However, analytical validity generally implies that the test has undergone thorough technical performance characterization. Clinical validity refers to the ability of a test to predict a clinical outcome given a set of events, irrespective of whether the test results can enable an effective therapy. Clinical utility indicates whether a test provides useful information, positive or negative, for the patient being tested. Tests that can either indicate a more effective therapy, such as a companion diagnostic, or provide information on avoiding some therapies may both have clinical utility.

Performance metrics and reference standards. To be analytically valid, a laboratory test must deliver accurate information with reproducible and robust performance. Accuracy is determined by evaluating a measured or calculated value compared to a reference 'gold standard', with evaluation of sensitivity (ability to detect true positives) and specificity (ability to detect true negatives). The test must also provide the same or similar results with repeat testing (reproducibility) and withstand small, deliberate changes in pre-analytic or analytic variables associated with testing (robustness).

Establishing reference standards and the best practices for measuring RNA-seq accuracy, reproducibility and robustness has initially been ad hoc with individual groups providing the initial steps. In 2008, Marioni and colleagues[8] provided some of the earliest technical assessments of reproducibility for measuring gene expression levels by RNA-seq, reporting high reproducibility across technical replicates for a single RNA sample. Of the genes denoted significantly differentially expressed by microarray analysis, 81% were also differentially expressed with RNA-seq, with fold-change correlations between the two technologies similar or better than those reported in comparisons of different microarray platforms[8]. RNA-seq detected ~30% more differentially expressed genes than microarray analysis, with qRT-PCR confirmation for a subset, suggesting a large proportion may represent true positives and this difference may be a result of the broader dynamic range of RNA-seq and/or the ability to resolve splicing changes[8]. In 2013, the Genetic European Variation in Disease (GEUVADIS) consortium demonstrated the feasibility and reproducibility of performing RNA-seq across multiple laboratories, sequencing lymphoblastoid cell lines for 465 individuals, in seven sequencing centres using a single platform[91]. On the basis of this study, the consortium proposed a set of quality checks to assess technical biases in RNA-seq data, including differences in GC content, fragment size, transcript length and the percentage of reads mapped to annotated exons[91].

Although providing necessary guidance on RNA-seq assay metrics, early assessments often used collection methods that may not reflect the conditions observed

Companion diagnostic
In vitro diagnostic tests that provide information critical for the safe and effective use of a corresponding therapeutic agent. These tests are used to select patients for treatment with specific agents, including identifying patient populations with predicted efficacy as well as those that should not receive the agent due to a low likelihood of effectiveness or possible serious adverse events from the therapy.

