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A comprehensive overview of computational tools for RNA-seq analysis

Authors and affiliations

† These authors contributed equally to the work.

Dhrithi Deshpande†
Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Room 713. Los Angeles, CA 90089-9121, USA
ddeshpan@usc.edu

Karishma Chhugani†
Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Room 713. Los Angeles, CA 90089-9121, USA
chhugani@usc.edu

Yutong Chang
Department of Pharmacology and Pharmaceutical Sciences, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Room 713. Los Angeles, CA 90089-9121, USA
yutongch@usc.edu

Aaron Karlsberg
Department of Clinical Pharmacy, School of Pharmacy, University of Southern California, 1540 Alcazar Street, Los Angeles, CA 90033, US
akarlsbe@usc.edu
Caitlin Loeffler
Department of Computer Science, University of California, Los Angeles, 404 Westwood Plaza, Los Angeles, CA 90095, USA
cloeffler@ucla.edu

Jinyang Zhang
Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China
zhangjinyang@biols.ac.cn

Agata Muszyńska
Małopolska Centre of Biotechnology, Jagiellonian University, Gronostajowa 7A, 30-387 Krakow, Poland
Institute of Automatic Control, Electronics and Computer Science, Silesian University of Technology, Akademicka 16, 44-100 Gliwice, Poland
a.muszynska@uj.edu.pl

Jeremy Rotman
Department of Clinical Pharmacy, School of Pharmacy, University of Southern California, 1540 Alcazar Street, Los Angeles, CA 90033, USA
rotman@usc.edu

Laura Tao
Department of Computational Medicine, David Geffen School of Medicine at UCLA, 73-235 CHS, Los Angeles, CA 90095, USA
laura.k.tao@gmail.com

Lana S. Martin
Department of Clinical Pharmacy, School of Pharmacy, University of Southern California, 1540 Alcazar Street, Los Angeles, CA 90033, USA
lana.mart@usc.edu
Brunilda Balliu
Department of Computational Medicine, David Geffen School of Medicine at UCLA, 73-235 CHS, Los Angeles, CA 90095, USA
bballiu@ucla.edu

Elizabeth Tseng
Pacific Biosciences
1305 O’Brien Drive, Menlo Park, CA 94025, USA
etseng@pacificbiosciences.com

Eleazar Eskin
Department of Computer Science, University of California, Los Angeles, 404 Westwood Plaza, Los Angeles, CA 90095, USA
Department of Human Genetics, David Geffen School of Medicine at UCLA, 695 Charles E. Young Drive South, Box 708822, Los Angeles, CA 90095, USA
Department of Computational Medicine, David Geffen School of Medicine at UCLA, 73-235 CHS, Los Angeles, CA 90095, USA
eeskin@cs.ucla.edu

Fangqing Zhao
Beijing Institutes of Life Science, Chinese Academy of Sciences, Beijing 100101, China
Key Laboratory of Systems Biology, Hangzhou Institute for Advanced Study, University of Chinese Academy of Sciences, Hangzhou 310024, China
zhfq@biols.ac.cn

Pejman Mohammadi
Department of Integrative Structural and Computational Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
Scripps Research Translational Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
pejman@scripps.edu
Abstract

RNA-sequencing (RNA-seq) has become an exemplar technology in modern biology and clinical applications over the past decade. It has gained immense popularity in the recent years driven by continuous efforts of the bioinformatics community to develop accurate and scalable computational tools. RNA-seq is a method of analyzing the RNA content of a sample using the modern sequencing platforms. It generates enormous amounts of genomic data in the form of nucleotide sequences, known as reads. RNA-seq analysis enables the probing of genes and corresponding transcripts which is essential for answering important biological questions, such as detecting novel exons, transcripts, gene expressions, and studying alternative splicing structure, to name a few. However, obtaining valid biological signals using these computational methods is challenging due to the limitations of modern sequencing technologies. These technological challenges have pushed the rapid development of many novel computational tools which have evolved and diversified in accordance with technological advancements, leading to the current
myriad population of RNA-seq tools. Our review provides a systemic overview of 235 available RNA-seq analysis tools across various domains published from 2008 to 2020. Additionally, we discuss the interdisciplinary nature of bioinformatics involved in RNA sequencing, analysis, and software development without laboring over the biological details.

**Keywords** RNA-sequencing, Transcriptome quantification, Differential gene expression, High-throughput sequencing, Read alignment, Bioinformatics

1. Introduction

Advances in high-throughput sequencing technologies, also known as next-generation sequencing (NGS), have enabled cost-effective probing of gene sequences in living organisms\(^1\). These sequencing technologies have been adapted to probe the RNA expression (mRNA or totalRNA) of an organism, known as RNA-seq. As a result of the continued efforts of the bioinformatics community, methods to analyze RNA-seq data have reshaped biomedical research to providing the unprecedented ability to robustly measure the expression levels of genes, alleles, and alternatively spliced isoforms directly from sequencing data. Biomedical researchers are often tasked with using RNA-seq computational methods which are typically available wrapped as software tools and packages. These methods require diverse computational skills to be used effectively. The lack of computational skills among the biomedical researchers limit the ability to unlock the full potential of RNA-seq analysis.
To address this barrier, we provide a comprehensive overview of the fundamentals of the RNA-sequencing and its associated computational methods. We also provide an overview of modern sequencing technologies and discuss the advantages and limitations associated with each type. We performed a survey of 235 computational tools designed for various domains of RNA-seq analysis that were developed between 2008 and 2020 (Figure 1a).

The average annual growth rate of computational tools developed for RNA-seq analysis was 114.4% from 2008 to 2014 (Figure 1a), but the rate of new tool development slowed after 2015; the average annual growth rate in tools from 2015 to 2020 was 8.97%. Of the 235 RNA-seq tools, most tools were developed for the transcriptome quantification (n=30) (Figure 1b) and the highest number of citations were in the domains of differential expression, followed by data quality control and transcriptome quantification (Figure 1c). Each category of RNA-seq analysis was dominated by a few of the most popular tools. In the Visualization category, the most cited tool (CummeRbund) has the largest proportion of citations per year (n=92.2%). On an average the most popular tool in each category accounted for 41.7% of citations every year. Additionally, we assessed the usability and archival stability of the computational tools designed for various types of RNA-seq analysis.

Our review intends to bridge the gap between computational and biological domains in RNA-seq analysis by providing a systematic review of the computational foundations coupled with technological aspects. This review will enhance the existing knowledge in computational and algorithmic foundations of RNA-seq analyses, leading to a more deliberate use of existing computational tools.
2. RNA sequencing

RNA sequencing (RNA-seq) uses high-throughput sequencing to determine the order of nucleotides and gene expression. The analysis of RNA-seq data requires specialized computational tools able to address shortcomings of sequencing technologies, including sequencing errors, length biases, and fragmentation. The ability of the computational tools to effectively analyze RNA-seq data has enabled novel therapeutic discoveries, detailed understanding of the regulatory regions that control genes, and identification of biomarkers or mutations in a gene that are known to cause disease onset or progression.

RNA-seq begins with preparation of RNA-seq libraries, which are curated collections of RNA fragments where each fragment is sequenced from one or both ends, and the sequence obtained is known as reads. Preparation of an RNA-seq library starts with extracting and isolating RNA from a biological sample, such as a cell line or a frozen tissue sample. The RNA then undergoes reverse transcription and is converted into cDNA, which is then amplified by polymerase chain reaction (PCR) and fragmented into short sequences (either before or after PCR) (Figure 2). After the RNA molecules are processed, the RNA-seq library becomes the input for the sequencing machine.

2.1. Modern high throughput RNA-seq technologies
Modern high-throughput sequencing techniques are capable of deriving millions of nucleotide sequences from an individual transcriptome. The data provides multifold coverage of the whole transcriptome including specific transcriptomic regions that are essential for identifying the underlying causes of common diseases and disorders. High-resolution RNA-seq data can help us identify active genes and quantify the magnitude at which the genes’ alternative transcripts are transcribed. The output of these modern sequencing technologies have lengths ranging from hundreds of base pairs (usually referred to as short reads) to thousands of base pairs (i.e., long reads). Illumina, Nanopore, and PacBio technologies are among the most commonly used sequencing platforms.

