Twenty years of RNA research and diagnostic development...

What have we learnt?

By Niamh McGivern, PhD[1], Nicholas Forsythe, PhD[1], Cheryl McFarlane, PhD[1], D. Paul Harkin, PhD[1,2], Richard D. Kennedy, PhD[1,2], and Nuala McCabe, PhD[1,2]

1. Almac Diagnostic Services, 19 Seagoe Industrial Estate, Craigavon, BT63 5QD, UK.
2. Queen's University Belfast, The Patrick G Johnston Centre for Cancer Research, 97 Lisburn Road, Belfast, BT9 7AE.

Introduction

Our understanding of the molecular drivers of disease has grown exponentially over the past two decades, largely due to continuous advancements in sequencing technology. DNA mutations, copy number variations, translocations, and fusions have been extensively profiled and have largely dominated the biomarker discovery field. However, information derived from DNA alone is limited and does not provide a full picture of underlying biology. RNA provides information on gene expression and represents active biologies that can also be used as biomarkers. Like most biotechnology areas, we see growth in RNA biomarkers reflected in the increasing number of related publications over the past 20 years (**Figure 1**).

mRNA and beyond

The RNA transcriptome consists of two major categories: coding and non-coding RNAs. Coding messenger RNA (mRNA) has been (and continues to be) the most studied RNA molecule. Unlike DNA aberrations that remain largely constant, mRNA levels may vary depending on a variety of internal and external stimuli, thereby providing quantifiable and dynamic information relating to a biological state, disease progression and/or response to treatment, thus making mRNA molecules ideal biomarkers. Non-coding RNAs (ncRNAs) account for approximately 80% of the transcriptome and include ribosomal RNA (rRNA), transfer RNA (tRNA), micro-RNA (miRNA), long non-coding RNA (lncRNA), small nucleolar RNAs (SnoRNAs), circular RNA (circRNA) and piwi [protein]-interacting RNA (piRNA); many of which have already shown promise as biomarkers.[1] Properties of different RNA species and their applications as biomarkers are summarised in (**Figure 2**).

Measures of RNA biomarkers range from single genes to multi-gene expression signatures (GES). In the case of infectious diseases, for example, the expression of relatively few pathogen-related genes can be used for diagnosis. In more complex diseases such as cancer, a gene expression signature may be needed. A signature refers to a group of mRNAs whose combined expression profile, usually calculated using an algorithm, provide a score that can be used as a biomarker.

RNA expression data can be used for molecular subtyping of oncology and non-oncology diseases. In the oncology setting, statistical methods brought to bear on expression data can tease out and identify groups of genes that may lead to understanding a tumour in context of its environment. Using methods such as unsupervised hierarchical clustering of gene expression can then group together clusters of tumours with similar characteristics, thereby identifying the molecular basis of several types of cancer that can arise from the same anatomical site.[2–4] GES can be developed to detect each subtype and can then be used to tailor therapies that target the molecular pathways driving each subtype. Discovery and validation of molecular subtypes of cancer has been aided by the availability of large data sets and data repositories, examples of which are listed in (**Figure 3**).

Transcriptomics data can also be used to detect RNA fusion events. Traditionally, the chromosomal aberrations underlying chimeric RNA transcripts are detected using fluorescent *in-situ* hybridisation (FISH) and immune-histochemistry (IHC) approaches, however non-concordance and ambiguities between these methods raise challenges for therapeutic decision-making. A direct measure of aberrations using RNA-seq has the advantages of requiring less tumour material for fusion detection, and also provides information on the expression level and fusion partners. Furthermore, unlike DNA-based tests, RNASeq assays can accommodate sequences with large intronic regions (for example, *NTRK* genes).[5]

RNA biomarkers in oncology

Multiple RNA biomarkers have now been approved for use as prognostic and predictive biomarkers across multiple cancer types. For example, several gene expression signatures are available to guide the management of early breast cancer including OncotypeDX® (Genomic Health Inc.) MammaPrint® (Agendia BV) and Prosigna® (NanoString Technologies). MammaPrint® is an FDA approved 70 gene expression signature that identifies patients at high risk of developing metastatic disease.[6] The Prosigna Breast Cancer Prognostic Gene Signature Assay (Veracyte) is an FDA approved

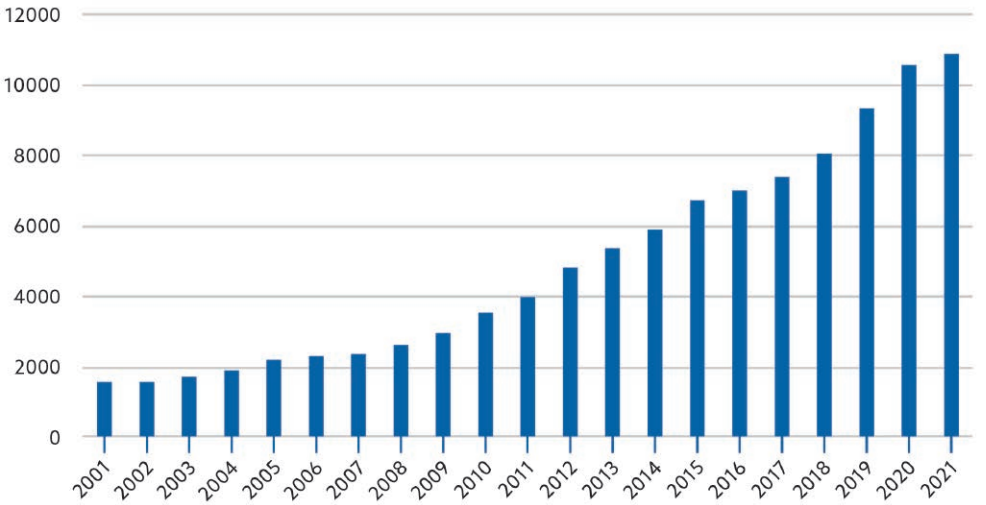


Figure 1: Numbers of publications on RNA biomarkers.

