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Standardising RNA profiling based biomarker application in cancer - the need for robust control of technical variables

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Standardising RNA profiling based biomarker application in cancer - the need for robust control of technical variables

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Abstract

Histopathology-based staging of colorectal cancer (CRC) has utility in assessing the prognosis of patient subtypes, but as yet cannot accurately predict individual patient's treatment response. Transcriptomics approaches, using array based or next generation sequencing (NGS) platforms, of formalin fixed paraffin embedded tissue can be harnessed to develop multi-gene biomarkers for predicting both prognosis and treatment response, leading to stratification of treatment. While transcriptomics can shape future biomarker development, currently <1% of published biomarkers become clinically validated tests, often due to poor study design or lack of independent validation. In this review of a large number of CRC transcriptional studies, we identify recurrent sources of technical variability that encompass collection, preservation and storage of malignant tissue, nucleic acid extraction, methods to quantitate RNA transcripts and data analysis pipelines. We propose a series of defined steps for removal of these confounding issues, to ultimately aid in the development of more robust clinical biomarkers.

Keywords: Microarray, RNA profiling, NGS, transcriptome, FFPE, biomarker

Abbreviations: CRC, colorectal cancer; AJCC, American Joint Committee on Cancer; TNM, Tumour Node Metastasis; GEP, Gene expression profile; CRCSC, CRC Subtyping Consortium; CMS, consensus molecular subgroups; MSI, microsatellite instability; FFPE, formalin fixed paraffin embedding; S:CORT, Stratification in COloRecTal cancer; PAC, probably approximately correct; SOP, standard operating procedures; FF, fresh frozen; IVT, in vitro transcription; RIN, RNA integrity number; BBRB, Biorepositories and Biospecimen Research Branch; CDP, Cancer Diagnosis Program; TCGA, The Cancer Genome Atlas;

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ERCC, External RNA Controls Consortium; LDT, laboratory developed test; CLIA, Clinical Laboratory Improvement Amendments.

Variability in colorectal cancer transcriptomic studies

Colorectal cancer (CRC) is the United Kingdom's second most common cause of cancer deaths [1]. Diagnostic staging of CRC relies on the American Joint Committee on Cancer (AJCC) Tumour Node Metastasis (TNM) staging system [2]. Classification into AJCC staging groups is based on extent of local tumour invasion, regional lymph node involvement and evidence of distant metastasis. While this staging system has intrinsic prognostic value, this initial categorisation is insufficient for predicting outcome following treatment [3]. Several clinical and histopathological markers have been reported to be 'prognosis predictors' for Stage II patients, by indicating benefit from adjuvant chemotherapy. These include extramural vascular invasion, grade 3/poor differentiation, serum carcinoembryonic antigen levels, T4 stage/perforation, perineural invasion, *CDX2*, obstructive tumours, mucinous tumours, tumour budding and microenvironmental factors such as immune cell infiltration [1,3-9].

Biomarker development aimed at guiding clinical decision-making for adjuvant therapy has been undermined by a lack of translation to clinical applicability, leading to a high attrition rate in effective cancer biomarkers. This is due in part to confounding issues such as inadequate study design, poorly selected clinical cohorts, technically inadequate assays, invalidated technology, inappropriate statistical analysis or lack of clinical relevance [10]. Thus, additional molecular-based biomarkers, informed by underlying CRC biology and the contributing role of the microenvironment, are required to stratify patients for biology-informed treatment.

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Comparison of single and multi-gene diagnostic testing: The EGFR paradigm

Molecular DNA-based biomarker testing, traditionally involving analysis of a single gene/mutation, have made important contributions in modern therapeutic decision-making in CRC, but may also have certain limitations. An example of the limitations associated with low throughput-gene testing is evident from the patient stratification approach used for cetuximab and panitumumab, monoclonal antibodies which target non-identical epitopes on the extracellular domain of *EGFR* [11]. Screening for mutations in *KRAS* exon 2 (codon 12/13) was initially employed to predict lack of response to anti-EGFR therapeutics, but recently studies postulate potential greater clinical utility in assessing additional mutations in *KRAS* exon 2, 3, and 4 and *NRAS* exons 2, 3, and 4, *PIK3CA* and *BRAF* mutations and *HER2* amplification [12-14]. Multi-gene assays, which encompass screening of at least *NRAS*, *KRAS*, *PIK3CA* and *BRAF* mutations, could enable better characterisation and ultimately more precise targeting of several druggable EGFR pathway components using therapeutic combinations to circumvent chemoresistance [15]. Transcriptional outlier analysis can be used to identify high expression of mRNA transcripts in individual samples, in comparison to the remainder of the cohort, which encode proteins that can be therapeutically targeted enabling individualised treatment options. Transcriptional outlier analysis of cetuximab resistant CRC cell lines, which were wild-type for KRAS, NRAS and BRAF has enabled the identification of tyrosine kinase that can be therapeutically targeted to overcome cetuximab resistance [16].

Requirement for a common approach to transcriptomic analysis of clinical specimens

In recent years, numerous efforts to precisely define the molecular landscape of CRC using transcriptional array-based studies have enabled classification of patients into 3-6 subgroups based on their underlying gene expression [17-22]. Recently, a CRC Subtyping Consortium (CRCSC) established a consensus molecular CRC classification comprising four subgroups

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(CMS1-CMS4) based on gene expression profiling (GEP) data from six key molecular taxonomy papers [23]. The CRCSC classifier demonstrated a robust performance across GEP platforms and sample collections, although the clinical value of prospective patient classification according to CMS subgroup for treatment selection remains to be tested. Importantly, while each study has identified prognostic biology associated with their identified subtypes, with the exception of the microsatellite instability (MSI) subtype (CMS1) and another subtype defined by high expression of mesenchymal genes (CMS4) [23] there are very few similarities between biological characteristics of the biomarker-driven classifiers that select these subgroups. On detailed examination, of the six classification signatures used in the CRCSC study, we identified only one gene, Quinolinate phosphoribosyltransferase (*QPRT*), present in all signatures [24] (Figure 1). The protein encoded by the *QPRT* gene is involved in *de novo* NAD biosynthesis using quinolinic acid [25]. The lack of common genes in different molecular, predictive or prognostic classifiers is often due to a combination of (i) small training sets, (ii) cohorts being limited to one institution or (iii) samples from patients with differing percentages of AJCC stages being used in signature generation, resulting in low reproducibility in independent datasets [26,27].