The Illumina sequencing platform, based on sequencing-by-synthesis chemistry, was commercialized in 2006. Illumina ligates cDNA fragments to adapters and immobilizes the fragments on a solid surface before PCR amplification. Next, a reaction mixture containing the primer, reversible nucleotide terminator for each base (labelled with different fluorescent dyes), and the DNA polymerase enzyme, are added to the immobilized solid surface. Each incorporated nucleotide is detected using the CCD camera based on the color of the fluorescent dye. The reversible nucleotide terminator and the dye are then removed from the base, and this cycle is repeated until all bases are identified and labelled. The sequences of more than 10 million colonies can be simultaneously determined in parallel, giving rise to a higher sequencing throughput rate when compared to other sequencing platforms.
Nanopore sequencing technology (including devices MinION, GridION, PromethION), introduced in 2014 by Oxford Nanopore Technologies, can produce short or long reads by sequencing native DNA and RNA fragments of any length. Nanopore technology uses a nanopore, which is a very small hole created by a pore-forming protein or in a synthetic membrane of silicon. The Nanopore sequencing platform simultaneously sends an ionic current and a single strand of DNA or RNA through the nanopore. As the ionic current passes through each nucleotide (e.g., A, C, G, or T in DNA), it experiences disruptions and variations in a pattern unique to the base pair. These observed patterns of disruption and variation in the current can be interpreted to identify the molecule and for further analysis. Data gathered via Nanopore sequencing technology can address a wide range of research areas including studies that involve transcriptome analysis, population-scale analysis, and clinical research\textsuperscript{11}. While short read sequencing technologies, such as Illumina, require chemical modification or PCR amplification, Nanopore technology is capable of sequencing DNA or RNA without these additional steps\textsuperscript{12}.

The PacBio sequencing method, commercially known as SMRT (Single-molecule, real-time) Sequencing, was introduced in 2010 and generates full length cDNA sequences (i.e., long reads) that characterize transcripts within targeted genes or across entire transcriptomes. PacBio eliminates the need for transcript assembly and interference by using long reads to sequence transcripts from the 5’ end to their poly-A tails. It can also be used to produce complete, unambiguous information about alternative splicing events (exons), and improve genome annotation to identify the structure, regulatory elements, and coding regions. Long reads generated by PacBio are accurate at the scale of a single molecule through circular consensus sequencing, effectively reading the same cDNA insert many times\textsuperscript{13,14}. The comparatively high sensitivity of
PacBio (Iso-Seq) can be limited by external factors; for example, Iso-Seq has a limitation of being able to produce full-length cDNA during the library preparation step. It can only generate high quality reads (HiFi reads) if the target cDNA is short enough to be sequenced in multiple passes.

Each sequencing technology exhibits inherent advantages and limitations unique to specific research tasks resulting in no sequencing technology being suitable for each step of RNA-seq analysis (Box1). Typically, short read sequencing technologies can generate data with a lower error rate and higher throughput. However, short length of the read makes reconstruction and quantification of the transcriptome challenging\textsuperscript{15–18}. Long read sequencing technologies improve the accuracy of assembly, or even eliminate the need for assembly as each read often covers the entire transcript. Long reads currently produce a higher error rate and lower throughput compared to short read technologies (Figure 3)\textsuperscript{19–21}. Hybrid approaches combining the long and short reads can eliminate the limitations of each platform and can be used to accurately quantify and assemble known and novel transcripts\textsuperscript{18,19,22}. 
Box 1. Advantages and limitations of short and long reads

i. **Error rate** - Short read sequencing technologies have a lower error rate when compared to long read sequencing technologies (a, b).

ii. **Throughput** - The throughput of long read sequencing technologies is typically lower than the throughput of short read sequencing technologies (c).

iii. **Alignment** - Short reads suffer from multi-mapping issues, whereas longer reads, by nature of having more information, can be more accurately mapped to its origin. Due to a high error rate, pairwise alignment between the read, the reference transcriptome, and/or genome is more challenging for long reads compared to short reads.

iv. **Assemble novel transcripts** - Longer reads are preferred for de novo assembly, because they make the assembly step efficient. Most short reads do not span the shared region or shared exon junction, making the assembly step ambiguous. Full-length transcript sequencing eliminates the need for assembly.

v. **Estimate transcripts and gene expression** - Shorter reads are preferred for quantification of transcripts due to their higher throughput. However, assigning short reads to the transcripts requires more advanced probabilistic and statistical approaches. Longer reads have lower throughput, but they can usually cover the entire transcript and make determination of the transcript for each read a straightforward process.
During the sequencing process, potential errors are introduced into reads that can bias the results of downstream analyses such as read mapping and transcriptome assembly. Data quality control and read trimming is an essential step after the reads have been generated using sequencing technologies. Trimming removes portions of the reads that have lower accuracy (i.e., removes the reads that are unable to be mapped precisely). In general, read trimming tools 23,24 aim to increase the accuracy of read alignment and/or transcriptome assembly by removing the bases with a low PHRED score present at the beginning or end of the read. Data quality control tools have been developed to filter and assess the quality of raw reads25. Tools designed for data quality control (Supplementary Table S.4) are one of the most popular computational tools used for RNA-seq analysis (Supplementary Fig. S.6).

3. Read alignment

Read alignment is an essential step in RNA-seq, often required for downstream analysis. The goal is to find the section of the genome or transcriptome from which the read originated (Figure 2). RNA-seq data typically lacks information about the order of the nucleotides and the reads’ origin, including the specific part, homolog, or strand of the subject genome from which they originate, and this can be established computationally.
Mismatches between the read and the aligned reference can be caused by either sequencing errors or biological variation\textsuperscript{26}. Often the read alignment tools provide a customizable threshold for the number of mismatches allowed.

The coverage represents the average number of reads that overlap on the same position of the reference sequence. Several different bioinformatics tools are capable of estimating the coverage rate and do not require mapping reads to the reference sequence; most of the overlap between reads is preserved with or without the reference sequence\textsuperscript{27} (Figure 2).

Assembling the individual transcriptome could be challenging and requires increased computational time and resources\textsuperscript{28}; instead, known transcripts generated by genome annotation are curated and available from reputable sources such as RefSeq\textsuperscript{29}, UCSC genome browser, Ensembl, GENCODE\textsuperscript{30} and AceView\textsuperscript{31}. However, even the human reference transcriptome remains incomplete\textsuperscript{32}, and unmatched RNA-seq reads should be aligned to the reference genome to recover novel transcripts. Alignment of the individual RNA-seq reads to their complementary reference sequences can help determine which transcripts are expressed and the strength of an expressed transcript’s signal.

Genes create multiple mRNA transcripts through alternative splicing, and, as a result, one or more exons are combined or skipped in different ways and have alternative start/end sites. This varying combination creates multiple transcripts, known as isoforms. As a biological process, alternative splicing is evolutionarily advantageous, facilitating efficient cellular reproduction since different protein variants can be generated from the same genetic code (Figure 3). One particular
computational challenge for aligning RNA-seq reads to the reference sequence is the handling of spliced junctions – one part of the read may map to one end of an exon, while the rest of the read can begin at another exon, often thousands of base pairs apart. When genome annotations are present, existing exon structures can be used in mapping across known splice junctions; however, this knowledge-guided approach may be biased towards mapping only the known junctions and failing to discover novel ones.

In cases where reads align to multiple transcripts, we may not be able to discern from which transcript a read originates. When the transcript of origin is unclear for a particular read, our goal is to identify the various alternative splicing arrangements and quantify the transcripts (Figure 3). Splice alignment software packages\textsuperscript{33–35} are designed to minimize multi-mapping by correctly aligning these reads across the exon-intron junction of the reference genome (Figure 3). Such capabilities can be a crucial first step of reference-guided assembly, wherein transcripts, present in the sample but not annotated in the reference, are assembled using the spliced read alignments to the reference genome.

RNA-seq alignment tools are typically equipped with a customizable threshold for mismatch or sequencing errors, yet these errors can be due to underlying mutations (genomic variants) in the RNA. Therefore, it is important to distinguish between ‘real’ deviations from the reference sequence from the sequencing errors. Identifying gene fusions (a type of genomic variant) – transcripts that cross over two or more genes - require specialized computational tools. Specialized computational tools\textsuperscript{36,37} can identify and classify genes using strategies such as \textit{de novo} assembly
of reads to genes, identification of the reads that span fusion junctions, and filtering, based on many different criteria, of the gene fusion candidates (Supplementary Table S.4).