50 gene expression signature used to assign tumour samples to one of 4 subtypes, Luminal A, Luminal B, HER2 Enriched and Basal-like and estimates the risk of recurrent disease.[7] Oncotype Dx® is a 21 gene expression signature that generates a Recurrence Score that represents the likelihood of benefit from adjuvant chemotherapy.[8]

Several RNA fusion biomarkers have also achieved FDA approval, including the targeted NGS panel FoundationOne® which measures *NTRK* and *ROS1* fusions that predict benefit from larotrectinib and entrectinib in lung cancer.[9,10] In addition to NGS, RT-PCR can be used to measure RNA fusions. The Therascreen FGFR CE-IVD (Qiagen) assay identifies two-point mutations and two gene fusions in *FGFR3* that predict benefit from Erdafitinib in advanced or metastatic urothelial carcinoma.[11] The FDA also maintains an updated list of Pharmacogenomic Biomarkers* and Companion Diagnostic Devices.†

RNA biomarkers- beyond cancer

RNA biomarkers are not limited to use in oncology, and blood-based and swab-based assays are routinely used for diagnosis of infectious diseases including HIV, hepatitis C and more recently COVID-19.[12] Quantitative mRNA measurement allows the biologies active in chronically diseased states to be measured and has shown promise across a wide range of conditions including Alzheimer's disease, Parkinson's disease, arthritis, IBD, cardiovascular disease, kidney disease and diabetes.[13–19]

Estimation of neutrophil populations from bulk RNA-seq data combined with expression of key 'hub' genes can predict risk of Alzheimer's disease.[13] Multiple microRNAs (miRNAs) have also been identified as potential biomarkers of Alzheimer's disease prediction and prognosis.[20] In Parkinson's disease downregulation of four circRNAs can be used predict disease development compared to healthy controls.[21] There is evidence that non-coding RNAs, including miRNAs, snoRNAs, circRNAs and lncRNAs, play a role in osteoarthritis development through modulation of various cellular processes and have potential as biomarkers for early disease.[22] Circulating mRNA, miRNA and lncRNAs have also been implicated in the progression of inflammatory bowel disease and may be useful as biomarkers for diagnosis and disease surveillance.[17,18]

The approval of RNA-based biomarkers in the oncology and infectious disease fields has paved the way for potential applications in the chronic disease setting. No validated RNA-based diagnostic, prognostic or companion diagnostic tests, however, have been approved in this setting yet.

Technologies for RNA biomarkers

Multiple technologies have been developed to detect and characterize RNA biomarkers, that are used for discovery and/or the final delivery platform. Next generation sequencing is the current platform of choice to support *de novo* biomarker discovery. Formerly, cDNA microarray technology was commonly used but has largely been superseded by NGS. A more targeted approach such as RT-qPCR or NanoString may be the platform of choice for clinical delivery, depending on the number of transcripts measured. The advantages and disadvantages of each technology for RNA biomarker detection are outlined in (**Figure 4**).

RNA Type	Length (nucleotides)	Function	Biomarker examples
mRNA (messenger RNA)	Average-2200	Translated to protein	**Approved prognostic mRNA biomarkers:** Prosigna®, Oncotype Dx®, Mammaprint®, Endopredict® **Approved predictive mRNA biomarkers:** FoundationOne®, Oncomine Dx®, Therasacreen FGFR CE-IVD
miRNA (microRNA)	18-22	RNA interference, translational regulation	**Demonstrated diagnostic and prognostic utility:** Breast, Ovarian, and Ovarian cancer, Osteoarthritis, Renal Fibrosis, Inflammatory Bowel Disease, Alzheimers Disease[54]
snoRNA (small necleolar RNA)	60-200	Processing of Ribosomal RNA	**Demonstrated diagnostic utility:** NSCLC[56], clear cell renal carcinoma[57], Gastric cancer[58], Osteoartritis[59]
lncRNA (long non-coding RNAs)	Average-1000	Transcriptional regulation, Regulation of DNA methylation and acetylation	**Demonstrated diagnostic utility:** Prostate cancer, Breast cancer, Gastric cancer[52], Rheumatoid arthritis[60], Demonstated prognotic utility in NSCLC[61]
circRNA (circular RNA)	100-999	Transcriptional regulation, miRNA decoy	**Demonstrated diagnostic utility:** Lung, Gastric, Colorectal and Breast cancer, Rheumatoid arthritis, Heart failure, Hypertension, Tuberculosis infection[62]
piRNA (PIWI-interacting RNAs)	26-31	Regulation of transposable elements	**Demonstrated diagnostic utility:** Breast cancer[63] and Colorectal cancer[64], Sporadic ALS[65]

Figure 2: Overview of RNA biomarkers

cDNA Microarray

Following the first publication referencing microarrays in 1995,[23] cDNA microarrays quickly became the primary method of choice for high throughput gene expression profiling. Unlike RT-qPCR technology, cDNA microarrays utilise a target hybridisation approach, typically with fluorescently labelled probes derived from the sample for quantification, enabling thousands of genes to be analysed simultaneously.[24] This platform is sufficiently robust that low quality FFPE samples can be used thereby allowing analysis of archived samples. Multiple RNA based assays have been developed with this technology, including the Almac ColDx, assay which can identify high risk early colon cancer.[25]

Despite the clear utility of cDNA microarrays for RNA biomarker discovery and validation, this approach has several limitations. While generally versatile, cDNA microarrays are restricted to the detection of known transcripts and have a lower dynamic range than NGS or PCR-based approaches at approximately 10^3. By comparison NGS methods offer a large dynamic range of $>10^5$.