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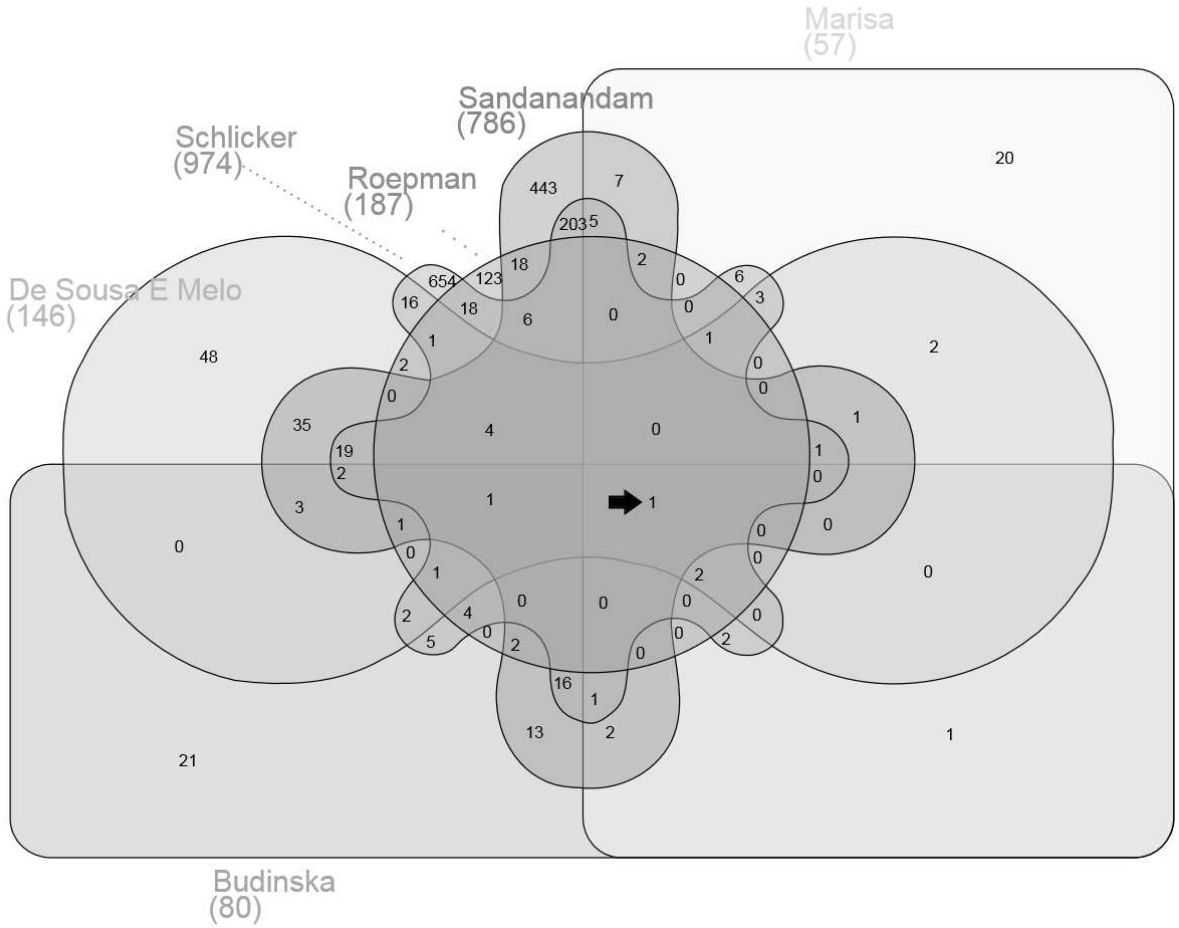

Figure 1. Lack of commonality between molecular taxonomy classification signatures. A Venn diagram, using InteractiVenn, was created, incorporating six published molecular taxonomy classification signatures used to create the CMS classification system [24]. Only one gene, highlighted by the black arrow, encoding Quinolinate Phosphoribosyltransferase was shown to be common among all six signatures.

The lack of commonality that we have highlighted between gene expression signatures may potentially be one of the reasons why multi-gene biomarker development rarely translates to a validated clinical test. Therefore, there is a requirement to examine the multi-gene biomarker development pathway to identify study design issues or technical variability that compromise robust gene expression signatures development for stratification of patients by prognosis or response to treatment. The multi-step biomarker validation pathway involves

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assessment of; (1) availability and quality of clinical specimens, (2) transcriptomic assay performance characteristics, (3) data pre-processing algorithms, (4) mathematical predictor model development (5) and assessment of its performance, (6) clinical interpretation of the test result, (7) clinical trial design, (8) ethical, legal, and regulatory issues [28].

In this review, we focus on the early steps of this biomarker validation pathway and examine in detail the level of scrutiny an investigator, looking to initiate a transcriptomic profiling study, should achieve to negate the introduction of study and/or technical variability. To help the reader understand the complexity involved in transcriptome profiling studies, we have highlighted the different methods published by independent CRC transcriptome profiling studies[29-63]. In the online supplementary table 1, we provide a summary of 57 transcriptome profiling studies and detail relevant information regarding the study cohorts such as small cohort sizes, different proportions of patients with stages 1-4 and number of study sites. We also highlighted that groups have differences in the level of tumour content they found acceptable for the study and the way in which the tissue was preserved. There are two common tissue preservation methods currently used for GEP studies; fresh frozen (FF) and formalin fixed paraffin embedding (FFPE), with FFPE almost universally used, due to retention of morphological features. FFPE is a well-established preservation method for histopathological assessment but results in extensive RNA fragmentation and cross-linking, impacting on high quality GEP. It is advantageous if GEP studies can address the RNA fragmentation and cross-linking issues, thus enabling FFPE samples to be utilised, as it is estimated that a billion FFPE samples are archived in hospitals and tissue banks, often with substantial clinical follow up [64].

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Aside from variables related to collection, preservation and storage of the tissue we noted that investigators often used different clinical endpoints to define good and poor prognosis which could potentially confound validating gene expression signatures on independent cohorts. To complicate matters, investigators have employed different RNA extraction protocols and microarray platforms from a number of different vendors (Affymetrix, Illumina, Agilent, Almac) to perform the quantitation of the RNA transcripts and the probe sets utilised by different array manufacturers can differ in size and also the target region they anneal to. Therefore, it is not uncommon to find in some validation studies that expression data derived from specific probe sets is often omitted when comparing a gene signature established using one microarray platform with gene expression data derived from a different vendor's platform. Of note, published studies utilised different pre-processing algorithms such as RMA, MAS 5.0, iterPLIER and other vendor specific packages which can affect the outcome of whether a gene is included or excluded in a multi-gene biomarker. We also detail the number of studies which do not conduct an independent validation of their developed gene expression signature. In Table 1A-C, we provide a synopsis of the online supplementary data to highlight the technical variability that occurs within multi-gene signature based biomarker CRC studies aimed at defining molecular taxonomy [17-22], patient prognosis [6,13,65-73] or treatment response [74-78]. Standardisation of these studies was commonly confounded by an incomplete control of pre-analytical variables, variable performance on different high-throughput technologies, use of an assortment of bioinformatics curation approaches. Additionally, the resulting multi-gene signatures were often not subjected to validation on an independent cohort of patient samples. While the studies detailed in Tables 1A-C primarily focus on array-based studies, several reports in the last 3-4 years have begun to describe the quantitation of RNA transcripts using a next generation sequencing approach, (RNA-seq), on FF resected tissue from CRC patients [79-82].

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This review identifies sources of technical variability in the GEP array-based biomarker development pipeline using FFPE CRC tumours. We categorise pre-analytical and analytical variables into nine distinct sections which can confound development and validation of published molecular signatures, to aid in the standardisation of the pipeline. This standardised approach is currently being implemented in the transcriptional profiling of 2000 CRC FFPE samples within the Medical Research Council and Cancer Research UK funded Stratification in COloRecTal cancer (S:CORT) consortium [83]. We also detail how eight of the nine categories of technical variability that we identify, also have application to quantitation of RNA transcripts using a NGS RNA-seq approach.