4. Estimation of transcript and gene expressions

Computational methods can effectively leverage matches between individual reads and reference transcripts by counting the number of reads to estimate expression levels of gene and transcripts. Among all the RNA-seq tools developed between 2008-2020, transcriptome quantification has had the largest number of tools developed, with an average of 3 quantification tools published per year. The total number of citations for this domain is 39,279 (Figure 1c). Counting-based methods\textsuperscript{38–40} can effectively estimate gene expression levels by counting aligned reads compatible with gene structure. However, counting-based tools are typically ill-equipped to estimate the expressions of isoforms of each expressed transcript using the short reads, as the majority of isoforms share a large percentage of exons and thus reads cannot be uniquely assigned to the transcripts (Figure 3). The shorter the reads, the greater the probability that they will match multiple transcripts, presenting a challenge to the identification of the origin of transcript. A conservative approach to tackle this challenge is to consider only the reads that are uniquely mapped to a single transcript\textsuperscript{41}. An alternative approach able to utilize larger fraction of RNA-seq is to probabilistically assigns reads to the isoforms from which they originated\textsuperscript{42–45}.

A number of approaches to quantifying gene expression do not require read alignment; instead, these tools use raw reads for transcriptome quantification (i.e., reads produced directly by the sequencing machine which are not aligned to the transcriptome/genome). These ultra-fast methods
with small computational footprint can estimate expansion of genes and transcripts using pseudoalignment\textsuperscript{46–48}. Sailfish\textsuperscript{48} pioneered pseudoalignment, based on the abundance of the k-mers present in each of the annotated transcripts. K-mers are substrings of length ‘k’ in a nucleotide sequence. For example, a dinucleotide sequence is a k-mer, where \( k = 2 \). In the sequence CCTAGTGTACCGTACC, CC is a 2-mer with a frequency of three. Tools such as Salmon\textsuperscript{49} and Kallisto\textsuperscript{47} also use pseudoalignment to quantify the isoforms of each expressed transcript\textsuperscript{46}. A more detailed explanation of these tools can be found in the Supplementary Note S.2.

4.1 Differential gene expression analysis

After the gene and the transcript expression levels are estimated, statistical approaches can be employed to detect expression levels of various genomic features (such as genes, exons, transcripts) significantly different between experimental groups. In order to perform robust Differential Expression (DE) analysis, one needs to reduce noise and false positives to account for measured sources of expression variation that are unrelated to our variable of interest such as batch effects. For example, the technical replicate of a sample can be different depending on the day or place where it was sequenced. Existing statistical methods\textsuperscript{50–52} can effectively determine such hidden factors\textsuperscript{53}.

The most common approach to DE involves statistical testing and choosing genes with a multiple hypothesis testing-corrected p-value below a certain threshold, typically 5%. Other approaches to DE, which may produce more accurate results, use different metrics such as the Minimum Significant Difference, a combination of p-value with log fold change, and, most recently, machine
learning approaches. DE is complemented by expression quantitative trait loci (eQTL) analyses, which formally compares the expression levels between different copies (0, 1, or 2) of the minor allele. Each read alignment technique produces different results which may impact which gene will be identified as the DE gene. The power to detect DE and eQTLs depends on the sequencing depth of the sample, the minor allele frequency of the genetic variant being tested, the expression level of the gene, and the length of the gene.

There are various tools (n=24) that perform DE analyses including DESeq2 which was cited 15586 times making it one of the most popular RNA-seq tools. The category of Differential expression also has the largest number of citations per year (n=6758.4) (Supplementary Fig. S.6). The results of these transcriptomic analyses can be validated using independent techniques such as qPCR, which is statistically assessable. The measurement of gene expression obtained by qPCR is relatively similar to the measurement obtained by RNA-seq analysis, where a value can be calculated for the concentration of a target region in a given sample. Additional information about the Quantification of RNA splicing and sQTL analyses can be found in Supplementary Note S.3.

5. Measuring allele specific expression from RNA-seq

RNA-seq can also be used to study allele specific expression (ASE or allelic expression) to study the cis-regulatory effects of genetic variants. ASE represents gene expression independently measured for the paternal and maternal allele of a gene. In a typical RNA-seq experiment, ASE can be measured only in genes where the sequenced individual is carrying a heterozygous SNP.
within the transcribed region. This SNP, referred to as the aseSNP, can be used as a tag to identify RNA-seq reads that originate from each of the gene alleles and produce ASE data (Figure 4).

The allelic imbalance—the ratio between paternal and maternal allele gene expressions—is, perhaps, the most interesting signal in ASE data and identifies genetic cis-regulatory differences between the two haplotypes. The magnitude of allelic imbalance can be quantified by log allelic Fold Change (aFC)\(^76\), and its significance is tested using a binomial distribution or the over dispersed generalizations\(^77-81\). An aseSNP is not itself a regulatory variant and should not induce an imbalanced ASE signal. However, there can be a bias in ASE data that falsely implies the haplotype carrying the reference allele for the aseSNP has a slightly higher expression across all genes. This issue, known as allelic bias or reference bias, can be mitigated by aligning reads to personalized reference genomes, excluding likely biased sites\(^33,73,82-84\) or aggregating the ASE signal from multiple aseSNPs in each gene\(^85\). ASE data can also be used to improve statistical power for identifying eQTL\(^78,82-84\), and fine mapping the causal regulatory variants in eQTL data\(^88-90\). ASE data is inherently robust to noise; thus, it can also be used to identify gene environment interaction effects\(^91\), or to identify the effect of rare genetic variants on gene expression in order to improve diagnostic accuracy for Mendelian diseases\(^92,93\). Tools and best practices\(^73\) for generating and analyzing ASE data are discussed in more detail in Supplementary Table S.4.

6. Profiling circular RNA from RNA-seq
Circular RNA (circRNA) is a large class of RNA molecules that plays important roles in various biological processes and metabolic mechanisms. In recent years, a variety of computational tools have been developed for circRNA study (Supplementary Table S.4). As an initial step in circRNA analysis, identification of circRNAs is currently based on detection of reads spanning back-splice junction (BSJ). Most tools employ aligners to detect putative back-spliced events from fusion-reads or split alignment results, while others splice-aware aligners can align circular reads and detect back-splice junctions directly.

Considering that most circRNAs are derived from exonic regions where computational methods are unable to accurately distinguish linear and circular reads, BSJ read count becomes the most reliable measurement of circRNA expression levels. For count-based methods, BSJ read count is inferred from alignment results, and different filters and statistical strategies have been employed to improve the accuracy and sensitivity of these methods. Similar to the quantification of linear transcripts, pseudoalignment-based tools for circRNA quantification substantially increased the computational efficiency compared to regular alignment-based methods. To effectively compare the expression levels of circRNAs and their host genes, the junction ratio, defined as the ratio of BSJ reads and linear reads mapped to BSJ site, is often used for comparative analysis. Several computational methods have been developed for accurate estimation of junction ratios. Moreover, circRNAs also exhibit alternative splicing patterns, and a number of specific tools have been developed for circular transcript assembly, internal structure visualization and differential expression analysis. Additionally, several comprehensive databases have been constructed for circRNA annotation and prioritization analysis.
7. Usability and archival stability of RNA-seq tools

Maintaining the archival stability of bioinformatics tools is increasingly important in preserving scientific transparency and reproducibility. We accessed the archival stability of 235 RNA-seq tools. The majority of RNA-seq tools are stored on archivally stable repositories (e.g., GitHub) and other tools are hosted on personal or academic webpages (Figure 5b), which often have limited archival stability.117

We have also accessed the computational expertise required to install and use RNA-seq tools. A vast majority of tools require the user to operate command line interface (Figure 5a). Web-based tools accounted for 8.09% of tools. There has been an increasing number of tools with available web-based interface designed for small-RNA detection and cell deconvolution categories (71.4% and 31.2%, respectively). We have also compared the availability of package manager across RNA-seq tools. Package managers are platforms that automate the installation, configuration, and maintenance of a software tool, promising to expedite and simplify the installation of a software tool and any required dependencies. The majority of RNA-seq tools lack a package manager implementation (Figure 5c and 5d). For the tools with available package manager implementation, Anaconda118 was the most commonly used package manager platform. The second most popular platform was Bioconductor119 and CRAN120.

Lastly, we evaluated the effect of usability on the popularity of RNA-seq tools. We found that tools that are available as package managers had significantly more citations per year compared with tools which are not available as package managers (Figure 5e; Mann–Whitney U test, p-value = 1.85 × 10⁻⁷).
8. Discussion

As technology has advanced, RNA-seq methods have become increasingly popular and has revolutionized modern biology and clinical applications in the recent past, driven by continuous efforts of the bioinformatics community to develop accurate and scalable computational tools. On an average, across the domains, there have been 18 tools developed each year between 2008-2020. This review provides an insight into the methods used for RNA-seq analysis mostly for researchers and others who are not bioinformaticians by training. We discuss the basic sequencing, mapping, and quantification techniques without laboring over biological details.