RNA-Seq

Since the first publications in 2008,[26–28] RNA-Seq has become the RNA analysis technology of choice. RNA-Seq can detect novel transcripts and structural variants (such as alternative splicing events and gene fusions) as well as identify allele-specific expression single nucleotide polymorphisms (SNPs). RNA-seq also allows for the discovery and validation of many gene expression signatures simultaneously from low input (20-50ng) RNA using a single tumour sample. For example, Almac Diagnostic Services Ltd. have developed clara^T, a software solution that can simultaneously report 108 gene expression signatures and 100 drug targets linked to each of the 10 hallmarks of cancer[29] using RNA-seq data generated from a single sample.[30]

With advances in technology and computational power, the cost of RNA-Seq continues to fall, increasing accessibility for biomarker discovery. With this platform, several methodologies are available for RNA sequencing, including whole transcriptome sequencing (WTS), mRNA sequencing and targeted RNA-sequencing. WTS is the most comprehensive RNA-seq approach, and involves sequencing of all RNA transcripts, including coding and non-coding RNA molecules. Particularly noteworthy is that WTS can be used for low quality FFPE samples. Targeted RNA-Seq can be achieved via target enrichment or amplicon-based approaches, both of which enable the analysis of specific RNA sequences with greater read depth and sensitivity. This allows detection of mRNA and gene fusions using low RNA input.[31]

Several challenges, however, are associated with RNA-seq technology. For example, to make templates for sequencing, cDNA libraries are generated from RNA using reverse transcription

RightMed for Populations® from **OneOme®** is an end-to-end PGx solution with proven results. We work with leading healthcare systems, insurers, and payers to identify and engage patients most likely to benefit from the **RightMed®** test. The results provide clinicians with actionable medication decision support. Resulting prescriptions may be more likely to work for patients, have less risk of adverse drug reactions and lower total cost of care.

Learn more or request a demo at
oneome.com/pmls-2022

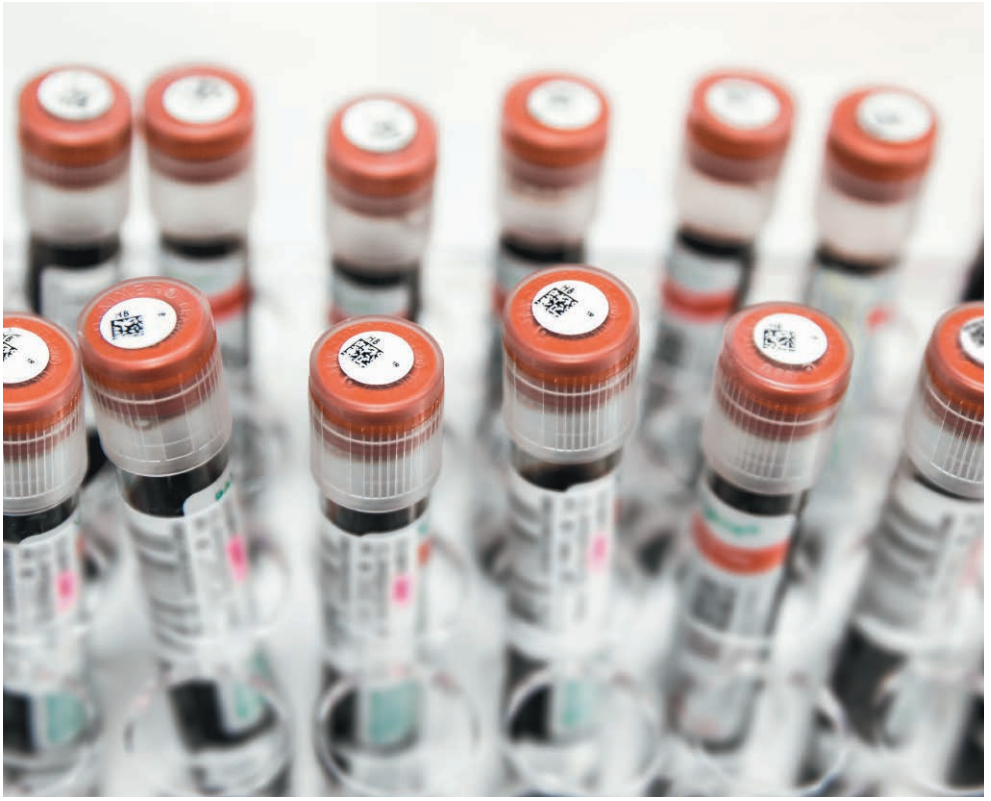
OneOme®

Start with the End in Mind: From PGx Pilot to Program
Panel featuring speakers from Blue Care Network, Mayo Clinic and OneOme
Live at PMLS - Wednesday, September 28 at 4pm

807 Broadway St NE #100, Minneapolis, MN 55413 | support@OneOme.com | (844) 663-6635

and PCR amplification steps. Biases and artefacts associated with these steps may be introduced that can affect data quality (false readings or over-representation of sequences).[32] Interpretation of RNA-Seq data requires sophisticated computational programs that may result in inconsistent interpretation of differential gene expression analysis, detection of fusion genes, alternative splicing, and variants detection, therefore requiring careful performance validation. A wealth of mature tools exists to meet basic requirements (e.g., applications hosted on Illumina BaseSpace Sequence Hub). Some challenges, however, remain where there is scope for more advanced algorithms.

Despite these challenges, RNA-Seq remains at the forefront of RNA biomarker research and development. As noted in the footnote in Section 3, the FDA has approved multiple RNA-Seq based companion diagnostic tests (Foundation One, Oncomine Dx, MI Transcriptome™ CD). Several more NGS based RNA biomarkers have been analytically validated, including the CE marked Agendia Mammaprint® and BluePrint® NGS Assays.