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Table 1A Molecular Classification Studies

Validation Group Within Original Paper	Spec (%)	ND	ND	ND	ND	ND	ND
	Sens (%)	ND	ND	ND	ND	ND	ND
	Acc (%)	ND	ND	ND	ND	ND	ND
	Cohort (n=)	1472	744	997	1643	2102	543
	Independent	Yes	Yes	Yes	Yes	Yes	Yes
Platform and method to derive multi-gene signature	Gene Signature	57	786	146	1042	83	187
	Pre-Processing Algorithm	RMA	RMA	fRMA	RMA	quantile normalization	Agilent Feature Extraction Software
	Array Platform(s)	Affy (U133A Plus 2.0)	Affy (Exon 1.0 ST, U133, U133Plus2.0), Agilent (G4502A, Human 44K), Rosetta human 23K array	Affy (U133Plus2.0, U133A, U133A2), Agilent (G4502A)	Affy (U133A Plus 2.0)	Affy (U133Plus2.0, U133A, U133A&B) Almac (Colorectal DSA) & Agilent (G4502A)	Agilent (4x44K Microarrays)
RNA Profiling Objectives	End point used for analysis	N/A	N/A	N/A	N/A	N/A	N/A
	Prognostic / Predictive/ Mol Class	Molecular Classification	Molecular Classification	Molecular Classification	Molecular Classification	Molecular Classification	Molecular Classification
Sample Details	Preservation	FF	INP	INP	INP	FF and FFPE	FF
	Tumour (%)	INP	INP	INP	INP	INP	INP
	Tissue Used	T	T	T	T	NM + T	T
Patient Cohort Information	Training (n=)	443	445	90	62	1177	188
	Treat Naive	Yes	INP	INP	INP	INP	INP
	Coll Sites	Multiple	Multiple	Multiple	Multiple	Multiple	Multiple
	Adenoma	0	INP	INP	INP	INP	0
	Stage 4	143	INP	INP	INP	INP	8
	Stage 3	318	INP	INP	INP	INP	279
	Stage 2	469	INP	INP	INP	INP	420
	Stage 1	85	INP	INP	INP	INP	24
	Total Pt No.	1472*	1189	1087	1705	925	731
Ref No.		23	26	25	27	22	24

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Table 1B Predictive Multi-gene Signature Studies

Validation Group Within Original Paper	**Spec (%)**	ND	ND	ND	ND	INP
	Sens (%)	ND	ND	ND	ND	INP
	Acc (%)	ND	ND	ND	ND	INP
	Cohort (n=)	7	0	0	0	39
	Independent	54 gene sig not validated	No - none performed	No - none performed	No - none performed	Yes
Platform and method to derive multi-gene signature	**Gene Signature**	54	54	7	14	miR-107 & miR-99a-3p
	Pre-Processing Algorithm	Normalised using BRB Array-Tools package	Normalised using BRB Array-Tools package	RMA	MAS 5.0	normalized to MammU6
	Array Platform(s)	NCI cDNA & oligo microarrays	NCI cDNA & oligo microarrays	Affy (U133A Plus 2.0) TaqMan (Low-Density Arrays)	Affy (U133A&B)	TaqMan (Human MicroRNA Card set v2.0)
RNA Profiling Objectives	**End point used for analysis**	preoperative 5-FU based CT/RT response	preoperative 5-FU based CT/RT response	5FU-based chemo response	FOLFIRI-based chemo response	FU-based chemo response
	Prognostic / Predictive/ Mol Class	Predictive	Predictive	Predictive	Predictive	Predictive
Sample Details	**Preservation**	FF	FF	FF	FF	FF
	Tumour (%)	INP	INP	> 50	INP	> 50
	Tissue Used	T	T	T	T	T
Patient Cohort Information	**Training (n=)**	23	23	90	21	39
	Treat Naive	No	No	No	Yes	Yes
	Coll Sites	1	1	4	1	4
	Adenoma	0	0	0	0	0
	Stage 4	0	0	90	21	78
	Stage 3	22	21	0	0	0
	Stage 2	8	9	0	0	0
	Stage 1	0	0	0	0	0
	Total Pt No.	30	30	90	21	78
Ref No.		42	43	44	45	46

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Table 1C Prognostic Multi-gene Signature Studies

Validation Group Within Original Paper	**Spec (%)**	ND	ND	ND	85	ND	86
	Sens (%)	ND	ND	ND	65	ND	88
	Acc (%)	ND	ND	ND	INP	ND	86
	Cohort (n=)	780	0	0	110	0	29
	Independent	Yes	No - none performed	No - none performed	Yes	51 gene sig not validated	Yes
Platform and method to derive multi-gene signature	**Gene Signature**	CDX2	30	Recurrence (7) FU/LV benefit (6)	7	51	10
	Pre-Processing Algorithm	RMA	RMA	normalized relative to 5 reference genes	MAS 5.0	RMA and iterPLIER	Median normalised, GeneSpring GX version 7.3
	Array Platform(s)	Affy (U133 Plus 2.0, U133A, U133A2 & U133A 2.0)	Affy (U133A)	RT-qPCR	Affy (U133A)	Affy (U133A, U133A Plus 2.0, Exon 1.0 ST)	Affy (U133A Plus 2.0)
RNA Profiling Objectives	**End point used for analysis**	5 yr disease free	5 yr disease free	3 yr disease free	3 yr disease free	recurrence associated transcription factors	Metastasis-prone gene classifier
	Prognostic / Predictive/ Mol Class	Prognostic	Prognostic	Prognostic (Genomic Health - OncotypeDX)	Prognostic	Prognostic	Prognostic
Sample Details	**Preservation**	INP	FF	FFPE	FF and FFPE	INP	INP
	Tumour (%)	INP	> 80	INP	≥ 70	INP	INP
	Tissue Used	NM + T	T	T	T	NM + T	T
Patient Cohort Information	**Training (n=)**	2329	18	1851	123	424	160
	Treat Naive	INP	Yes	Yes	Yes	Yes	INP
	Coll Sites	Multiple	1	4	3	INP	INP
	Adenoma	0	0	0	0	20	INP
	Stage 4	27	0	0	0	12	INP
	Stage 3	330	8	844	0	49	INP
	Stage 2	327	10	1007	233	340	INP
	Stage 1	96	0	0	0	23	INP
	Total Pt No.	780*	18	1851	233	444	189
Ref No.		8	34	3	35	36	37

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Table 1C Prognostic Multi-gene Signature Studies (Cont'd)

Validation Group Within Original Paper	**Spec (%)**	ND	ND	INP	ND	86
	Sens (%)	ND	ND	INP	ND	96
	Acc (%)	ND	ND	INP	ND	ND
	Cohort (n=)	206	0	460	0	134
	Independent	Yes	No - none performed	Yes	No - none performed	Yes
Platform and method to derive multi-gene signature	**Gene Signature**	18	8	six miRNAs	6 lncRNA	64
	Pre-Processing Algorithm	Agilent Feature Extraction Software	quantile normalization	quantile normalization	quantile method by GeneSpring GX v12.1	RMA
	Array Platform(s)	Agilent Whole Human Genome 4x44K Microarrays	Illumina (DASL Cancer Panel)	Sun Yatsen University (1894 miRNA probe array)	Arraystar (Human LncRNA Microarray V3.0)	Almac (Colorectal Cancer DSA)
RNA Profiling Objectives	**End point used for analysis**	5 yr disease free	3 yr disease free	5 yr disease free	Metastasis lncRNA prognosis classifier	BRAF-mutant gene prognosis classifier
	Prognostic / Predictive/ Mol Class	Prognosis (Agendia NV - ColoPrint)	Prognostic	Prognostic	Prognostic	Prognostic
Sample Details	**Preservation**	FF	FF	FFPE	FF	FFPE
	Tumour (%)	INP	> 80	> 80	INP	INP
	Tissue Used	T	T	T	T	T
Patient Cohort Information	**Training (n=)**	188	95	275	12	688
	Treat Naive	INP	Yes	Yes	Yes	Yes
	Coll Sites	4	1	4	1	Multiple
	Adenoma	0	0	0	0	0
	Stage 4	8	0	0	5	0
	Stage 3	118	60	0	3	580
	Stage 2	214	35	735	3	108
	Stage 1	54	0	0	1	0
	Total Pt No.	394	95	735	12	822
Ref No.		38	39	40	41	18

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Table 1. Summary of CRC GEP studies. Variables which can impact on the success of gene signature development were detailed and for the purposes of the table, grouped into 5 overarching categories: patient cohort information; sample details; RNA profiling objectives; array platform and methodology used to derive multi-gene signatures; and validation group within original paper. Data were recorded for a select number of studies relating to (A) molecular taxonomy/classification studies (B) predictive signatures and (C) prognostic signatures. Abbreviations used in table are as follows; specimen collections sites (coll sites), treatment naïve (treat naïve), molecular classification (mol class), accuracy (acc), sensitivity (sens), specificity (spec), non-malignant tissue (NM), malignant tissue (T), robust multiarray analysis (RMA), frozen RMA (fRMA), Affymetrix (affy) and information not provided (INP). The full list of publications reviewed for this study can be found in Supplementary table 1.