RNA-seq data analyzed by computational tools can be used to effectively tackle important biological problems such as estimating gene expression profiles across various phenotypes and conditions or detecting novel alternative splicing on specific exons. Specialized analyses of RNA-seq data can also help detect changes in concentration, function, or localization of transcription factors that affect splicing and can cause onset of neurodegenerative diseases and cancers\textsuperscript{121,122}. More recently developed computational tools\textsuperscript{112–116} are capable of repurposing RNA-seq data characterizing the individual adaptive immune repertoire and microbiome\textsuperscript{128}. Additionally, RNA-seq data can also be used to study cell type compositions using computational deconvolution, a process of estimating cell type proportions in tissue samples\textsuperscript{129,119}.

This interdisciplinary nature of bioinformatics involved in RNA sequencing, analysis, and software development introduces undefined terms that challenge novice researchers in the wider scientific and medical research community. The current literature on RNA-seq methods have
traditionally assumed that the reader is familiar with fundamental concepts of RNA-seq technologies and the implied bioinformatics analyses\textsuperscript{131–136}, therefore requiring a review that explains rudimentary concepts and defines discipline-specific jargon.

In addition to an overview on the fundamentals of RNA-seq, our review also includes survey results of 235 computational tools developed from 2008 to 2020 for various domains of RNA-seq analysis are available in this easy-to-use resource - https://github.com/Mangul-Lab-USC/RNA-seq. In addition to information about usability and archival stability of the tools, this resource will engage the biomedical community through sharing the feedback on utilizing the tools. We hope our resources will help researchers make a more informed decision when selecting a tool for a specific type of data and research question.

**Methods**

**Determine features of RNA-seq tools**

We compiled 235 RNA-seq tools published between 2008 and 2020, which have varying purposes and capabilities based on the type of analysis one is conducting or the biological questions one is answering.

We identified 15 primary areas of application in RNA-seq analysis (Data quality control, read alignment, gene annotation, transcriptome assembly, transcriptome quantification, differential expression, RNA splicing, cell deconvolution, immune repertoire profiling, allele specific expression, viral detection, fusion detection, small RNA detection, detecting circRNA, and
visualization tools). After assigning each tool a category based on its area of application, we highlighted the “Notable Features” for each tool. These notable features encompassed a range of functionalities: purpose of the tool; features that render the tool unique or not unique within its category; the form of receiving RNA-seq data as an input; the form of presenting output.

We documented whether each tool was web-based or required of the user one or many programming languages for installation and/or utilization (“Programming Language”). In addition to the programming languages, we highlighted whether a package manager (e.g., Anaconda, Bioconductor, or CRAN) was available. Based on the combination of which programming language was required and which package manager was available for each tool, we assessed the required expertise needed to be able to install or run the tool. If the tool was a web-based tool with no package manager available or a web-based tool along with programming languages and package manager present, we assigned the tool the little-to-none required expertise of “+”. If the tool required only R as a programming language or along with other programming languages and had a package manager present, the tool was assigned a required expertise of “++”. In addition, if programming languages other than R were required, and a package manager was present, the tool was assigned a “++”. Lastly, for tools that required a single programming language (other than R) or multiple programming languages, and lacked a package manager to aid installation, were assigned a “+++” for the most required expertise.

Each published tool had a designated software link where the tool can be downloaded and installed. Based on the type of platform hosting the URL, we assigned the tools a “1” or “2”. An assignment of “1” meant that the tool’s software was hosted on a more archivally stable web service designed
to host source code. An assignment of “2” meant that the tool’s software was hosted on a less archivally stable web service (e.g., personal and/or university web services).

**Code and Data availability**

https://github.com/Mangul-Lab-USC/RNA-seq

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**Competing Interests**

The authors declare no competing interests.

**References**

1. Shendure, J. & Ji, H. Next-generation DNA sequencing. *Nat. Biotechnol.* **26**, 1135–1145 (2008).

2. Han, Y., Gao, S., Muegge, K., Zhang, W. & Zhou, B. Advanced Applications of RNA Sequencing and Challenges. *Bioinform Biol Insights* **9**, 29–46 (2015).
3. Prakash, C. & Haeseler, A. V. An Enumerative Combinatorics Model for Fragmentation Patterns in RNA Sequencing Provides Insights into Nonuniformity of the Expected Fragment Starting-Point and Coverage Profile. *J Comput Biol* **24**, 200–212 (2017).

4. Kukurba, K. R. & Montgomery, S. B. RNA Sequencing and Analysis. *Cold Spring Harb. Protoc.* **2015**, 951–969 (2015).

5. Gerstein, M. B. *et al.* What is a gene, post-ENCODE? History and updated definition. *Genome Res.* **17**, 669–681 (2007).

6. Haas, B. J. & Zody, M. C. *Advancing RNA-Seq analysis*. *Nature Biotechnology* vol. 28 (2010).

7. Pollard, M. O., Gurdasani, D., Mentzer, A. J., Porter, T. & Sandhu, M. S. Long reads: their purpose and place. *Hum Mol Genet* **27**, R234–R241 (2018).

8. Ye, H., Meehan, J., Tong, W. & Hong, H. Alignment of Short Reads: A Crucial Step for Application of Next-Generation Sequencing Data in Precision Medicine. *Pharmaceutics* **7**, 523–541 (2015).

9. *RNA Sequencing | RNA-Seq methods and workflows.*

10. Morganti, S. *et al.* *Next Generation Sequencing (NGS): A Revolutionary Technology in Pharmacogenomics and Personalized Medicine in Cancer. Translational Research and Onco-Omics Applications in the Era of Cancer Personal Genomics* (2019).

11. *Oxford Nanopore Technologies.*

12. Bharagava, R. N., Purchase, D., Saxena, G. & Mulla, S. I. *Applications of Metagenomics in Microbial Bioremediation of Pollutants. Microbial Diversity in the Genomic Era* (2019).

13. Eid, J. *et al.* Real-time DNA sequencing from single polymerase molecules. *Science* **323**, 133–138 (2009).
14. *From RNA to full-length transcripts: The PacBio Iso-Seq method for transcriptome analysis and genome annotation - PacBio. PacBio.*

15. Engström, P. G. *et al.* Systematic evaluation of spliced alignment programs for RNA-seq data. *Nature Methods* **10**, 1185–1191 (2013).

16. Assessment of transcript reconstruction methods for RNA-seq | Nature Methods. https://www.nature.com/articles/nmeth.2714.

17. Korf, I. Genomics: the state of the art in RNA-seq analysis. *Nature Methods* **10**, 1165–1166 (2013).

18. Amarasinghe, S. L. *et al.* Opportunities and challenges in long-read sequencing data analysis. *Genome Biol.* **21**, 30 (2020).

19. Maio, N. D. *et al.* Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. *Microbial Genomics* vol. 5 (2019).

20. Sedlazeck, F. J., Lee, H., Darby, C. A. & Schatz, M. C. Piercing the dark matter: bioinformatics of long-range sequencing and mapping. *Nature Reviews Genetics* **19**, 329–346 (2018).

21. Mahmoud, M. *et al.* Structural variant calling: the long and the short of it. *Genome Biology* **20**, 246 (2019).

22. Berbers, B. *et al.* Combining short and long read sequencing to characterize antimicrobial resistance genes on plasmids applied to an unauthorized genetically modified Bacillus. *Scientific Reports* vol. 10 (2020).

23. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
24. Dodt, M., Roehr, J., Ahmed, R. & Dieterich, C. *FLEXBAR—Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms*. Biology vol. 1 (2012).

25. Yang, X. *et al.* HTQC: a fast quality control toolkit for Illumina sequencing data. *BMC Bioinformatics* **14**, 33 (2013).

26. Mitchell, K. *et al.* Benchmarking of computational error-correction methods for next-generation sequencing data. *Genome Biology* **21**, 71 (2020).

27. Vurture, G. W. *et al.* GenomeScope: fast reference-free genome profiling from short reads. *Bioinformatics* **33**, 2202–2204 (2017).

28. Grabherr, M. G. *et al.* Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652 (2011).

29. Pruitt, K. D., Tatusova, T. & Maglott, D. R. NCBI reference sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res* **35**, D61–D65 (2007).

30. GENCODE - Home page. https://www.gencodegenes.org/.

31. Larsson, T. P., Murray, C. G., Hill, T., Fredriksson, R. & Schiöth, H. B. Comparison of the current RefSeq, Ensembl and EST databases for counting genes and gene discovery. *FEBS Lett.* **579**, 690–698 (2005).