RT-PCR

Reverse transcription quantitative PCR (RT-qPCR) has long been considered the gold standard for detection and quantification of RNA targets.[33] RT-qPCR is highly sensitive and specific, allowing detection of a single or few transcripts within a given sample. This methodology is the most amenable to delivery of clinical tests, with a familiar workflow, easy standardisation, necessary equipment typically available and accessible in most labs, alongside relatively quick turn-around times and low costs. Furthermore, recent advances in digital droplet PCR (ddPCR) have seen improvements in sensitivity and limits of detection (LOD) for gene expression analysis and RNA biomarker detection.[34] The success of RT-qPCR for diagnostic testing is exemplified by the rapid set up, delivery and success of SARS-CoV-2 testing around the globe during the COVID-19 pandemic.[35] Several RT-qPCR based assays have also been analytically validated for predictive and prognostic testing in the field of oncology, including the Oncotype Dx Recurrence Score® and EndoPredict® for breast cancer.[8,36]

RT-qPCR is only a suitable, however when the aim is to quantify known variants. The technology is also limited by low sample throughput and the number of genes that can be analysed simultaneously. Multiplex RT-qPCR can overcome some of these disadvantages; it can be used for detection of multiple gene targets in a single reaction, therefore increasing sample throughput,

Data Repository	Acronym	Reference
The Cancer Genome Atlas	TCGA	https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga
The Cancer Protein Atlas	TCPA	https://www.tcpaportal.org/tcpa/
Gene Expression Omnibus	GEO	https://www.ncbi.nih.gov/geo/
Cancer Cell Line Encyclopaedia	CCLE	https://www.sites.broadinstitute.org/ccle/
Genomics and Drug Sensitivity in Cancer	GDSC	https://www.cancerrxgene.org/
Arrayexpress	AE	https://www.ebi.ac.uk/arrayexpress/
Genomic Data Commons Data Portal	GDC	https://www.portal.gdc.cancer.gov/
cBioPortal for Cancer Genomics	cBioPortal	https://www.cbioportal.org/datasets
Catalogue of Somatic Mutations in Cancer	COSMIC	https://www.cancer.sanger.ac.uk/cosmic
International Cancer Genome Consortium	ICGC	https://www.dcc.icgc.org/
Firehose Broad Genome Data Analysis Center	GDAC	https://www.gdac.broadinstitute.org/
The Encyclopedia of DNA Elements	Encode	https://www.encodeproject.org/
SRA raw sequence data repository	SRA	https://www.ncbi.nlm.nih.gov/sra
European Genome-Phenome Archive	EGA	https://www.ega-archive.org/

Figure 3: Publicly Available Data Set Examples

reducing turn-around time and reagent costs and minimizing the required amount of starting material required.[37] The Fluidigm Biomark™ HD system has further built upon the concept of multiplex RT-qPCR, and utilises microfluidic chip-based technology, allowing the analysis of 96 target genes in 96 samples within a single run.[38]

NanoString nCounter

NanoString Technologies nCounter® is a multiplex nucleic acid hybridisation platform that bridges the gap between genome wide and targeted gene expression analysis. It uses digital detection of individual mRNA transcripts using target specific, fluorescently labelled barcodes[39] and is suitable for low quality, low input RNA samples. Unlike RT-qPCR and RNA Seq, it does not require conversion of mRNA to cDNA, or PCR amplification,[40] thereby reducing the potential for error. Each gene panel measures expression of approximately 800 genes, allowing for targeted multiplex gene expression analysis, which is suitable for a curated biomarker discovery approach, or RNA biomarker delivery.

The NanoString nCounter panels provide data delivery with a shorter turnaround time compared to standard NGS analysis, and with less requirement and reliance on bioinformatic specialists for data processing and interpretation. The platform is best suited to biomarker delivery or investigation of specific biologies, as each panel is focussed on specific content and does not provide enough information for large scale RNA analysis. This technology is currently used to deliver the Prosigna™ Breast Cancer Prognostic Gene Signature Assay, described above.

ISH

Tumours are not uniform collections of malignant cells, but rather a diverse assembly of tumour and associated cells within the tumour microenvironment (TME). This spatial heterogeneity can result in non-uniform distribution of genetically distinct tumour-cell subpopulations.[41] Traditionally spatially resolved RNA profiling is performed using low-plex methods such as in-situ hybridisation (ISH). More recently RNA-scope has been developed which utilises a unique probe design that allows simultaneous signal amplification and background suppression to achieve direct single-molecule visualization while preserving tissue morphology.[42]

RNAscope is compatible with FFPE samples and clinical laboratory workflows, however, it is limited by its low plex nature, with a maximum of 4 target genes analysed per sample. Novel spatially resolved high throughput molecular profiling technologies are now emerging such as the Nanostring GeoMx which utilises *in-situ* hybridisation probes linked to indexing oligo barcodes via a photocleavable linker to allow visualisation of RNA targets in a region of interest.[43] This technology is suitable for fresh frozen and FFPE material, and is suitable for a range of samples sizes, from core-needle biopsies, tissue microarrays and resected tumours. To date, no RNA-ISH biomarkers have been validated or commercially approved, however this emerging technology has potential to further aid the understanding of spatially heterogenous transcriptomics and RNA biomarker discovery and development.

Technology	Advantages	Disadvantages
cDNA micro-array	Suitable for high-throughput gene expression analysis Applicable to FFPE/degraded sample types	Limited detection of known variants Lower dynamic range than NGS
RNA-Seq	Suitable for high-throughput gene expression analysis Suite for *de novo* biomarker discovery High dynamic range Workflow can be tailored to work with degrated/FFPE samples	Amplification induced bias Higher cost and TAT Large data output requires storage and expertise for interpretation
RT-qPCR	Quick TAT Low cost Applicable to clinical delivery	Low sample throughput Limited to detection of known variants
Nanostring	Suitable for multigene expression analysis Quick TAT Low levels of data interpretation required Direct hybridization of RNA removed amplification induced bias	Limited to specialised labs Targeted panels minimise number of biologies that can be simultaneously analysed
ISH	Provides spatial representation	Low dynamic range Requires visual interpretation

Figure 4: RNA Technology Review

Sample Type

RNA biomarkers can be detected from many sample types, including solid tissue biopsies collected as either FFPE or fresh frozen tissue, or liquid biopsies such as urine, blood, saliva and synovial fluid. Each sample type has unique advantages and challenges.