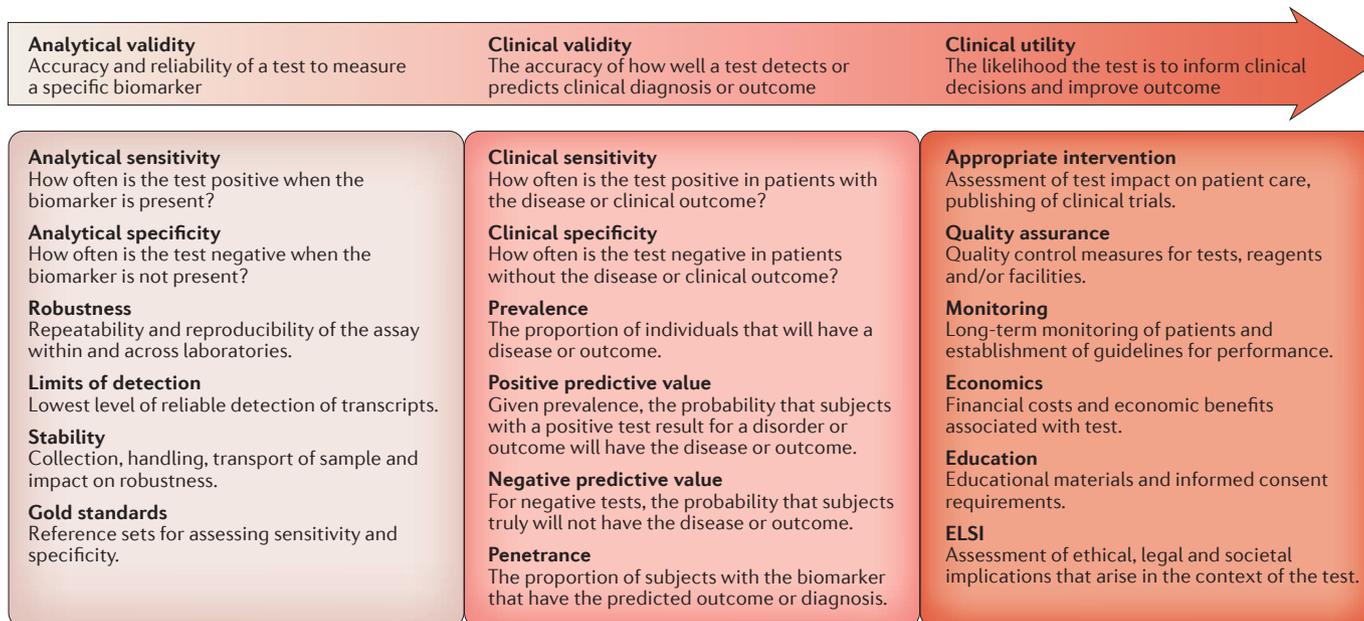| Analytical validity Accuracy and reliability of a test to measure a specific biomarker	Clinical validity The accuracy of how well a test detects or predicts clinical diagnosis or outcome	Clinical utility The likelihood the test is to inform clinical decisions and improve outcome
Analytical sensitivity How often is the test positive when the biomarker is present? **Analytical specificity** How often is the test negative when the biomarker is not present? **Robustness** Repeatability and reproducibility of the assay within and across laboratories. **Limits of detection** Lowest level of reliable detection of transcripts. **Stability** Collection, handling, transport of sample and impact on robustness. **Gold standards** Reference sets for assessing sensitivity and specificity.	**Clinical sensitivity** How often is the test positive in patients with the disease or clinical outcome? **Clinical specificity** How often is the test negative in patients without the disease or clinical outcome? **Prevalence** The proportion of individuals that will have a disease or outcome. **Positive predictive value** Given prevalence, the probability that subjects with a positive test result for a disorder or outcome will have the disease or outcome. **Negative predictive value** For negative tests, the probability that subjects truly will not have the disease or outcome. **Penetrance** The proportion of subjects with the biomarker that have the predicted outcome or diagnosis.	**Appropriate intervention** Assessment of test impact on patient care, publishing of clinical trials. **Quality assurance** Quality control measures for tests, reagents and/or facilities. **Monitoring** Long-term monitoring of patients and establishment of guidelines for performance. **Economics** Financial costs and economic benefits associated with test. **Education** Educational materials and informed consent requirements. **ELSI** Assessment of ethical, legal and societal implications that arise in the context of the test.

Figure 2 | **Criteria for clinical test development and adoption.** Before initial clinical introduction, a clinical test must demonstrate analytical validity, showing sufficient assay performance to produce accurate and reproducible technical results. Demonstration of analytical validity involves several measures, including sensitivity (true technical positives), specificity (true technical negatives), robustness and limits of detection. Clinical validity follows analytical validity and, depending on the approval path, demonstration of clinical validity can come before (US Food and Drug Administration (FDA) *in vitro* diagnostic device) or after (Clinical Laboratory Improvement Amendments (CLIA) laboratory-developed test) test clearance or approval. Clinical validity refers to the concordance between the test result and the clinical diagnosis or outcome and involves measures of sensitivity (true clinical positives) and specificity (true clinical negatives), as well as determination of positive and negative predictive values. Demonstration of both analytical validity and clinical validity occurs before that of clinical utility. Clinical utility requires clinical evidence that use of the test has an impact on patient care and includes evaluation of patient outcomes and the economic benefits associated with the test. ELSI, National Human Genome Research Institute's Ethical, Legal and Social Implications Research Program.