32. Nellore, A. *et al.* Rail-RNA: scalable analysis of RNA-seq splicing and coverage. *Bioinformatics* **33**, 4033–4040 (2017).

33. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

34. Kim, D., Paggi, J. M., Park, C., Bennett, C. & Salzberg, S. L. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* **37**, 907–915 (2019).
35. Wang, K. et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. *Nucleic Acids Res.* **38**, e178 (2010).

36. Fernandez-Cuesta, L. et al. Identification of novel fusion genes in lung cancer using breakpoint assembly of transcriptome sequencing data. *Genome Biol.* **16**, 7 (2015).

37. Abate, F. et al. Pegasus: a comprehensive annotation and prediction tool for detection of driver gene fusions in cancer. *BMC Syst. Biol.* **8**, 97 (2014).

38. Anders, S., Pyl, P. T. & Huber, W. HTSeq—a Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).

39. Schmid, M. W. & Grossniklaus, U. Rcount: simple and flexible RNA-Seq read counting. *Bioinformatics* **31**, 436–437 (2015).

40. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).

41. Conesa, A. et al. A survey of best practices for RNA-seq data analysis. *Genome Biol* **17**, (2016).

42. Trapnell, C. et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578 (2012).

43. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).

44. Nicolae, M., Mangul, S., Mândoiu, I. I. & Zelikovsky, A. Estimation of alternative splicing isoform frequencies from RNA-Seq data. *Algorithms Mol. Biol.* **6**, 9 (2011).

45. Pertea, M. et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* **33**, 290–295 (2015).
46. Alser, M. et al. Technology dictates algorithms: Recent developments in read alignment. *arXiv:2003.00110 [q-bio]* (2020).

47. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol. 34*, 525–527 (2016).

48. Patro, R., Mount, S. M. & Kingsford, C. Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. *Nat. Biotechnol. 32*, 462–464 (2014).

49. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods 14*, 417–419 (2017).

50. Leek, J. T. svaseq: removing batch effects and other unwanted noise from sequencing data. *Nucleic Acids Res. 42*, (2014).

51. Risso, D., Ngai, J., Speed, T. P. & Dudoit, S. Normalization of RNA-seq data using factor analysis of control genes or samples. *Nat Biotechnol 32*, 896–902 (2014).

52. Stegle, O., Parts, L., Piipari, M., Winn, J. & Durbin, R. Using probabilistic estimation of expression residuals (PEER) to obtain increased power and interpretability of gene expression analyses. *Nat Protoc 7*, 500–507 (2012).

53. Li, S. et al. Detecting and correcting systematic variation in large-scale RNA sequencing data. *Nature Biotechnology 32*, 888–895 (2014).

54. IJzendoorn, D. G. P. van et al. Machine learning analysis of gene expression data reveals novel diagnostic and prognostic biomarkers and identifies therapeutic targets for soft tissue sarcomas. *PLOS Computational Biology 15*, e1006826 (2019).

55. Simoneau, J., Gosselin, R. & Scott, M. S. *Factorial study of the RNA-seq computational workflow identifies biases as technical gene signatures*. 


56. Soneson, C. & Delorenzi, M. *A comparison of methods for differential expression analysis of RNA-seq data*. *BMC Bioinformatics* vol. 14 (2013).

57. Tarazona, S., García, F., Ferrer, A., Dopazo, J. & Conesa, A. *NOIseq: a RNA-seq differential expression method robust for sequencing depth biases*. *EMBnet.journal* vol. 17 (2012).

58. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. *edgeR: a Bioconductor package for differential expression analysis of digital gene expression data*. *Bioinformatics* **26**, 139–140 (2010).

59. Love, M. I., Huber, W. & Anders, S. *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2*. *Genome Biol.* **15**, 550 (2014).

60. Fang, Z. & Cui, X. *Design and validation issues in RNA-seq experiments*. *Brief. Bioinformatics* **12**, 280–287 (2011).

61. Wolf, J. B. W. *Principles of transcriptome analysis and gene expression quantification: an RNA-seq tutorial*. *Mol. Ecol. Resour.* **13**, 559–572 (2013).

62. Lowe, R., Shirley, N., Bleackley, M., Dolan, S. & Shafee, T. *Transcriptomics technologies*. *PLoS Comput. Biol.* **13**, e1005457 (2017).

63. Garber, M., Grabherr, M. G., Guttman, M. & Trapnell, C. *Computational methods for transcriptome annotation and quantification using RNA-seq*. *Nature Methods* vol. 8 (2011).

64. Castel, S. E., Levy-Moonshine, A., Mohammadi, P., Banks, E. & Lappalainen, T. *Tools and best practices for data processing in allelic expression analysis*. *Genome Biol.* **16**, 195 (2015).

65. GATK. https://gatk.broadinstitute.org/hc/en-us.

66. Raghupathy, N. *et al.* *Hierarchical analysis of RNA-seq reads improves the accuracy of allele-specific expression*. *Bioinformatics* **34**, 2177–2184 (2018).
67. Mohammadi, P., Castel, S. E., Brown, A. A. & Lappalainen, T. Quantifying the regulatory effect size of cis-acting genetic variation using allelic fold change. bioRxiv 078717 (2016) doi:10.1101/078717.

68. Mayba, O. et al. MBASED: allele-specific expression detection in cancer tissues and cell lines. Genome Biol. 15, 405 (2014).

69. Skelly, D. A., Johansson, M., Madeoy, J., Wakefield, J. & Akey, J. M. A powerful and flexible statistical framework for testing hypotheses of allele-specific gene expression from RNA-seq data. Genome Res. 21, 1728–1737 (2011).

70. Romanel, A., Lago, S., Prandi, D., Sboner, A. & Demichelis, F. ASEQ: fast allele-specific studies from next-generation sequencing data. BMC Med. Genomics 8, 9 (2015).

71. Xie, J. et al. Modeling allele-specific expression at the gene and SNP levels simultaneously by a Bayesian logistic mixed regression model. BMC Bioinformatics 20, 530 (2019).

72. Harvey, C. T. et al. QuASAR: quantitative allele-specific analysis of reads. Bioinformatics 31, 1235 (2015).

73. van de Geijn, B., McVicker, G., Gilad, Y. & Pritchard, J. K. WASP: allele-specific software for robust molecular quantitative trait locus discovery. Nat. Methods 12, 1061–1063 (2015).

74. Castel, S. E., Mohammadi, P., Chung, W. K., Shen, Y. & Lappalainen, T. Rare variant phasing and haplotypic expression from RNA sequencing with phASER. Nat. Commun. 7, 12817 (2016).

75. Sun, W. A statistical framework for eQTL mapping using RNA-seq data. Biometrics 68, 1–11 (2012).
76. Hu, Y.-J., Sun, W., Tzeng, J.-Y. & Perou, C. M. Proper Use of Allele-Specific Expression Improves Statistical Power for eQTL Mapping with RNA-Seq Data. *J. Am. Stat. Assoc.* **110**, 962–974 (2015).

77. Kumasaka, N., Knights, A. J. & Gaffney, D. J. Fine-mapping cellular QTLs with RASQUAL and ATAC-seq. *Nat. Genet.* **48**, 206–213 (2016).

78. Zou, J. *et al.* Leveraging allelic imbalance to refine fine-mapping for eQTL studies. *PLoS Genet.* **15**, e1008481 (2019).

79. Wang, A. T. *et al.* Allele-Specific QTL Fine Mapping with PLASMA. *Am. J. Hum. Genet.* **106**, 170–187 (2020).

80. Knowles, D. A. *et al.* Allele-specific expression reveals interactions between genetic variation and environment. *Nat. Methods* **14**, 699–702 (2017).

81. Mohammadi, P. *et al.* Genetic regulatory variation in populations informs transcriptome analysis in rare disease. *Science* **366**, 351–356 (2019).

82. Ferraro, N. M. *et al.* Diverse transcriptomic signatures across human tissues identify functional rare genetic variation.

83. Kristensen, L. S. *et al.* The biogenesis, biology and characterization of circular RNAs. *Nat. Rev. Genet.* (2019) doi:10.1038/s41576-019-0158-7.

84. Gao, Y. & Zhao, F. Computational Strategies for Exploring Circular RNAs. *Trends Genet.* **34**, 389–400 (2018).

85. Chen, L. *et al.* The bioinformatics toolbox for circRNA discovery and analysis. *Brief. Bioinform.* (2020) doi:10.1093/bib/bbaa001.