FFPE

Formalin-fixation and paraffin embedding (FFPE) is the current (and legacy) gold standard for clinical sample archiving. To take advantage of the mRNA in these sample archives, therefore, most RNA extraction methods and biomarkers need to be validated for this material. FFPE samples present multiple technical challenges: formalin fixation causes fragmentation of RNA transcripts to 100-200 nts (on average), as well as base modifications and crosslinking of RNA to nucleic acids and proteins. Any downstream analysis therefore needs to be suitable for low yields of heavily fragmented RNA.[44]

Several extraction methods are available to maximise yield and quality of RNA from FFPE samples; these methods may be used either individually or in combination and spin-column based extractions that use liquid phase separation, and the use of silica coated magnetic beads for high-throughput RNA extraction. All methods require deparaffinisation of the FFPE samples, which requires using solvents such as xylene, commercially-available deparaffinisation solutions, or mechanical disruption such as ultrasonication. This is followed by protein digestion to disrupt cellular structures and release nucleic acid from protein crosslinks. DNAse treatment is required to ensure any remaining genomic DNA is removed from the sample, before RNA purification and elution.[45]

Variation in handling of FFPE samples across clinical sites – e.g., time to fixation, tissue thickness, duration of fixation, and composition of paraffin for embedding – can also impact quality of RNA retrieval and subsequent biomarker analysis. Large tumour samples take longer for formalin

to penetrate and may undergo fixation slower than small biopsies resulting in differences in transcriptional profiles.[46] Additionally, intra-tumour heterogeneity and the presence of high levels of stromal cells such as fibroblasts, endothelial and immune cells, often results in the collection of FFPE specimens that are not entirely representative of the entire tumour. These variables result in challenges such as data replication and validation of RNA biomarkers using datasets collected across multiple, different sites.[46] Macrodissection (removal of non-tumour material) and microdissection (specific dissection of tumour epithelial component using microscopy) can help to ensure some standardisation of gene expression data from the tumour compartment prior to RNA extraction.[47]

Fresh Frozen

Fresh frozen (FF) tissue offers several advantages over FFPE tissue. Fixation and mounting of FF tissue using a cryostat is more rapid than the FFPE process. FF samples also preserve the integrity of genetic material and for this reason are the ideal sample type for RNA sequencing. FF tissue, however, will rapidly degrade at room temperature, so the sample needs to be frozen as soon as possible after surgery and maintained at a low temperature. FF tissue also requires specialised ultra-low temperature freezers for long-term storage, making sample storage expensive, with higher risk of sample degradation upon thawing prior to nucleic acid retrieval. Since it can be challenging to section and visualise frozen samples microscopically, it can be difficult to standardise the tumour/stromal content through macrodissection. This can potentially result in inconsistency of gene expression profiles from samples taken from the same tumour.

Circulating/liquid biopsies.

Liquid biopsies typically assess disease markers in blood, urine, saliva and sputum samples. This has advantages over surgical approaches as it is less risky to patient heath, can represent metastatic disease which is difficult to sample directly and is amenable to serial sampling over time. Highly sensitive liquid biopsy approaches have been developed that can detect and characterize minimal residual disease (MRD), reflecting the presence of tumour cells disseminated in patients who do not display any clinical or radiological signs of metastasis or residual tumour cells left behind after therapy.[48]

To date, the liquid biopsy field has been dominated by proteins (e.g., PSA and CA125), metabolites and more recently circulating tumour DNA and circulating tumour cells. However, the circulating transcriptome also represents a rich source of potential disease biomarkers. The measurement of tumor-associated mRNA has advantages over ctDNA in this application, as tumour cells have multiple copies of the mRNA transcript compared to limited copies of the source gene at DNA level.

RNA species in the bloodstream, however, have been considered a challenge for biomarker discovery due to highly unstable nature owing to high levels of circulating RNAse and low abundance transcripts.[49] There is however evidence that RNA molecules may be specifically released by tumour cells, protected by circulating vesicles, and may therefore be protected from degradation.[50] Several non-coding RNAs have shown promise as suitable biomarkers from liquid biopsies. Cancer specific miRNAs can be detected in blood samples collected from a range of cancer indications,[51] and multiple studies are ongoing to assess the potential of miRNA as a biomarker for MRD. Circulating lncRNA has also shown promise as diagnostic tool for a range of cancer types.[52]

Regulatory Considerations of RNA-based Biomarkers

The performance specifications of any laboratory assay need to be verified prior to use in clinical testing.[53] Verification and validation is particularly important in RNA-based assays where several factors can introduce errors such as: poor sample quality and mRNA degradation, differences in reagent batches, different technologies, differences in instrument calibration and complex procedures that may be predisposed to operator mistakes. In contrast to DNA-based molecular diagnostic assays, multi-gene RNA assays often require complex algorithms with reporting software which must be fully validated prior to use in clinical testing. The scale and scope of analytical validation is determined by the intended use of the assay and must comply with guidance from the relevant regulatory jurisdiction. For example, quantitative gene expression assays are typically validated for specificity, sensitivity, linearity, reference range, stability and precision in compliance with the relevant regulatory jurisdiction.

RNA-based assays also require the inclusion of positive and negative controls. These are used for both the quality control of clinical testing runs and the surveillance of assay performance over time. This internal check ensures the identification of issues such as reagent changes, technological failures or lab staff errors; should discrepancy or errors be detected, corrective action can be taken. The identification of suitable controls can be particularly challenging for multi-gene signatures. In this instance, cell line models are a valuable resource for the generation of complex gene signature controls, however, their utility is somewhat limited for inflammatory or immunological signatures as basal gene expression tends to be low in the absence of stimulation. One potential solution is a qualified, manufactured positive control in a format compatible with distribution in kits such as Armored RNA.[54] Of highest value would be a control that is accepted by professional and regulatory groups that would serve as a standard across application platforms and clinical use.