Variable 1 – Quality and quantity of samples

Prior to commencing array-based or RNA-seq studies, it is important to determine if sufficient samples (with adequate malignant tissue percentages) are available for analysis. As discussed earlier, gene lists developed for classification, prediction or prognosis or treatment response exhibit little commonality between studies and often are heavily dependent on the training group selected from the overall cohort. From reviewing supplementary table 1 there is a marked difference in the number of genes included within a gene signature with some signatures having less than 10 genes whereas one publication used 1042 genes to perform molecular classification. Some papers suggest to have a small number of genes within a gene signature to enable the test to be transferred to other platforms such as quantitative PCR[27]. One group has used a mathematical model called the "probably approximately correct" learning technique to predict the number of patients required within a randomly assigned

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training cohort to enable a 50% overlap between 70-gene signatures generated from the randomly assigned training cohorts[27]. This study indicated that 2000-3000 samples are required in order to generate a robust 70-gene signature, yet of the 57 publications we identified from PubMed searches, only one study had over 2000 samples in the training cohort[27]. Two studies used only cell lines and 33 studies had fewer than 100 patients in the training cohort, providing a potential explanation as to why many signatures fail to demonstrate clinical value during validation on independent cohorts.

Transcriptomic-based biomarker development must conform to appropriate ethical approval, attention to clinical and pathological characteristics, inclusion/exclusion study criteria and consultation with clinicians and statisticians. We noted a large disparity in tumour staging and tumour content, preservation method, treatment status and metastatic status in studies reviewed in Table 1. In addition, some studies were performed on a single site whereas others were across multiple sites, with little evidence of standard operating procedures (SOPs) being applied across study centres. Introduction of sampling bias must be considered as tissue blocks selected based on availability of tissue may be biased towards more bulky tumour subtypes. Defining sample type, method of preservation/storage, transportation, tissue area to macrodissect and volume of malignant tissue required by the transcriptomic facility is essential in order to account for all these variables at the analytic stage [84]. Determining the projected attrition rates throughout the analytical pipeline, by firstly performing a pilot study, will allow an informed assessment of minimum tissue requirements and a better estimation of whether sufficient samples exist [85]. Before study commencement, SOPs should be developed to describe tissue procurement, processing, storage, purification and quantitation of nucleic acids and microarray protocols employed.

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Variable 2 - Tissue Resection

During surgical resection, the type of drugs employed (including anaesthetics) and the duration of exposure in patients, can have substantial effects on subsequent RNA profiles [86]. In surgery, reducing warm (disruption of blood supply while removing the tissue) and cold (time between tumour removal and formalin fixation) ischemia times can improve RNA integrity. Some studies indicate CRC tissue shows little variation in GEP with changes in warm or cold ischaemia times whereas other studies have demonstrated a tissue-specific degradation of RNA with extended ischaemia times [87-90]. Regulator of G-protein signalling 1 (RGS1) and eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) have been identified as potential markers of primary colorectal carcinoma tissue quality and post-operative tissue quality respectively [91]. Therefore warm/cold ischaemia duration, type of drugs administered during surgery, along with type and location of resected tissue should be recorded as potential confounding variables for array-based or RNA-seq studies of tissue derived from different organs.

Variable 3 - Formalin fixation and paraffin embedding effect on mRNA

AJCC staging requires histopathological examination of resected tumours. Only six of the 57 (11%) studies that we reviewed were exclusively performed on FFPE tissue, while two studies (4%) used both FF and FFPE material from different cohorts, with the remainder of studies being FF or the information was not provided. A FF tissue GEP signature may also require validation with FFPE material to ensure probesets are not adversely affected by formalin fixation [17]. Formalin fixation alternatives do exist (Z7, RCL2®, PAXgene®, Allprotect® and RNAlater®), although formalin fixation is considered the standard processing method in clinical practice, and the reference approach for routine diagnostics validations [92,93].

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Increasing tumour size leads to increased time for the formalin to penetrate the inner core, resulting in potential tissue autolysis [94]. Therefore, potential differences in gene expression profiles observed between small biopsy material and large resection material may simply arise from differences in formalin penetration and fixation times that are dependent on tumour size. FFPE recommendations for nucleic acid studies include ensuring pre-fixation time is minimised, use of cold (4^{o}C) 10% neutral buffered formalin and limiting the duration of fixation (3 to 6 hours) [94]. RNA integrity from FFPE tissue is poorer in comparison to snap frozen, although some studies indicate that having a quick, uniform FFPE method can lead to comparable results [95]. However, working with FF samples also has its difficulties in routine practice, and a degree of attrition is equally expected with this type of sample.

While SOPs should be implemented in transcriptomic studies, investigators need to be wary, particularly in retrospective studies where clinical material is being received from multiple sites, that processes such as tissue fixation are often not standardised and can lead to variation in resulting molecular studies[96,97]. Therefore, variations in tumour blocks from different hospital sites, surgical resection of the tumour, pre-fixation times and methods should be recorded, and the effects of these variables on subsequent array-based or RNA-seq derived molecular signatures examined.

Variable 4 - FFPE Tumour Block Storage

Following FFPE processing, initial guidelines recommended blocks are stored without protection from light or air, provided the uppermost section is discarded for RNA preparations and extractions are performed within a year [98]. Improvements in RNA extraction, *in vitro* transcription (IVT) and microarray platforms have led an increasing

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number of studies utilising FFPE blocks which have been stored for longer than a year, with a successful RNA profiling outcome [99]. The S:CORT programme has performed RNA profiling on FFPE tumour blocks from randomised control trials which have been stored at room temperature for up to 21 years. Figure 2 illustrates that we did not identify a correlation between tumour block age and the following parameters; total RNA 260/280 and 260/230 ratios, total RNA yield, cRNA yield, QC metrics such as 3'/5' ratio of actin or the overall success rate for transcriptional profiling of the sample using the FFPE-optimised Almac Xcel Array. On the Xcel array, probes which have been shown to be stable or having comparable longitudinal stability during formalin fixation or subsequent storage were included. For arrays containing probes which have not been optimised for FFPE material, we recommend recording FFPE tumour block storage duration and using material for RNA extraction within 2 weeks of sectioning from the block to ensure storage-dependent RNA degradation does not further compromise any array-based or RNA-seq study derived signature.