86. Zhang, X.-O. *et al.* Diverse alternative back-splicing and alternative splicing landscape of circular RNAs. *Genome Res.* **26**, 1277–1287 (2016).
87. Cheng, J., Metge, F. & Dieterich, C. Specific identification and quantification of circular RNAs from sequencing data. *Bioinformatics* **32**, 1094–1096 (2016).

88. Gao, Y., Zhang, J. & Zhao, F. Circular RNA identification based on multiple seed matching. *Brief. Bioinform.* **19**, 803–810 (2018).

89. Haas, B. J. *et al.* Accuracy assessment of fusion transcript detection via read-mapping and de novo fusion transcript assembly-based methods. *Genome Biol.* **20**, 213 (2019).

90. Kim, D. & Salzberg, S. L. TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. *Genome Biol.* **12**, R72 (2011).

91. Li, H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *arXiv [q-bio.GN]* (2013).

92. Hoffmann, S. *et al.* A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection. *Genome Biol.* **15**, R34 (2014).

93. Ji, P. *et al.* Expanded Expression Landscape and Prioritization of Circular RNAs in Mammals. *Cell Rep.* **26**, 3444-3460.e5 (2019).

94. Wu, W., Ji, P. & Zhao, F. CircAtlas: an integrated resource of one million highly accurate circular RNAs from 1070 vertebrate transcriptomes. *Genome Biol.* **21**, 101 (2020).

95. Szabo, L. *et al.* Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. *Genome Biol.* **16**, 126 (2015).

96. Li, M. *et al.* Quantifying circular RNA expression from RNA-seq data using model-based framework. *Bioinformatics* **33**, 2131–2139 (2017).

97. Ma, X.-K. *et al.* CIRCexplorer3: A CLEAR Pipeline for Direct Comparison of Circular and Linear RNA Expression. *Genomics Proteomics Bioinformatics* **17**, 511–521 (2019).
98. Zhang, J., Chen, S., Yang, J. & Zhao, F. Accurate quantification of circular RNAs identifies extensive circular isoform switching events. *Nat. Commun.* **11**, 90 (2020).

99. Gao, Y. *et al.* Comprehensive identification of internal structure and alternative splicing events in circular RNAs. *Nat. Commun.* **7**, 12060 (2016).

100. Wu, J. *et al.* CircAST: Full-length Assembly and Quantification of Alternatively Spliced Isoforms in Circular RNAs. *Genomics Proteomics Bioinformatics* **17**, 522–534 (2019).

101. Zheng, Y., Ji, P., Chen, S., Hou, L. & Zhao, F. Reconstruction of full-length circular RNAs enables isoform-level quantification. *Genome Med.* **11**, 2 (2019).

102. Humphreys, D. T., Fossat, N., Demuth, M., Tam, P. P. L. & Ho, J. W. K. Ularcirc: visualization and enhanced analysis of circular RNAs via back and canonical forward splicing. *Nucleic Acids Res.* **47**, e123 (2019).

103. Zheng, Y. & Zhao, F. Visualization of circular RNAs and their internal splicing events from transcriptomic data. *Bioinformatics* (2020) doi:10.1093/bioinformatics/btaa033.

104. Dong, R., Ma, X.-K., Li, G.-W. & Yang, L. CIRCpedia v2: An Updated Database for Comprehensive Circular RNA Annotation and Expression Comparison. *Genomics Proteomics Bioinformatics* **16**, 226–233 (2018).

105. Xia, S. *et al.* CSCD: a database for cancer-specific circular RNAs. *Nucleic Acids Res.* **46**, D925–D929 (2018).

106. Mangul, S. *et al.* Challenges and recommendations to improve the installability and archival stability of omics computational tools. *PLOS Biology* **17**, e3000333 (2019).

107. Grüning, B. *et al.* Bioconda: sustainable and comprehensive software distribution for the life sciences. *Nature Methods* **15**, 475–476 (2018).
108. Reimer, M. & Carey, V. J. [8] Bioconductor: An Open Source Framework for Bioinformatics and Computational Biology. in Methods in Enzymology vol. 411 119–134 (Academic Press, 2006).

109. The Comprehensive R Archive Network. https://cran.r-project.org/.

110. Tollervey, J. R. et al. Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. Genome Research vol. 21 (2011).

111. Tazi, J., Bakkour, N. & Stamm, S. Alternative splicing and disease. Biochim. Biophys. Acta 1792, 14–26 (2009).

112. Mangul, S. et al. Profiling immunoglobulin repertoires across multiple human tissues by RNA Sequencing.

113. Li, B. et al. Landscape of tumor-infiltrating T cell repertoire of human cancers. Nature Genetics vol. 48 (2016).

114. Mose, L. E. et al. Assembly-based inference of B-cell receptor repertoires from short read RNA sequencing data with V’DJer. Bioinformatics vol. 32 (2016).

115. Bolotin, D. A. et al. MiXCR: software for comprehensive adaptive immunity profiling. Nat. Methods 12, 380–381 (2015).

116. Xu, G. et al. RNA CoMPASS: a dual approach for pathogen and host transcriptome analysis of RNA-seq datasets. PLoS One 9, e89445 (2014).

117. Mangul, S. et al. ROP: dumpster diving in RNA-sequencing to find the source of 1 trillion reads across diverse adult human tissues. Genome Biol. 19, 36 (2018).

118. Nadel, B. B. et al. The Gene Expression Deconvolution Interactive Tool (GEDIT): Accurate Cell Type Quantification from Gene Expression Data. bioRxiv 728493 (2020) doi:10.1101/728493.
119. Kang, K. et al. CDSeq: A novel complete deconvolution method for dissecting heterogeneous samples using gene expression data. *PLoS Comput. Biol.* **15**, e1007510 (2019).

120. Byron, S. A., Van Keuren-Jensen, K. R., Engelthaler, D. M., Carpten, J. D. & Craig, D. W. Translating RNA sequencing into clinical diagnostics: opportunities and challenges. *Nat. Rev. Genet.* **17**, 257–271 (2016).

121. Szabo, L. & Salzman, J. Detecting circular RNAs: bioinformatic and experimental challenges. *Nat. Rev. Genet.* **17**, 679–692 (2016).

122. Ozsolak, F. & Milos, P. M. RNA sequencing: advances, challenges and opportunities. *Nat. Rev. Genet.* **12**, 87–98 (2011).

123. Licatalosi, D. D. & Darnell, R. B. RNA processing and its regulation: global insights into biological networks. *Nat. Rev. Genet.* **11**, 75–87 (2010).

124. Hwang, B., Lee, J. H. & Bang, D. Single-cell RNA sequencing technologies and bioinformatics pipelines. *Exp. Mol. Med.* **50**, 96 (2018).

125. Stark, R., Grzelak, M. & Hadfield, J. RNA sequencing: the teenage years. *Nat. Rev. Genet.* **20**, 631–656 (2019).

126. Salzman, J., Jiang, H. & Wong, W. H. Statistical Modeling of RNA-Seq Data. *Stat Sci* **26**, (2011).

127. Turro, E. et al. Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biol.* **12**, R13 (2011).

128. Varadhan, R. & Roland, C. Simple and Globally Convergent Methods for Accelerating the Convergence of Any EM Algorithm. *Scandinavian Journal of Statistics* **35**, 335–353 (2008).

129. Melsted, P. et al. Fusion detection and quantification by pseudoalignment.
130. Reppell, M. & Novembre, J. Using pseudoalignment and base quality to accurately quantify microbial community composition. *PLoS Comput. Biol.* **14**, e1006096 (2018).

131. Srivastava, A., Sarkar, H., Gupta, N. & Patro, R. RapMap: a rapid, sensitive and accurate tool for mapping RNA-seq reads to transcriptomes. *Bioinformatics* **32**, i192–i200 (2016).

132. Zakeri, M., Srivastava, A., Almodaresi, F. & Patro, R. Improved data-driven likelihood factorizations for transcript abundance estimation. *Bioinformatics* **33**, i142–i151 (2017).

133. Nariai, N., Hirose, O., Kojima, K. & Nagasaki, M. TIGAR: transcript isoform abundance estimation method with gapped alignment of RNA-Seq data by variational Bayesian inference. *Bioinformatics* **29**, 2292–2299 (2013).

134. Vaquero-Garcia, J., Norton, S. & Barash, Y. *LeafCutter vs. MAJIQ and comparing software in the fast moving field of genomics*.

135. Green, C. J., Gazzara, M. R. & Barash, Y. MAJIQ-SPEL: web-tool to interrogate classical and complex splicing variations from RNA-Seq data. *Bioinformatics* **34**, 300–302 (2018).