within the clinic. For example, within oncology, most studies of transcriptome analysis by RNA-seq come from fresh-frozen tumour samples that are stringently collected in terms of cellularity, tumour necrosis and RNA quality, whereas most pathological samples are collected through formalin fixation to preserve the protein and cellular structure. As RNA-seq libraries are typically prepared from total RNA using polyadenylation (poly(A)) enrichment of mRNAs, this method does not adequately capture partially degraded mRNAs, as are found in FFPE samples. Given the clear challenge of low-quality RNA from clinical FFPE samples, concerted effort has focused on optimization and evaluation of protocol modifications, including rRNA depletion (ribo-depletion) protocols to remove rRNA without poly(A) enrichment[92] and the use of capture sequencing, such as with oligonucleotide probe hybridization[93]. The utility of cDNA-Capture sequencing (exome capture and RNA-seq) was demonstrated for differential gene expression analysis from FFPE samples[94]. In addition to differential expression, this capture protocol was recently applied in the Michigan Oncology Sequencing Center (MI-ONCOSEQ) clinical sequencing programme, which demonstrated that the use of capture libraries improved performance for fusion and splice junction detection, compared to typical poly(A)-enriched RNA-seq using low-quality RNA (FFPE) samples[95]. Analytical

tools are also being developed and evaluated for their effect on test performance. In a comparison study of several common software tools (Cufflinks-Cuffdiff2, DESeq and edgeR) used to analyse differential expression by RNA-seq, using qRT-PCR and microarray results as benchmark standards, Zhang and colleagues[96] found that each tool had different strengths and recommended an ensemble-based approach combining two or more tools to reduce false-positives.

Although these early efforts advanced the field and indeed proposed reference standards and best practices, the most substantial large-scale efforts have mainly emerged in the past few years and include consortium members spanning public regulatory bodies (such as the FDA, NIST and the Centers for Disease Control and Prevention), academic groups and industry. One of the first sets of reference standards for RNA-seq was the development of synthetic RNA spike-in controls by the External RNA Controls Consortium (ERCC)[97]. The ERCC RNA spike-in controls contain 92 polyadenylated transcripts pre-formulated into two sets (Mix1 and Mix2), each with the full complement of transcripts spanning approximately 10^6-fold concentration range, present at defined Mix1/Mix2 molar ratios in four subgroups. These stock solutions can be diluted and added to each RNA sample, thus providing a post hoc measurement of assay performance from a set

of synthetic transcripts[98]. Synthetic gene-fusion spike-ins, composed of polyadenylated RNA transcripts corresponding to known oncogenic gene fusions, are also available with corresponding RNA-seq data[99]. In late 2014, the Sequencing Quality Control (SEQC) project (Phase III of the Microarray Quality Control (MAQC) Consortium)[100] and the Association of Biomolecular Resource Facilities (ABRF)[101] independently reported on large-scale efforts to define the performance characteristics of RNA-seq. Both studies represented remarkable coordinated analysis of inter-sample, cross-platform and inter-site variability for RNA-seq, compared to gold-standard methods such as microarrays and qRT-PCR, with extensive use of ERCC RNA spike-in controls. Both studies extensively examined how assay differences such as poly(A)-enrichment selection or random priming plus ribo-deletion affect reproducibility. Specifically, the FDA-led SEQC study examined the reproducibility and accuracy of RNA-seq in a multi-site study using different sequencing platforms. The SEQC study found that the correlation of relative gene expression measurements between different RNA-seq platforms (SOLiD, Life Technologies; HiSeq 2000, Illumina) and Affymetrix HuGene U133 Plus 2.0 microarrays with TaqMan qRT-PCR was high (Spearman and Pearson correlation coefficients of >0.9), although none of the platforms provided accurate absolute quantification of transcript levels, based on evaluation of ERCC spike-in control titration values[98]. Sensitivity, specificity and reproducibility of differential expression calls across sites were dependent on the analysis pipeline and the use of filters. Applying filters for P value, fold-change and expression level improved the false discovery rate for differential gene expression analysis, with most pipelines showing high reproducibility for differential expression calls across sites and greatest concordance for the most highly expressed genes. Consistent with previous reports, the SEQC study found that RNA-seq was seen as fundamentally superior at detecting novel transcripts, such as those resulting from alternative splicing, validating over 80% by qRT-PCR[100]. The ABRF study examined intra- and inter-laboratory reproducibility at 15 different laboratories, comparing different library preparation methods, sample variables (RNA integrity, size-specific fractionation), analysis algorithms and sequencing platforms. The ABRF study reported an overall high concordance for normalized gene expression measures within platforms (Spearman correlation >0.86) and between platforms (Spearman correlation >0.83). Deficiencies in cross-platform detection were identified and associated with read-length, analysis approaches and technology differences[101]. Similar to previous reports, library preparation methods influenced transcript enrichment, with poly(A) libraries containing more exonic reads and ribo-depletion libraries containing more intronic reads. Notably, although differences were observed, differential gene expression results were comparable between poly(A) enrichment and ribo-depletion library preparation methods, as well as between degraded and non-degraded RNA samples[101].