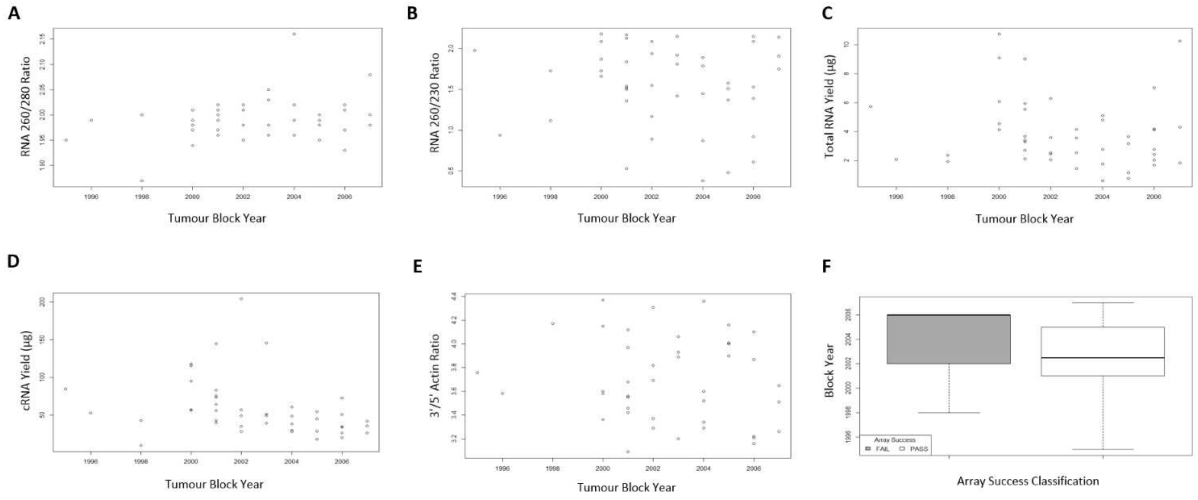

Figure 2. Assessment of the effect of tumour block age on RNA and cRNA parameters and success in generating a CEL file. RNA extraction was performed using the Roche High Pure RNA Paraffin Kit and the resulting 260/280, 260/230 and total RNA yield for each sample was recorded. During the Affymetrix 3' IVT PLUS Reagent Kit stage, 260/280, 260/230 and total yields for cRNA and double stranded cDNA were recorded. Following

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hybridisation, washing and scanning, the CEL files were normalised using Robust Multi-array Average (RMA) and QC metrics, including actin 3'/5' ratio, were examined using Affymetrix Expression Console Software. Dot plots examining the relationship between tumour block year and total RNA 260/280 **(A)**, total RNA 260/230 **(B)**, total RNA yield **(C)**, cRNA yield **(D)**, actin 3'/5' ratio **(E)** and whether the sample was subsequently hybridised to an array **(F)**.

Variable 5 – Tissue macrodissection

Prior to commencing the study, the minimum quantity of RNA that can be analysed using the laboratory's standard RNA profiling protocol and selected GEP platform, without confounding the resulting RNA profile, needs to be determined. This will enable investigators to estimate tissue quantity requirements and therefore determine how many sections, of a specified thickness, need to be prepared from each tumour block. In our current S:CORT transcriptome profiling study, when using biopsy material, the annotated tumour region can be very small (surface area < 20 mm^2 per section) and therefore require a larger number of sections to be prepared in comparison to the number of sections prepared from resection blocks. This is particularly relevant if the study involves limited biopsy material, or if a multi-omics approach is required from the same tissue block.

An additional section is prepared for H&E staining to enable a pathologist to mark areas of tumour presence and assess tumour content. Tumour cell isolation from FFPE sections requires macrodissection of pathologist-annotated tumour regions, which is prone to individual subjectivity [100]. As tumours are not uniform in shape throughout the tissue block, many investigators prepare consecutive sections, placed onto numbered slides corresponding with the order that they were sectioned in, with the top, intermediate and bottom sections

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being used for H&E staining [101]. Table 1 highlights that the tumour content percentage acceptable for the published studies is not reported in over half the studies, or large variations in acceptable tumour content (mean 68% with a range of 30-90%) are reported. A high degree of variation exists between different pathologists' assessment of tumour content in defined samples, which could ultimately diminish the prognostic/predictive power of any resultant GEP signature [102]. In collaboration with an industrial partner, our laboratory has developed a digital pathology platform for automated tumour annotation and percentage tumour nuclei measurement, which correlates well with benchmark tumour cell counts [100].

Studies assessing the impact of lower tumour content (cellularity) highlight that pathological factors such as the presence of invasive cells and budding at the tumour margin can impact on the expression profile and be predictive of survival [103]. The tumour content threshold and the number and type of cells present in the non-tumour areas can have a major impact on the expression level of specific genes. Inaccurate assessment of tumour content may impact on GEP-based clinical diagnostics and GEP-based methods to evaluate tumour content may need to be developed to complement the pathologist assessment [104]. In CRC, two of the four consensus CMS groups are based on relative changes in the stromal content, namely increasing fibroblasts (CMS4) or immune cell infiltration (CMS1) in the tumour microenvironment, with the type and location of immune cells having a well-defined impact on clinical outcome [105]. Microarray profiling of multiple regions within individual CRC FFPE samples by our group has highlighted that the region of tumour used for GEP can confound patient CMS classification, further confirming how CRC classification signatures are fundamentally affected by intratumoural stromal heterogeneity as well as the underlying tumour biology [84]. A further complication is the differences in the GEP from primary tumour and metastatic sites, arising from the differing tumour microenvironment, underlying

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biological factors such as clonality in the primary tumour, or metastatic cells having undergone an epithelial mesenchymal transition [84]. Creating an electronic repository of the pathologist annotated H&E images will enable researchers to review images to validate array-based or RNA-seq derived multi-gene signatures, which may be indicative of immune or fibroblast cell infiltration.

Variable 6 - RNA Extraction

Developments in commercial total RNA extraction kits have led to more FFPE tumours becoming suitable for analysis. Although there are protocol variations between kits, they typically involve tissue dewaxing by a solvent such as xylene and sequential ethanol hydration steps. Recent non-toxic approaches have been reported, such as elevated heat, sonication or proprietary buffers to remove the paraffin. This is followed by macrodissection prior to enzymatic proteolysis of the tissue by a proteinase K incubation. It is reported that proteinase K can reverse mono-methyl nucleotide modifications and break the RNA–protein cross-links formed during formalin fixation [95,106]. A DNase step is often included to remove genomic DNA contamination from downstream applications, while some report additional steps to reverse RNA cross-linking which occurs during formalin fixation. RNA purification is commonly performed using spin column-based technology, utilising glass fibres or suitable matrices. Contaminants and/or carry-over of nucleic acid based carriers during RNA purification can inhibit downstream enzymatic applications and therefore compatibility with downstream array based technologies needs to be assessed.

Recent research also suggests other RNA populations, such as microRNA (miRNA), may be more stable in samples with heavily degraded mRNA, particularly from FFPE tissue [107]. Investigators may therefore require either array-based or RNA-seq platforms to quantitate

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levels of small RNA populations such as miRNA. For both array-based and RNA-seq studies, it is currently not possible to measure mRNA and miRNA transcripts simultaneously. RNA-Seq of mRNA and miRNA require different chemistries used for ligation of RNA-Seq adaptors during library preparation protocols and array based platforms use different chemistries for ligation of the biotin signalling molecule to miRNA or templates derived from mRNA. Therefore, separate protocols for analysis are required and the financial feasibility of running both mRNA and miRNA expression studies in tandem needs to be considered. For investigators looking to quantitate miRNA using either an array-based or RNA-seq approach, ensuring the RNA extraction protocol will retain small RNAs (<200 nucleotides) is essential, as it has been reported that commercial RNA extraction kits exhibit variable success in retaining small non-coding RNAs [108].