136. Li, Y. I. *et al.* Annotation-free quantification of RNA splicing using LeafCutter. *Nat. Genet.* **50**, 151–158 (2018).

137. Yang, Q., Hu, Y., Li, J. & Zhang, X. ulfasQTL: an ultra-fast method of composite splicing QTL analysis. *BMC Genomics* **18**, 963 (2017).

138. Monlong, J., Calvo, M., Ferreira, P. G. & Guigó, R. Identification of genetic variants associated with alternative splicing using sQTLseekeR. *Nat. Commun.* **5**, 4698 (2014).
Figures and figure legends

Figure 1: Landscape of RNA-seq tools (a) The cumulative number of current computational tools for RNA-seq analysis from 2008 to 2020. (b) The number of current computational tools for RNA-seq analysis in each category. (c) The total number of citations in each domain from 2008-2020. (d) The popularities of tools in different categories, determined by citations per year since initial release. The most popular tools in some of the categories are labeled.
RNA-seq is a process of creating short sequencing reads from RNA molecules. The steps consist of first converting the RNA (a) into cDNA (b) and then amplifying the cDNA by PCR (c - which is an optional step) and lastly fragmenting the cDNA into short pieces (known as fragments). After the sequencing library (d) is prepared, the fragments are used as input for the Next Generation Sequencer (e). The Next Generation Sequencer then sequences each fragment generating FASTQ files. (f) In read alignment, reads from a sequencing machine are provided. The modern high throughput sequencing machines are able to generate up to 150 million reads. The reference sequence, shown as a thick black line, is known. The goal of alignment is to find the loci in the reference genome that mostly matches a read sequence. A read is shown to align to the position x1, x2, x3 and the position is recorded.
Figure 3: Alternative splicing and RNA-Seq technologies.

The flow of genetic information begins with the DNA, which consists of introns and exons. DNA is transcribed into pre-mRNA, further processed into mature mRNA, splicing out the introns and leaving the exons glued together. mRNA is further translated into a protein.

Different arrangements of exons (transcripts) may be formed in a process called alternative splicing or exon skipping. An RNA-seq read is a short sequence sampled from a transcript. These reads are generated using modern sequencing technologies such as (a) the Illumina platform - a short-read sequencing technology, (b) Nanopore and PacBio platforms - long-read sequencing technologies. The figure depicts two scenarios which illustrate the alignment of the uniquely mapped reads to the transcriptome (c) and the genome (d). A few of the reads are multicolored indicating that, when aligned, they span across the exon-exon junction. Some of the shorter reads (single-colored) are aligned only to a single exon and do not span across the junction.
Figure 4: Measuring allele specific expression from RNA-seq.

RNA sequencing can be used for generating ASE data for genes in which an individual is carrying a heterozygous SNP in the transcribed region (aseSNP). aseSNP alleles allow for mapping sequencing reads back to the haplotype the originate from. Imbalance in ASE data is a functional indicator of cis-regulatory difference between the two haplotypes that is driven by heterozygous regulatory variants. Data from multiple aseSNPs can be aggregated to improve ASE data quality.
Figure 5. Usability and archival stability of RNA-seq tools analysis (a) The percentage of required computational expertise of current computational tools that are used for RNA-seq analysis in different categories. (b) The percentages of type of URLs for tools collected. (c) The percentages of package managers for all tools collected. (d) The percentage of availability of package managers for tools in different categories. (e) Tools that are available as package managers exhibit increased citations per year compared with tools that are not available as package managers (Mann–Whitney U test, p-value = 1.85 × 10^{-7}).
Supplementary Materials

Glossary

1. High throughput: High throughput refers to the sequencing methods which are capable of or enable the sequencing of multiple DNA molecules (thousands of strands) at a time.

2. cDNA: complementary DNA or cDNA is the DNA produced by reverse transcription of the template RNA strand by the action of the enzyme reverse transcriptase. The cDNA being synthesized remains single stranded (mRNA) until PCR is carried out. In PCR, aided by the DNA polymerase, it gets its double stranded form.

3. Genome: Genome is the collection of all the DNA present in the nucleus and mitochondria of the cell.

4. Transcriptome: Transcriptome is the collection of all the mRNA molecules derived from or expressed in the genes of an organism.

5. Transcript: A transcript is the single stranded RNA molecule produced as a result of transcription (copying of a particular region of the DNA into RNA by an enzyme RNA polymerase)

6. Isoform: Isoforms are transcripts produced from the same gene but have different TSS and potentially different functions as a result.

7. Loci: Locus (plural - loci) is a specific location or position on a gene.

8. PCR: Polymerase chain reaction is a method used to amplify or create multiple copies of a specific DNA sample rapidly.

9. Nucleotide fluorescence detection: Nucleotide fluorescence detection is a method of fluorescently labelling the nucleotide bases for detection of each base and deducing the nucleotide sequence.
10. Phred quality scores: Phred quality score is a measure of accuracy of sequencing or the estimate of probability of an error in the base call; also called Q score.

11. CCD camera: Charge-coupled device camera used for digital imaging in sequencing.

12. Poly A tails: Poly-A tail is a long chain of Adenine nucleotides added to mRNA for protection from enzymatic degradation, increasing its stability.

13. Pseudoalignment: Pseudoalignment of a read is a set of target sequences the read is compatible with without any base level alignment.

14. qPCR: quantitative PCR measures the amplification of the reverse transcribed DNA code. It is used for the quantification and analysis of nucleic acids for numerous applications.

15. eQTL: expression quantitative trait loci focusing on amount and not the type of trait.

16. Batch effect: Batch effect is the difference that can be observed within the technical replicates of a sample which could be due to various factors such as the day or place where it was sequenced, the researcher conducting the experiment, etc.

17. Normalization: Normalization is the process of adjusting or removing confounders and variations in the data caused due to batch effect.

S.1 Supplementary Note 1

Alternative Splicing:

The presence of alternative splicing in RNA presents a challenge to aligning reads to a reference genome for RNA-seq reads. Alternative splicing begins during transcription, when DNA is used as a template to create a single strand of RNA. Typically for protein coding transcripts, first, the DNA molecule is copied by RNA polymerase into a form known as pre-mRNA, which contains all the bases of the original DNA strand. Next,
portions of the pre-mRNA, called introns, are removed and the remaining portions, called exons, are combined. The final mRNA referred to as a transcript, is ultimately translated into a protein, or has other functions as a noncoding RNA (ncRNA). Introns are excised from the RNA transcripts, but the order of exons that comprise these transcripts vary. Accounting for this loss of sequence information from the intron requires that we look to the junction regions of where any two exons are combined (Figure 3).

S.2 Supplementary Note 2

Sailfish, Salmon and Kallisto

Sailfish\textsuperscript{48} pioneered pseudoalignment, based on the abundance of the k-mers present in each of the annotated transcripts. This reformulation replaces the problem of read alignment with the method of k-mer counting, which is considerably faster. K-mers are substrings of length ‘k’ in a nucleotide sequence. For example, a dinucleotide sequence is a k-mer, where k = 2. In the sequence CCTAGTGACCGTACC, CC is a 2-mer with a frequency of three. This approach represents a reduction of the EM algorithm from modeling the set of observed fragments, as is done in \textsuperscript{43} to modeling the set of fragment counts; such a reformulation was also introduced by \textsuperscript{50} at the per-gene level and by \textsuperscript{51} at a transcriptome-wide level, where the equivalence classes are defined over RNA-seq fragments rather than k-mers. To further accelerate EM convergence, Sailfish makes use of the SQUAREM algorithm \textsuperscript{52}. While vastly accelerating quantification, this approach neglects accounting for coherence between the k-mers within a read or read pair, which can diminish accuracy.
Most recent methods also leverage raw reads for quantifying gene expression, are computationally efficient, and use lesser memory than alignment-based approaches comparable to methods \(^{42,46,47}\). Kallisto’s algorithm deciphers the compatibility of reads with transcripts but does not map the entire read \(^{53}\). This pseudoalignment approach increases the speed of computation and decreases the memory requirement. Each k-mer is matched with a group of transcripts, and the intersection of the sets of transcripts represent the best possible matches\(^{54}\). These transcript compatibility sets define equivalence classes of fragments, and the equivalence classes and counts are used as sufficient statistics, as in \(^{51}\), to estimate transcript-level abundances using an EM algorithm.