The importance of these studies goes well beyond the initial reporting, as companion papers helped to frame the specific areas where analytical validity can be substantially improved. Particularly relevant to clinical specimens, a concordance between RNA-seq and microarray was reported for 27 different chemical treatments, showing that differentially expressed genes correlated with the effect size of the treatment given and, whereas both platforms showed high concordance with qRT-PCR data for highly expressed genes, RNA-seq showed higher concordance than microarrays for genes with low expression[102]. Furthermore, multiple groups have examined the role of normalization methods towards the controlling biases resulting from GC content, sequencing coverage and insert size[103,104].

Analysis paralysis and other bioinformatic challenges. The SEQC–MAQC[100] and ABRF[101] consortium papers identified numerous substantial bioinformatic challenges that must be addressed for RNA-seq to become broadly adopted into clinical laboratories. Recognizing that excellent bioinformatics reviews are available elsewhere[105], here we attempt only to highlight the major overarching bioinformatics challenges. We find three large themes that frequently contribute to 'analysis paralysis' during the development of bioinformatics solutions to RNA-seq: first is the lack of consensus by governing bodies advocating best practices and reference standards for validating RNA-seq pipelines; second is the overabundance of software tools, options and combinations thereof for RNA-seq analysis; and third are highly complex pipelines consisting of chaining together multiple tools that are independently developed, maintained and licensed.

First and probably most relevant, RNA-seq analysis has largely grown organically without extensive standards or dominating governing bodies. By comparison, standards were established early on for DNA-based NGS by the 1,000 Genomes Project, including variant call format (VCF), binary alignment/map format (BAM format) and genotype likelihoods, essentially providing the 'best practice approaches' (REFS 106–111). Before 2014, reference standards, ERCC spike-in controls and the general MAQC were major contributors towards building a reproducible RNA-seq pipeline, but they pale in comparison to having a consensus germline-genomic reference standard, such as the NA12878 human reference genome[112]. As a consequence of this lack of standards, RNA-seq provided fertile grounds for the emergence of a multitude of software tools and other options, all competing for relevance as part of a broad range of RNA-seq solutions. RNA-seq pipelines represent the laboratory-specific wrapper scripts, chaining together collections of software tools with the goal of reporting hundreds to thousands of test results from gigabases of data. Unfortunately, having RNA-seq pipelines composed of several independently developed components, each with continual versioning and variable licenses, can be challenging in a clinical testing laboratory. Although excellent for agile software development in a non-regulated and rapidly changing field,

Variant call format
Standard text file format for storing genomic sequence variant data, with each line of the file describing a variant present at a specific genomic region or position.

Binary alignment/map format
(BAM format). Standard file format for storing sequencing reads with alignments. BAM files are binary representations of the sequence alignment/map (SAM) format.

this type of fertile environment creates major challenges in a clinical laboratory. For example, whereas genome builds change every few years, transcript definitions often change quarterly.

Although the fundamental challenges of bioinformatics are unlikely to be easily solved, the framework for how they are managed has improved considerably. With the ERCC[97], SEQC–MAQC[100] and ABRF[101] consortia providing initial models and reference standards, the emergence of 'bake-offs' and 'best practices' will be an essential next step in reducing some of the bioinformatics challenges of clinical RNA-seq. Moreover, the emergence of tools that allow containerization, such as Docker[113], provide a platform for distributing fully contained pipelines such that a pipeline run in one facility could be reproducibly deployed in another laboratory. Some full-packaged pipelines have recently emerged with the expressed intent of being relevant for clinical applications[114,115].