Variable 7 - Quantitation and Assessment of RNA Quality

The FFPE process leads to extensive RNA fragmentation and cross-linking, impacting on RNA profiling success. A combination of RNA quantitation and integrity/quality assessments are warranted. There are a number of spectrophotometric (Nanodrop) and fluorescence based (Qubit and Quant-iT) methods for RNA quantitation. Spectrophotometric methods require little preparation time and function by measuring absorbance at 260nm to determine nucleic acid quantity, with measurements at 230nm and 280nm indicative of solvent and protein contamination respectively [109]. Unfortunately, spectrophotometric methods can report artificially high concentrations, due to simultaneous measurement of both RNA and DNA readings within the sample. Fluorescence-based methods have suitable levels of sensitivity for RNA, although the dynamic measurement range is smaller than for spectrophotometric methods, with a frequent requirement for sample dilution, resulting in longer assay times [110]. Due to FFPE-dependent fragmentation of RNA, a separate method to examine RNA integrity

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is often reported, using the Agilent 2100 Bioanalyser along with a proprietary algorithm to establish a RNA integrity number (RIN). There is no consensus threshold RIN value for downstream applications and we noted large variations in this threshold value between studies [40,53,54,63,111]. From our microarray experience, we have generated RNA profiling data, deemed successful by assessment of QC metrics such as 3'-5' ratio of housekeeping genes, from samples with RIN values as low as 1.3. FFPE also results in chemical modification of nucleic acids and crosslinking with proteins, which would not be detected by the Agilent 2100 Bioanalyser [98]. Performing a PCR-based screen to assess RNA sample quality prior to proceeding to analysis on a comparatively expensive array is recommended [112].

Variable 8 - RNA Profiling Platform

The Biorepositories and Biospecimen Research Branch (BBRB) of the Cancer Diagnosis Program (CDP) at the National Cancer Institute advises the use of validated assays, standardized training of technical staff in the performance of the assay, lot uniformity of reagents, inclusion of appropriate type and number of quality control samples, randomisation when possible and standardised methods for documenting and interpreting testing results [113]. If a GEP signature is to be developed that can harness the power of large archived clinically annotated cohorts of FFPE tumour blocks, then inclusion of samples with moderate to severe RNA degradation will need to be accommodated, impacting on array platform selection. Studies have demonstrated specific mRNA transcripts are more susceptible to degradation, due to FFPE block processing or storage [61,114]. The 5' end of the mRNA transcript is more sensitive to FFPE-dependent RNA degradation, resulting in development of arrays with probe designs focused on the extreme 3' of mRNA (Affymetrix Human X3P Array & Almac Xcel Array) as these regions are more stable in FFPE tissue. The use of IVT reagents specifically designed for FFPE-extracted RNA which include both oligo-dT primers (bind to polyA tail

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and specific for mRNA) and random hexamers (anneal throughout the target molecule) for first strand cDNA synthesis. This enables amplification at the 3' end and throughout the whole transcript, which is more applicable to partially degraded mRNA. In GEP studies, recording the type of IVT, labelling and hybridisation kits and associated lot numbers, performance at intermediate steps (such as cRNA and cDNA quantity), dates of IVT/hybridisation/scanning, hybridisation time, chip lot number and operator ID is advised (Table 2).

Category	Recorded Variables
Tumour Block	Hospital Site
	Anaesthetic Used
	Warm & Cold Ischaemia Times
	Sample Type (Biopsy / Resection)
	Tumour Block Age
	Tumour Block ID
Macrodissection	Macrodissection Date
	Pathologist Responsible for Annotation
	Electronic repository location of annotated images
	Section Thickness (µM)
	No. of Sections Macrodissected
RNA Extraction	RNA Extraction Kit Manufacturer
	RNA Extraction Kit Catalogue No.
	RNA Extraction Kit Lot No.
	RNA Extraction Date
RNA Quantitation / Integrity Assessment	RNA Quantitation Method
	RNA Concentration (ng/µl)
	RNA 260/280 ratio
	RNA 260/230 ratio
	RNA Quantitation Date
	RNA Integrity Measurement Date
	RIN Value
Microarray Dates	IVT Date
	Technician Performing IVT Process
	Hybridisation Date
	Scanning Date
IVT Information	IVT Starting Amount of RNA (ng)
	cRNA Concentration (ng/µl)
	cRNA 260/280 ratio
	cRNA 260/230 ratio
	ds-cDNA Concentration (ng/µl)
	ds-cDNA 260/280 ratio

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	ds-cDNA 260/230 ratio
Hybridisation	Array Type
	Array Catalogue No.
	Array Lot No.
	Hybridisation Start Time
	Hybridisation Finish Time
	Hybridisation Total Time
Scanning Information	DAT Image
	Electronic Location of CEL File

Table 2. Pre-analytical and analytical variables. Suggested pre-analytical variables which can be recorded to help determine/minimise technical variable-dependent artefacts occurring during gene signature development. Analytical variables can be detailed to monitor performance of samples through RNA profiling and to enable development of exclusion/inclusion sample quality criteria.

Variable 9 - Quality Control (QC) Pipeline

Development of a QC pipeline for array-based or RNA-seq platforms, integrated into the SOP, enables monitoring of the successful preparation of a sample through pre-analytical and analytical steps. From a pre-analytical perspective, samples are blinded by assigning unique codes so operators are unaware of related clinical information. In large microarray studies, controlling batch effects is required to minimise artefacts occurring in transcriptomic signatures due to non-biological sample variation. Batch effects result from changes in room temperature, ozone level, sample degradation over time, changes in reagent lot numbers, different technical staff or equipment performance variation [115]. The Cancer Genome Atlas (TCGA) data has been generated from different platforms, laboratories and over different time periods, resulting in batch effects which has been problematic [115,116]. This led to TCGA developing statistical batch effect identification tools, enabling implementation of additional statistics to account for batch effects in downstream analyses [115].

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From an analytical perspective, a positive control is run alongside all batches, which can be sourced from the manufacturer or a third party. Alternatively, other investigators replicate the sample type being analysed in the study, which for the purposes of this review would be a FFPE treated CRC cell line. Employing a quality control chart such as a Levey-Jennings plot, to monitor the GEP performance of the RNA control over a period of time, highlights sample runs outside an operator defined number of standard deviations. Controls for the IVT and hybridisation stages are also advised to be included and more detail has been provided on the online supplementary files[117-122].

Examination of post-analytical variables commences with the initial QC following scanning by visually image inspection to identify abnormalities including dimness, uneven signal intensity, haze bands, crop circles, scratches, bubbles and areas of poor hybridisation (Figure 3).