Concluding remarks

RNA is a dynamically expressed molecule which changes depending on time, state, and disease of a patient. These properties make RNA a useful biomarker, and the last 20 years have therefore seen an exponential period of growth in the discovery, validation and clinical use of RNA biomarkers for multiple diseases. There have been significant advances in the technologies available for RNA biomarker discovery, from low throughput RT-qPCR to high throughout NGS platforms and spatially resolved, high throughput gene expression platforms, enabling researchers to gain deep insight into multiple biologies from a single sample.

Several challenges, however, remain in the field.

Professor Richard Kennedy

Global VP of Biomarker Development & Medical Director

Professor Richard Kennedy is the Global VP of Biomarker Development, Medical Director of Almac Diagnostic Services and CLIA compliant laboratory Director. He is responsible for the application of the company's technology into medical practice. He graduated in medicine from Queen's University Belfast in 1995. As a post-graduate he trained as a medical oncologist and received a PhD in Molecular Biology in 2004.

From 2004-2007 he worked as an instructor in oncology at Harvard Medical School, USA, where he identified novel biomarkers and drug targets for cancer treatment.

In August 2007 he joined Almac Diagnostic Services as the director of a CLIA compliant diagnostics laboratory and has been involved in the biomarker design for several international clinical trials. In 2012 he established a research group in Queen's University Belfast focussed on various aspects of stratified medicine.

Professor Paul Harkin

President and Managing Director

Professor Paul Harkin is founder of Almac Diagnostic Services, a company focused on the discovery, development and commercialisation of diagnostic and companion diagnostic tests. In 2004 Almac Diagnostic Services was incorporated as a Division of the Almac Group and Professor Harkin was appointed as President and Managing Director with overall responsibility for the strategic, financial and operational leadership of the company.

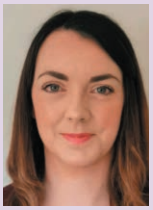
Paul is also a Professor in Molecular Oncology within the Centre for Cancer Research and Cell Biology at Queen's University Belfast where his research has focused on the role played by BRCA1 mutation in the development of hereditary breast cancer. Prior to this appointment he had been a Research Fellow in Medicine at Harvard Medical School and the Massachusetts General Hospital where he developed his interest in the emerging field of precision medicine.

Dr Nuala McCabe

Biomarker Research Manager

Dr Nuala McCabe graduated from Queen's University Belfast in 1997 with an Honours Degree in Biomedical Science. She then gained her Ph.D. training in the Department of Oncology, Queen's University Belfast in 2001 investigating the role of the BRCA1 tumour suppressor in DNA damage response. Following this, she then worked as a post-doctoral researcher Prof. Alan Ashworth in the Breakthrough Breast Cancer Research Centre (Institute of Cancer Research, London). During this time she was the involved in the identification of PARP1 inhibitors for the treatment of BRCA-associated breast and ovarian cancers, which are now approved by the EMA and FDA.

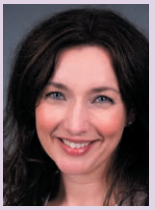
Dr McCabe is Biomarker Research Manager at Almac Diagnostic Services. Within this role she has gained over 15 years industrial experience in drug target identification and biomarker discovery. Dr McCabe has an honorary position at Queen's University Belfast and runs a team of both industrial and academic staff with a focus on the discovery and validation of biomarkers for clinical trials. Dr McCabe's work has led to a number of significant invited talks, patents and publications including Nature, Cancer Research, Cancer Cell and Oncogene.



Dr Niamh McGivern

Biomarker Research Team Leader

Niamh McGivern is a team leader in the Biomarker Research Unit at Almac Diagnostics Services. Prior to joining Almac, Niamh gained a BSc in Biological Sciences, followed by a PhD in molecular oncology from Queen's University Belfast. Following this, Niamh worked as a post-doctoral researcher at the University of Edinburgh, using high throughput technologies to investigate the role of adhesion proteins in cancer development and progression. In her role at Almac Diagnostic Services, Niamh is responsible for overseeing biomarker discovery projects within the laboratory and implementation of novel platforms and methodologies to support biomarker research.



Dr Cheryl McFarlane

Assay Development & Validation Manager

Dr. McFarlane (PhD) has worked for over 15 years in the field of translational molecular oncology and holds the post of Assay Development & Validation Manager at Almac Diagnostic Services where she has responsibility for assay development and validation activities supporting biomarker discovery, development and delivery for a range of Global Biopharma clients.

Prior to joining Almac in 2015, Dr McFarlane obtained a PhD from Queen's University Belfast. She subsequently completed postdoctoral fellowships where she specialised in the identification and validation of novel therapeutic targets in the Ubiquitin Proteasome system.

Cheryl is also a General Supervisor in the clinical laboratory supervising the day-to-day work of all CLIA certified laboratory testing.

Dr Nicholas Forsythe

Senior Scientist – Assay Development & Validation

Nicholas Forsythe is a senior scientist in the Assay development and Validation unit at Almac Diagnostic Services. He obtained his PhD in molecular oncology from Queen's University Belfast in 2017. Nicholas has over 9 years combined PhD and Post-Doctoral experience working in the colorectal and prostate cancer fields. His work has primarily focused on deriving novel and exploitable biology and biomarkers from RNA-seq data. His most recent work focused on epigenetic modifying agents and their use as novel therapeutics to overcome hormone-resistance in prostate cancer. Nicholas previously worked with Almac Diagnostic Services as part of the Stratified Medicine Group at Queen's University from 2017-2019. He continued his affiliation with Almac by joining the company in 2022.