Regulatory considerations. Deployment of a clinical assay in the United States involves two paths, each with accompanying regulations administered under the US Department of Health and Human Services (HHS). The first are those approved through the CLIA of 1988 that allow 'laboratory-developed tests' (LDTs). LDTs are *in vitro* diagnostic tests that are developed and used within a single approved laboratory and are not marketed towards any other laboratory. CLIA regulations monitor the laboratory process to ensure the accuracy, reliability and appropriateness of laboratory testing, from sample acquisition, handling and storage to the interpretation and reporting of test results. The guidelines for approving CLIA laboratories are established by accredited professional organizations, such as the College of American Pathologists or by other agencies approved by the Centers for Medicare and Medicaid Services (CMS), such as in the state of New York. CLIA regulations of LDTs do not address the clinical validity or clinical utility of an assay, but instead provide a framework whereby clinical laboratories validate analytical performance measures of the LDTs within their own laboratory facility. The second set of regulations for clinical assay deployment are the Medical Device Amendments of 1976, which expanded FDA oversight for the marketing of *in vitro* diagnostic devices (IVDs). FDA premarket review of IVDs assures the assay has established analytical and clinical validity; with the exception of companion diagnostics, the FDA does not typically require demonstration of clinical utility for clearance or approval of IVDs. Demonstration of clinical validity (for LDTs) and clinical utility (for LDTs and IVDs) can follow the initial clearance or approval of a diagnostic test; clinical utility, in particular, requires broader clinical evaluation across multiple sites and/or within clinical trials. For example, the Afirma (Veracyte) microarray-based gene expression classifier for thyroid nodule assessment was launched in 2011 as a CLIA-regulated LDT. Subsequent studies have reported on clinical validity[116] and clinical utility[117], the latter involving a multi-site study that

demonstrated the effects of the Afirma test on clinical care recommendations, which resulted in a reduction of unnecessary surgeries[117].

In the US, the distinction over when NGS assays are under regulatory oversight by the FDA or the CMS is emerging as an area of regulatory and legislative debate. In late 2014, the FDA proposed a regulatory framework for LDTs[118,119] that will, in all likelihood, alter the regulatory landscape discussion for RNA-seq assays moving to the clinic. The FDA also provided a perspective on the mammoth shifts created by technological advances associated with NGS, and the requirement for the agency to change from the current 'general enforcement discretion' — in which the FDA has generally not enforced regulations with respect to LDTs — to having a more active role, with proposed premarket review and quality system regulation requirements[120]. Under this proposed LDT framework, the CMS (under CLIA) would oversee the laboratory operations and testing processes and the FDA would monitor compliance with quality system regulations.

The effects of expanding regulatory oversight by the FDA on RNA-seq are predicated around the FDA approval process for the first FDA-cleared NGS instrument and NGS *in vitro* diagnostic tests, the Illumina MiSeqDx and the associated *in vitro* diagnostic assays for cystic fibrosis, the Illumina MiSeqDx Cystic Fibrosis 139-Variant and Cystic Fibrosis Clinical Sequencing Assays. Accuracy was evaluated using a representative subset of variants, rather than evaluating all possible variants, and relied on publicly available data to support clinical relevance of the variants. Although evaluation of analytical performance may continue to involve this subset-based approach, the proposed new standards, as outlined by the FDA[120], could include defined technical metrics for data quality, additional standards for computational approaches and standard best practices for quality assurance. The debate over FDA oversight is largely focused on the presence or absence detection of DNA variants, such as germline cystic fibrosis transmembrane conductance regulator (*CFTR*) or *BRCA2* testing. While the FDA guidance and debate is limited in use of examples, the broad scope of additional regulation on all NGS-developed tests, including RNA-seq, may provide regulatory uncertainty for RNA-seq and impede its adoption in the clinic. The proposed FDA regulations around NGS have not gone without debate, emphasizing that the limited enforcement capabilities and regulatory guidance could unnecessarily stifle adoption and innovation[121]. International regulatory frameworks vary across jurisdictions[122,123], with evolving practice guidelines and regulations for the clinical use of NGS[118,119,123,124]. For example, in the European Union (EU), IVD tests require a Conformité Européenne (CE) mark to indicate compliance with the EU IVD Directive (98/79/EC). Similar to the US, the EU is reviewing policy changes related to IVDs, with proposed changes to harmonize the IVD market and increase oversight, including the use of a risk-based classification scheme to define clinical evidence requirements, such as analytical and clinical performance, for IVD approval[125]. The pending regulatory