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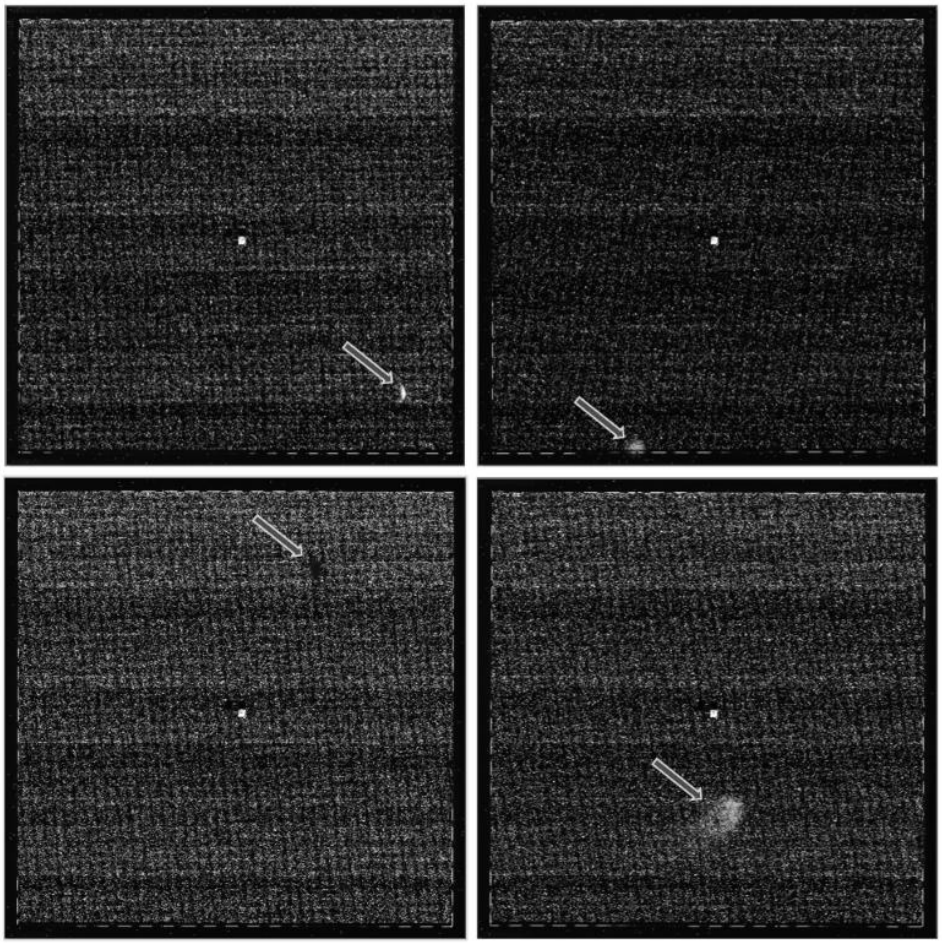

Figure 3: Assessment of the scanned Affymetrix Genechip array image for abnormalities. The images were accessed using the Affymetrix AGCC viewer function which are generated from the DAT file, which contains the pixel intensity values measured during scanning. Red arrows indicate areas of abnormality in the images from four CRC samples hybridised to the Almac Xcel array.

It is important that dry lab analytical processing methods have a similar degree of validation, maintenance, document-control and quality assurance as wet lab procedures[123]. Subsequently, data pre-processing is performed using commercial or open source analytical platforms to perform a three step process comprising (a) background adjustment, (b) normalisation and (c) summarisation. To ensure hybridisation differences between arrays are accounted for, measurements from different arrays undergo normalisation to ensure array

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results are comparable[124]. Normalisation algorithms (MAS5, RMA, GCRMA etc) are different normalisation methods which will lead to different final expression values and therefore the normalisation method should be reported[124-128]. For IVT QC, an assessment takes three different approaches (i) positive control performance assessment, (ii) assessing endogenous RNA integrity through RNA degradation plots or examination of 3' to 5' ratios of housekeeping genes such as GAPDH, (iii) assessing the signal intensity from spike-in polyA or ERCC controls. For hybridisation QC, assessing the signal intensity from the four hybridisation control transcripts ensures consistent hybridisation within batches. Additional QC metrics related to signal distribution and array comparison can be performed to ensure all samples in a batch are within established thresholds. In the absence of an external quality assessment/proficiency scheme we, similar to other RNA profiling studies, have examined concordance in gene expression profiles from 42 samples measured both at our department and an independent transcriptome profiling centre (Figure 4) [129,130].

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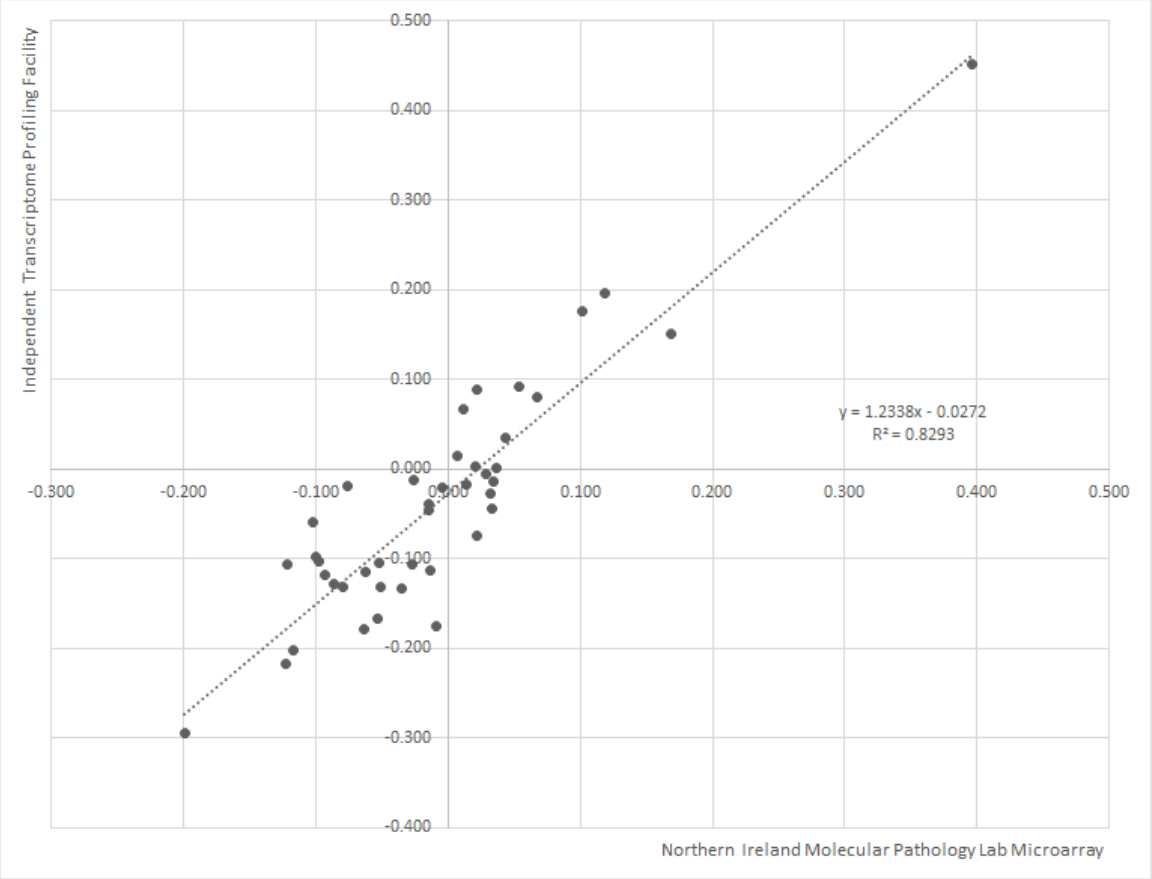

Figure 4: Concordance assessed using a published gene expression score, between RNA profiles measured at our laboratory and an independent transcriptome profiling facility using FFPE CRC samples. Consecutive sections from the same 42 FFPE tumour blocks were sent to two different RNA profiling facilities. RNA extraction was performed using the Roche High Pure RNA Paraffin Kit, 3' IVT target preparation and labelling was performed using the Affymetrix 3' IVT PLUS Reagent Kit and GeneChip Hybridization, Wash, and Stain Kit (Site 1) or the NuGEN Ovation FFPE WTA System and Encore Biotin Module (Site2). Hybridisation, scanning and CEL file generation was performed in an identical manner using the Almac Xcel array.