Due to its instability, attaining suitable RNA quality and quantity is more of an issue for RNA-based assays than DNA-based assays, and at this stage of development, reagents and equipment are more often designed and qualified only for research than clinical use. Since RNA is subject to handling issues and related measurement standardisation, variations can be introduced, resulting in errors requiring yet more stringent analytical and clinical validation to ensure robustness of any RNA-based clinical assay. The effort will result in dividends as RNA-based biomarkers, unlike DNA-based biomarkers, can provide a measurement of the molecular pathways underpinning a disease at any specific time without the need to understand the significance of mutations or variants in specific genes. We therefore continue to look to RNA assays to be important tools for the delivery of precision medicine. JoPM

References

1. Palazzo, A. F. & Lee, E. S. Non-coding RNA: What is functional and what is junk? Front. Genet. 5, (2015).
2. Guinney, J. et al. The consensus molecular subtypes of colorectal cancer. Nat. Med. 21, (2015).
3. Koboldt, D. C. et al. Comprehensive molecular portraits of human breast tumours. Nature 490, (2012).
4. Bell, D. et al. Integrated genomic analyses of ovarian carcinoma. Nature 474, (2011).
5. Marino, F. Z. et al. Ntrk fusions, from the diagnostic algorithm to innovative treatment in the era of precision medicine. International Journal of Molecular Sciences 21, (2020).
6. Van't Veer, L. J. et al. Gene expression profiling predicts clinical outcome of breast cancer. Nature 415, (2002).
7. Bernard, P. S. et al. Supervised risk predictor of breast cancer based on intrinsic subtypes. J. Clin. Oncol. 27, (2009).
8. Paik, S. et al. Gene expression and benefit of chemotherapy in women with node-negative, estrogen receptor-positive breast cancer. J. Clin. Oncol. 24, (2006).
9. https://www.foundationmedicine.com/press-releases/3fe223c4-f8ae-4c35-b138-47ad284f6452.
10. https://www.foundationmedicine.com/press-releases/c90da36c-205d-4b8d-9848-dfcb970da47a.
11. https://corporate.qiagen.com/newsroom/press-releases/press-release-details/2019/QIAGEN-launches-first-FDA-approved-companion-diagnostic-using-FGFR-alterations-to-help-guide-the-treatment-of-metastatic-urothelial-cancer/default.aspx.
12. Bukasov, R., Dossym, D. & Filchakova, O. Detection of RNA viruses from influenza and HIV to Ebola and SARS-CoV-2: a review. Analytical methods : advancing methods and applications 13, (2021).
13. Shigemizu, D. et al. Identification of potential blood biomarkers for early diagnosis of Alzheimer's disease through RNA sequencing analysis. Alzheimer's Res. Ther. 12, (2020).
14. Chou, C. H. et al. Synovial cell cross-talk with cartilage plays a major role in the pathogenesis of osteoarthritis. Sci. Rep. 10, (2020).
15. Rego-Pérez, I., Durán-Sotuela, A., Ramos-Louro, P. & Blanco, F. J. Genetic biomarkers in osteoarthritis: a quick overview. Fac. Rev. 10, (2021).
16. Vanhaverbeke, M. et al. Peripheral blood RNA biomarkers for cardiovascular disease from bench to bedside: a position paper from the EU-CardioRNA COST action CA17129. Cardiovasc. Res. (2021). doi:10.1093/cvr/cvab327
17. Ju, W., Smith, S. & Kretzler, M. Genomic biomarkers for chronic kidney disease. Translational Research 159, (2012).
18. Yang, B. et al. Identifying Biomarkers from Transcriptomic Signatures in Renal Allograft Biopsies Using Deceased and Living Donors. Front. Immunol. 12, (2021).
19. Ducharme, J. et al. A multi-mrna host-response molecular blood test for the diagnosis and prognosis of acute infections and sepsis: Proceedings from a clinical advisory panel. J. Pers. Med. 10, (2020).
20. Zhao, Y. et al. microRNA-Based Biomarkers in Alzheimer's Disease (AD). Frontiers in Neuroscience 14, (2020).
21. Ravanidis, S. et al. Differentially Expressed Circular RNAs in Peripheral Blood Mononuclear Cells of Patients with Parkinson's Disease. Mov. Disord. 36, (2021).
22. Kong, H., Sun, M. L., Zhang, X. A. & Wang, X. Q. Crosstalk Among circRNA/lncRNA, miRNA, and mRNA in Osteoarthritis. Frontiers in Cell and Developmental Biology 9, (2021).
23. Schena, M., Shalon, D., Davis, R. W. & Brown, P. O. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science (80-.). 270, (1995).
24. Russo, G., Zegar, C. & Giordano, A. Advantages and limitations of microarray technology in human cancer. Oncogene 22, (2003).
25. Kennedy, R. D. et al. Development and independent validation of a prognostic assay for stage ii colon cancer using formalin-fixed paraffin-embedded tissue. J. Clin. Oncol. 29, (2011).
26. Lister, R. et al. Highly Integrated Single-Base Resolution Maps of the Epigenome in Arabidopsis. Cell 133, (2008).
27. Nagalakshmi, U. et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. Science (80-.). 320, (2008).
28. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 5, (2008).
29. Hanahan, D. & Weinberg, R. A. Hallmarks of Cancer: The Next Generation | Elsevier Enhanced Reader. Cell (2011).
30. https://www.almacgroup.com/diagnostics/claratreport/.
31. https://emea.illumina.com/techniques/sequencing/rna-sequencing/targeted-rna-seq.html.
32. Shi, H. et al. Bias in RNA-seq Library Preparation: Current Challenges and Solutions. BioMed Research International 2021, (2021).