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In vitro diagnostic tests
Laboratory tests used to detect health conditions, infections or diseases. These diagnostic tests are performed using a sample collected from the patient without direct physical interaction between the test and the patient. In the United States, *in vitro* diagnostics are regulated by the US Food and Drug Administration (FDA).

Box 2 | **Selected examples of integrating DNA and RNA analysis in oncology**

Recent clinical sequencing reports from various groups point to the value of incorporating RNA sequencing (RNA-seq) with DNA sequencing to evaluate the expression of mutant alleles, to detect both known and novel gene fusions, and to detect splice variants[127,138,139]. For example, Mody and colleagues[139] recently reported results from the Pediatric Michigan Oncology Sequencing (MI-ONCOSEQ) programme, incorporating clinical exome-sequencing of tumour and germline DNA and transcriptome sequencing of tumour RNA into the management of children and young adults with refractory or relapsed cancer. The application of these integrative sequencing strategies resulted in changes to patient management in 46% (42 out of 91) of cases, changes to therapy in 15% (14 out of 91) and partial or complete clinical remissions in 10% (9 out of 91), including cases in which potentially actionable events (mainly gene fusions) were detected by RNA-seq, but absent in DNA sequencing[139]. In one reported case, RNA-seq identified a cryptic *ETV6–ABL1* fusion (involving ets variant 6 (*ETV6*) and *ABL1*, which was not detected by standard cytogenetics or fluorescence *in situ* hybridization (FISH), in a patient with precursor B cell acute lymphoblastic leukaemia; the patient maintained molecular remission following treatment with the ABL1 inhibitor imatinib. Using RNA-seq and whole-genome sequencing, Andersson and colleagues[127] reported fusion detection in infant mixed lineage leukaemia (*MLL*)-rearranged acute lymphoblastic leukaemia. In addition, they identified frequent activating mutations in tyrosine kinase–PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase)–RAS signalling pathway genes, at low DNA allele frequencies, which is suggestive of clonal populations; however, RNA-seq data demonstrated expression of the mutant allele in all cases[127].

The value of integrating DNA and RNA analysis is also evident in our own clinical research sequencing experience. In one example, whole-genome sequencing and RNA-seq was used to detect a highly expressed *CTLA4–CD28* fusion (involving cytotoxic T-lymphocyte associated protein 4 (*CTLA4*) and CD28 molecule (*CD28*)) in a patient with advanced Sézary syndrome, with rapid clinical response noted following treatment with ipilimumab[140], a monoclonal antibody targeting CTLA4. Integrated analysis of DNA sequencing and RNA-seq data in triple-negative breast cancer samples also revealed the consequence of a splice site alteration in the tumour suppressor retinoblastoma 1 (*RB1*), providing transcript evidence for an in-frame exon skipping event that was suggested to result in RB1 inactivation, indicating a lack of benefit from CDK4/CDK6 inhibitors[141]. In another study[142], integrated whole-genome and whole-transcriptome analysis of cholangiocarcinoma tumours revealed fibroblast growth factor receptor 2 (*FGFR2*) fusions in three of six cases, two of which received FGFR-targeted therapy with evidence of clinical response. In an additional case, preferential allele-specific expression of a loss-of-function mutation in ERBB receptor feedback inhibitor 1 (*ERRFI1*), a negative regulator of EGFR, was detected and the patient went on to experience marked disease regression following treatment with an EGFR inhibitor[142]. Together, these selected oncology examples illustrate the potential clinical value for integrating DNA- and RNA-based measures.