A QC metrics report, including thresholds, accompanied by justification for inclusion or exclusion of samples from analysis should be included in publications [Table 3]. Furthermore, in the development of RNA profiling-based clinical diagnostic biomarker(s),

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QC data may provide invaluable information for the creation of inclusion/exclusion sample quality criteria. Tables 2 and 3 detail pre-analytical and analytical variables we record within the S:CORT programme to enable us to identify factors which may impact on GEP success [Table 2].

Category	Recorded Variables
Data Image File	Bubbles, Scratches, Uneven brightness
	Intensity of Image Brightness
QC Array Metrics (IVT Process)	Positive Control (Commercial RNA or FFPE) Cell
	3'/5' for GAPDH and Actin
	RNA Degradation Plot
	lys Average Signal Intensity
	phe Average Signal Intensity
	tyr Average Signal Intensity
	dap Average Signal Intensity
QC Array Metrics (Hybridisation)	bioB Average Signal Intensity
	bioC Average Signal Intensity
	bioD Average Signal Intensity
	Cre Average Signal Intensity
QC Signal Quality/Distribution	Scale Factor
	Signal Histogram
	Feature Intensity Box Plot
	Signal Box Plot
QC Array Comparison	PCA Analysis
	Pearsons or Spearmans Correlation Plots
	Hierarchical Clustering

Table 3. Quality control metrics. A list of quality control metrics which can be examined to ensure robust high quality data is included during downstream analysis. Lys, phe, tyr and dap are bacterially expressed RNA transcripts which have been modified to include a poly-A tail. Four transcripts encoded from E.coli genes bioB, bioC, bioD and the bacteriophage P1 recombinase gene, Cre, are utilised for hybridisation controls. PCA, principal component analysis.

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Prior to commercialisation of any multi-gene prognostic test, the gene expression signature will need to be validated on independent clinical cohorts. As shown in Tables 1A-C, and also in the full table accessible through the online supplementary data, we detail the limited number of published studies which perform an independent validation. Aside from validation of the relevance of the genes involved in the biomarker signature and ensuring the sensitivity and specificity of the test, additional work is necessary to validate the assay performance prior to commercialisation. Intra- and inter-assay precision, accuracy, linearity and sensitivity are required to be investigated for the specific assay. While sensitivity assessment of an immunoassay for a protein or hormone is relatively simple to conduct, RNA profiling from a tissue biopsy has additional complications such as ascertaining the tissue limitations that may limit the generation of sufficient RNA. Factors such as macrodissected area of the biopsy FFPE section, tumour cellularity and the impact of infiltration from fibroblasts and immune cells will also impact on gene express this threshold will be of use.

Commercialisation of transcriptomic based biomarkers

Several commercial multi-gene CRC prognostic tests have been developed from transcriptomic studies and the commercial product names are listed in the Prognostic / Predictive column in supplementary table 1. Commercial and research-based GEP signatures often result in a gene score, which is a continuous variable, with an assigned threshold to dichotomise samples [131-134]. When technical variability, as detailed in the nine variables above, occurs during transcriptomic analysis, this can lead to misclassification of GEP-derived risk scores close to the assigned threshold [128,130]. Manufacturers suggest requesting an additional sample from a different area of the tumour/body to repeat RNA profiling or to consider the GEP risk score alongside clinical parameters of prognostic/predictive value before reaching a final decision that may impact on therapy [62,128]. Given the complexity and

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inherent variability of the technology, GEP companies adopt a centralised lab approach and perform the test as a laboratory developed test (LDT) [135]. The centralised lab operating the LDT is subject to Clinical Laboratory Improvement Amendments (CLIA) laboratory testing processes (the ability to perform laboratory testing in an accurate and reliable manner). The LDT currently falls outside of FDA scrutiny for both the analytical (specificity, sensitivity, accuracy and precision) and clinical validity of *in vitro* diagnostic tests [136]. Therefore, LDT manufacturers can avoid clinical validation costs and are not subject to the same stringent pre-market review or post market surveillance as an FDA approved *in vitro* diagnostic would be required to undergo [137].

Conclusion

In precision medicine, there are continued discussions on what are the most optimal actionable biomarkers (i.e. biomarkers that will indicate particular therapeutic intervention). DNA-based biomarker assays are robust, but the number of actionable mutations is modest, resulting in clinical benefit for a limited number of patients. Protein expression by immunohistochemistry is widely available and relatively affordable, but there are limited informative markers and QA/QC technique concerns [138]. Delineating the transcriptional landscape has the potential to identify robust signatures that may inform stratification of patients for different treatment options. Future molecular transcriptomic approaches, undertaken by international consortiums with large cohort sizes, will require high-throughput standardised methodologies.

From published CRC GEP studies, we report pre-analytical, analytical and post analytical variables within a GEP study, where adoption of a more robust and standardised process, within a transcriptomics laboratory, would lead to a GEP based signature which would be

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more likely to pass future independent validation studies (Figure 5). The technical variables should be considered as part of a comprehensive approach to RNA profiling studies requiring input from a multidisciplinary team (clinicians, statisticians, bioinformaticians, research scientists, health economists) both in the planning, execution and clinical impact assessment.

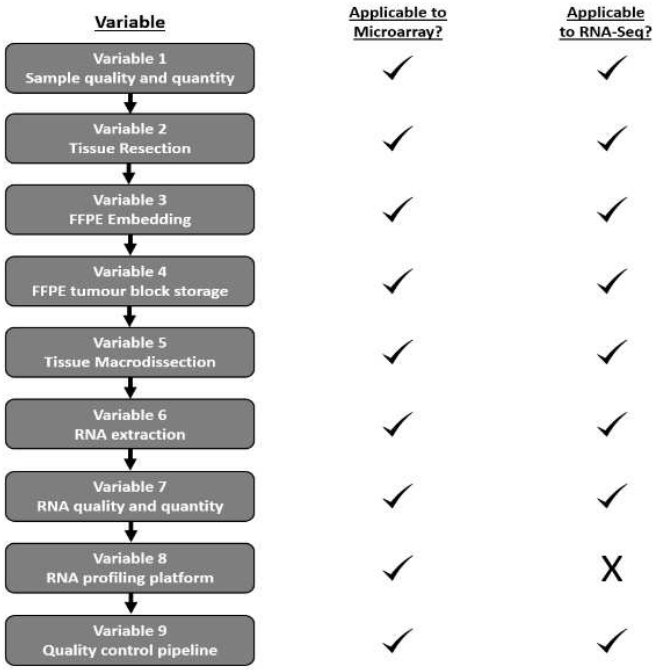

Figure 5: Flow diagram of the RNA profiling technical variables. This diagram depicts the nine different categories of technical variables that can affect results derived from high throughput RNA profiling technologies, such as microarray and RNA-Seq. It is recommended that investigators review the nine different categories and identify specific variables which are relevant to their particular RNA profiling studies.

Future development of *in vitro* diagnostic RNA profiling tests requires significant investment in technical and clinical validation by manufacturers, alongside development of an external quality assessment scheme. Economically, this investment needs to be balanced with considerations from the clinical diagnostics laboratory in purchasing and maintaining a RNA profiling platform, upskilling technical staff and considering RNA profiling test expenses

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which may limit the broad adoption of the test. Alternatively, RNA profiling, performed with consideration for the technical variables, could be used for GEP signature discovery before transferring to a more accessible technology such as a qPCR platform or miniarray for independent validation, test development and its commercialisation [62]. Irrespective of the approach, having a comprehensive understanding of RNA profiling technical variables, leading to consensus guidelines on how to identify and mitigate against them, will result in a more robust GEP signature suitable for clinical validation.

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