33. Bustin, S. A. & Mueller, R. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clinical Science 109, (2005).
34. Hindson, C. M. et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat. Methods 10, (2013).
35. Peeling, R. W., Heymann, D. L., Teo, Y. Y. & Garcia, P. J. Diagnostics for COVID-19: moving from pandemic response to control. The Lancet 399, (2022).
36. Filipits, M. et al. A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. Clin. Cancer Res. 17, (2011).
37. Henegariu, O., Heerema, N. A., Dlouhy, S. R., Vance, G. H. & Vogt, P. H. Multiplex PCR: Critical parameters and step-by-step protocol. Biotechniques 23, (1997).
38. Jang, J. S. et al. Quantitative miRNA Expression Analysis Using Fluidigm Microfluidics Dynamic Arrays. BMC Genomics 12, (2011).
39. Cesano, A. nCounter® PanCancer Immune Profiling Panel (NanoString Technologies, Inc., Seattle, WA). J. Immunother. Cancer 3, (2015).
40. Kulkarni, M. M. Digital multiplexed gene expression analysis using the nanostring ncounter system. Curr. Protoc. Mol. Biol. (2011). doi:10.1002/0471142727.mb25b10s94
41. Dagogo-Jack, I. & Shaw, A. T. Tumour heterogeneity and resistance to cancer therapies. Nature Reviews Clinical Oncology 15, (2018).
42. Wang, F. et al. RNAscope: A novel in situ RNA analysis platform for formalin-fixed, paraffin-embedded tissues. J. Mol. Diagnostics 14, (2012).
43. Zollinger, D. R., Lingle, S. E., Sorg, K., Beechem, J. M. & Merritt, C. R. in Methods in Molecular Biology 2148, (2020).
44. David, L. E., Fowler, C. B., Cunningham, B. R., Mason, J. T. & O'Leary, T. J. The effect of formaldehyde fixation on RNA: Optimization of formaldehyde adduct removal. J. Mol. Diagnostics 13, (2011).
45. Lewis, F., Maughan, N. J., Smith, V., Hillan, K. & Quirke E, P. Unlocking the archive – Gene expression in paraffin-embedded tissue. Journal of Pathology 195, (2001).
46. Srinivasan, M., Sedmak, D. & Jewell, S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. American Journal of Pathology 161, (2002).
47. https://sequencing.roche.com/en/blog/enriching-tumor-content-in-FFPE-sample-dissections.html.
48. Pantel, K. & Alix-Panabières, C. Liquid biopsy and minimal residual disease – latest advances and implications for cure. Nature Reviews Clinical Oncology 16, (2019).
49. Zaporozhchenko, I. A., Ponomaryova, A. A., Rykova, E. Y. & Laktionov, P. P. The potential of circulating cell-free RNA as a cancer biomarker: challenges and opportunities. Expert Review of Molecular Diagnostics 18, (2018).
50. Huang, X. et al. Characterization of human plasma-derived exosomal RNAs by deep sequencing. BMC Genomics 14, (2013).
51. Mishra, P. J. MicroRNAs as promising biomarkers in cancer diagnostics. Biomark. Res. 2, (2014).
52. Bolha, L., Ravnik-Glavac, M. & Glavac, D. Long Noncoding RNAs as Biomarkers in Cancer. Disease Markers 2017, (2017).
53. Halling, K. C., Schrijver, I. & Persons, D. L. Test verification and validation for molecular diagnostic assays. Arch. Pathol. Lab. Med. 136, (2012).
54. Pasloske, B. L., Walkerpeach, C. R., Obermoeller, R. D., Winkler, M. & Dubois, D. B. Armored RNA technology for production of ribonuclease-resistant viral RNA controls and standards. J. Clin. Microbiol. 36, (1998).
55. Condrat, C. E. et al. miRNAs as Biomarkers in Disease: Latest Findings Regarding Their Role in Diagnosis and Prognosis. Cells 9, (2020).
56. Liao, J. et al. Small nucleolar RNA signatures as biomarkers for non-small-cell lung cancer. Mol. Cancer 9, (2010).
57. Zhao, Y. et al. Expression signature of six-snoRNA serves as novel non-invasive biomarker for diagnosis and prognosis prediction of renal clear cell carcinoma. J. Cell. Mol. Med. 24, (2020).
58. Wang, X. et al. Identification of Eight Small Nucleolar RNAs as Survival Biomarkers and Their Clinical Significance in Gastric Cancer. Front. Oncol. 9, (2019).
59. Steinbusch, M. M. F. et al. Serum snoRNAs as biomarkers for joint ageing and post traumatic osteoarthritis. Sci. Rep. 7, (2017).
60. Miao, C., Bai, L., Yang, Y. & Huang, J. Dysregulation of lncRNAs in Rheumatoid Arthritis: Biomarkers, Pathogenesis and Potential Therapeutic Targets. Frontiers in Pharmacology 12, (2021).
61. Liu, Y. et al. A novel prognostic signature of immune-related lncRNA pairs in lung adenocarcinoma. Sci. Rep. 11, (2021).
62. Zhang, Z., Yang, T. & Xiao, J. Circular RNAs: Promising Biomarkers for Human Diseases. EBioMedicine 34, (2018).
63. Maleki Dana, P., Mansournia, M. A. & Mirhashemi, S. M. PIWI-interacting RNAs: New biomarkers for diagnosis and treatment of breast cancer. Cell and Bioscience 10, (2020).
64. Mai, D. et al. Serum piRNA-54265 is a new biomarker for early detection and clinical surveillance of Human Colorectal Cancer. Theranostics 10, (2020).
65. Abdelhamid, R. F. et al. piRNA/PIWI Protein Complex as a Potential Biomarker in Sporadic Amyotrophic Lateral Sclerosis. Mol. Neurobiol. 59, (2022).

* FDA Table of Pharmacogenomic Biomarkers in Drug Labelling: https://www.fda.gov/drugs/science-and-research-drugs/table-pharmacogenomic-biomarkers-drug-labeling

† List of Cleared or Approved Companion Diagnostic Devices (In Vitro and Imaging Tools): https://www.fda.gov/medical-devices/in-vitro-diagnostics/list-cleared-or-approved-companion-diagnostic-devices-in-vitro-and-imaging-tools