Triple-negative breast cancer
A breast cancer subtype characterized as oestrogen receptor-negative, progesterone receptor-negative and ERBB2 (also known as HER2)-negative.

changes, both in the US and internationally, may substantially impact the clinical utility of RNA, particularly until greater consensus is reached towards reference standards.

Integrating DNA and RNA sequencing

In clinical studies, integrative DNA and RNA analysis has provided additional evidence for dysregulation of mutated genes, as well as detection of gene fusions and splicing variants and, in some cases, helped prioritize variants for therapeutic intervention (BOX 2). In addition to associating specific genomic alterations with potential therapeutic response, exciting and emerging work suggests that integrated sequencing strategies may also aid in the identification of patient-specific immunogenic neoantigens expressed in the tumour. Recently, RNA-seq and exome sequencing were used to identify and filter for predicted immunogenic neoantigens that were expressed in melanoma tumours, demonstrating an association between these expressed neoantigens and response to the immune-checkpoint inhibitor ipilimumab[17].

Integration of DNA sequencing and RNA-seq holds promise beyond oncology. For example, in the transplant field, reports are emerging for the utility of circulating DNA in monitoring for transplant rejection[129,130]; as discussed earlier, RNA-based measures are already used for the early detection of rejection in heart transplant recipients. The integration of RNA and DNA sequencing to improve transplant rejection diagnosis is an important area currently being examined. The combination of genotyping circulating DNA from donor and recipient, and assessing changes in expression level may provide insights for the degree of rejection, the molecular mechanisms underlying rejection and could suggest possible therapeutic strategies to keep the transplant viable. In the same way, integration of DNA and RNA sequencing could benefit fetal medicine. DNA sequencing of the fetus from maternal blood, in combination with changes in transcript expression levels, could provide additional accuracy and insight for assessing developmental complications.

Although challenges exist, the demonstrated utility of integrative sequencing strategies in research studies is growing across broad health applications and points to the promise for incorporation of RNA-seq into clinical medicine.

Conclusion and perspectives

With its unprecedented ability to simultaneously detect global gene transcript levels and diverse RNA species, RNA-seq has the potential to revolutionize clinical testing for a wide range of diseases. Although recent efforts have set the stage for the establishment of benchmark standards for technical and analytical best practices in order to better standardize RNA-seq accuracy, reproducibility and precision, additional steps toward test proficiency and validation will be required to expand the utility of RNA-seq.

Conceptually, RNA-seq is shot-gun sequencing of the transcriptome, lending to both potential utility and considerable hurdles towards translating RNA to the clinic. The dynamic range and expanding approaches for sample preparation and analysis allow for incredible fine-tuning of sensitivity, specificity and reproducibility. To some extent, the same flexibility and seemingly infinite set of options for RNA-seq that has spurred incredible discoveries into the dynamic nature of human disease has also hindered its path to the clinic. In particular, the establishment of standards has lagged until recently. Several paths forward exist and it is likely that many will be taken towards the direct use of RNA-seq in clinical applications. Once the discovery phase is complete, many diagnostic tests will become targeted assays, sensitive enough to detect small numbers of rare transcripts. The fixed nature of probe sets with microarrays or qRT-PCR offer an accelerated path for clinical test development, as 'the data are the data' without the lure of the latest and newest analysis methods. Therefore, although RNA-seq as a platform has great promise, continuing studies are needed to demonstrate analytical validity and facilitate its adoption within the clinical laboratory setting.

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DATABASES
circBase: http://www.circbase.org/
Genomic tRNA Database: http://gtrnadb.ucsc.edu/genomes/eukaryota/Hsapi19/hg19-tRNAs.fa
miRBase: http://www.mirbase.org/
piRBase: http://regulatoryrna.org/database/piRNA/
piRNABank: http://pirnabank.ibab.ac.in/
SILVA ribosomal RNA database project: http://www.arb-silva.de/
The Ribosomal Database Project: https://rdp.cme.msu.edu/
The National Comprehensive Cancer Network: http://www.nccn.org/icl/default.aspx

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