Salmon offers two different modes for quantification of transcripts. One mode includes both mapping and quantification in a single step, with reads being mapped directly to the transcriptome using the quasi-mapping approach of RapMap\(^{55}\), without creating any intermediate alignment files \(^{49}\). Alternatively, transcriptome-aligned SAM or BAM files — such as those used by RSEM \(^{43}\) — can be provided. Salmon leverages a two-phase inference algorithm for quantification. The first phase consists of a stochastic Bayesian inference procedure that models the reads at fragment resolution (which can be used to estimate certain effects, like fragment GC-bias or alignment error profiles, that depend on the characteristics of individual read mappings), while the second phase operates on equivalence classes of fragments, either as in \(^{51}\) or using refined fragment equivalence notions that have been introduced subsequently\(^{56}\), and estimates abundance using either an EM or variational Bayesian EM \(^{57}\) algorithm.
S.3 Supplementary Note 3

Quantification of RNA splicing and sQTL analyses

Computational tools that capture alternative splicing events in RNA seq data can characterize the possible set of transcripts within the data that were missing from multi-mapped reads. Further insight regarding differentially expressed genes can then be obtained by comparing this broader set of transcripts between case and control groups.

Estimation of isoform ratios or exon inclusion levels is currently the standard approach to studying splicing events in RNA-seq data. Direct inference based on the excised introns is the second most common approach to study splicing events. Accurate quantification of splicing events can be obtained using methods such as LeafCutter\textsuperscript{137} or MAJIQ-SPEL\textsuperscript{138}. MAJIQ-SPEL\textsuperscript{138} is often used together with VOILA (a visualization package) to define, quantify, and visualize local splicing variations from RNA-seq data. LeafCutter\textsuperscript{137} quantifies RNA splicing variations using short read RNA-seq data; in other words, the program anchors reads that span an intron to quantify the intron usage across samples. By detecting novel introns, LeafCutter\textsuperscript{137} can help researchers avoid the additional burden of performing isoform abundance estimation\textsuperscript{137,139}.

Once the ratios of each isoform, exon, or excised intron are available, splicing levels across different treatment groups or different copies of the minor allele can be compared with differential splicing analyses and splicing QTL (sQTL) analyses. LeafCutter\textsuperscript{137} can detect differential splicing between sample groups and map sQTLs. ulfasQTL\textsuperscript{140} and sQTLseeker\textsuperscript{141} are commonly selected for mapping sQTLs.
Supplementary Table

S.4 Table

<attached as a separate document>

Supplementary Figures

S.5 Proportion of citations of RNA-seq tools per year.
S.6 The relationship between total number of citations per year of each category and the number of citations per year for the most cited tool in that category.
S.4 Table. Landscape of current computational tools for RNA-seq analysis

| Tool       | Year Published | Notable Features                                      | Programming language | Package manager | Required expertise | Software                                      | Type of URL                                                                 |
|------------|----------------|-------------------------------------------------------|----------------------|-----------------|-------------------|------------------------------------------------|----------------------------------------------------------------------------|
| 1. Web services designed to host source code                                                                                                   |
| 2. Others (e.g personal and/or university web services)                                                                                                           |
| a. Data quality control                                                                                                                                                 |
| iSeqQC¹    | 2020           | Expression-based raw data QC tool that detects outliers | R                    | N/A             | ++                | https://github.com/gkumar09/iSeqQC               | 1                                                                           |
| qsmooth²   | 2018           | Adaptive smooth quantile normalization                 | R                    | Bioconductor    | ++                | http://bioconductor.org/packages/release/bioc/html/qsmooth.html | 1                                                                           |
| Tool      | Year | Description                                                                 | Language | Distribution | Website                                      | Version |
|-----------|------|-----------------------------------------------------------------------------|----------|--------------|----------------------------------------------|---------|
| FastQC³   | 2018 | Raw data QC tool for high throughput sequence data                          | Java     | Anaconda     | https://github.com/s-andrews/fastqc/        | ++      |
| QC3⁴      | 2014 | Raw data QC tool detecting batch effect and cross contamination              | Perl, R  | Anaconda     | https://github.com/slzhaol/QC3              | ++      |
| kPAL⁵     | 2014 | Alignment-free assessment raw data QC tool by analyzing k-mer frequencies    | Python   | Anaconda     | https://github.com/LU/MC/kPAL              | ++      |
| HTQC⁶     | 2013 | Raw data QC read assessment and filtration                                  | C++      | N/A          | https://sourceforge.net/projects/htqc/     | +++     |
| Trimmomatic⁷ | 2014 | Trimming of reads and removal of adapters                                   | Java     | Anaconda     | http://www.usadellab.org/cms/index.php?page=trimmomatic | ++      |
| Skewer⁸   | 2014 | Adapter trimming of reads                                                   | C++      | Anaconda     | https://sourceforge.net/projects/skewer    | ++      |
| Flexbar⁹  | 2012 | Trimming of reads and adaptor removal                                       | C++      | Anaconda     | https://github.com/seqan/flexbar           | ++      |
| Tool   | Year | Description                                                                                           | Language | Dependency | Availability          | Note |
|--------|------|-------------------------------------------------------------------------------------------------------|----------|------------|------------------------|------|
| QuaCRS | 2014 | Post QC tool by performing meta-analyses on QC metrics across large numbers of samples.               | Python   | N/A        | +++                    | [link](https://github.com/kwkr0ll32/QuaCRS) | 1    |
| BlackOPS | 2013 | Post QC tool that simulates experimental RNA-seq derived from the reference genome and aligns these sequences and outputs a blacklist of positions and alleles caused by mismapping | Perl     | N/A        | +++                    | [link](https://sourceforge.net/projects/rnaseqvariantblacklist/) | 1    |
| RSeQC  | 2012 | Post QC evaluation of different aspects of RNA-seq experiments, such as sequence quality, GC bias, nucleotide composition bias, sequencing depth, strand specificity, coverage uniformity and | Python, C| Anaconda   | ++                     | [link](http://rseqc.sourceforge.net/) | 1    |
| Tool               | Year | Description                                                                 | Language | Environment | Quality | Website                                                      | Count |
|-------------------|------|-----------------------------------------------------------------------------|----------|-------------|---------|-------------------------------------------------------------|-------|
| RNA-SeQC          | 2012 | RNA-seq metrics for post-quality control and process optimization           | Java     | Anaconda    | ++      | https://software.broadinstitute.org/cancer/cga/rna-seqc      | 2     |
| Seqbias           | 2012 | Post QC tool using a graphical model to increase accuracy of de novo gene annotation, uniformity of read coverage, consistency of nucleotide frequencies and agreement with qRT-PCR | R        | Anaconda, Bioconductor | ++      | http://master.bioconductor.org/packages/devel/bioc/html/seqbias.html | 1     |
| SAMStat           | 2011 | Post QC tool which plots nucleotide overrepresentation and other statistics in mapped and unmapped reads in a html page | C        | N/A         | +++     | http://samstat.sourceforge.net                               | 1     |
| Tool         | Year | Description                                                                 | Language | Environment | License | Website                                      | Row |
|--------------|------|------------------------------------------------------------------------------|----------|-------------|---------|----------------------------------------------|-----|
| Samtools     | 2009 | Post QC tool using generic alignment format for storing read alignments against reference sequences and to visualize the Binary/Alignment Map (BAM). | C, Perl  | Anaconda    | ++      | https://github.com/samtools/samtools        | 1   |

b. Read alignment

| Tool         | Year | Description                                                                 | Language | Environment | License | Website                                      | Row |
|--------------|------|------------------------------------------------------------------------------|----------|-------------|---------|----------------------------------------------|-----|
| deSALT       | 2019 | Long transcriptomic read alignment with de Bruijn graph-based index          | C        | Anaconda    | ++      | https://github.com/ydliu-HIT/deSALT         | 1   |
| Magic-BLAST  | 2018 | Aligner for long and short reads through optimization of a spliced alignment score | C++      | N/A         | +++     | https://ncbi.github.io/magicblast/          | 1   |
| Minimap      | 2018 | Alignment using seed chain alignment procedure                               | C, Python| Anaconda    | ++      | https://github.com/lh3/minimap2            | 1   |
|        |      | Description                                                                 | Programming language | Environment | Score | Website                                                                 |
|--------|------|------------------------------------------------------------------------------|-----------------------|-------------|-------|------------------------------------------------------------------------|
| DART   | 2018 | Burrows-Wheeler Transform based aligner which adopts partitioning strategy to divide a read into two groups | C/C++                | Anaconda    | ++    | [https://github.com/hsinnan75/DART]                                   |
| MMR    | 2016 | Resolves the mapping location of multi-mapping reads, optimising for locally smooth coverage. | C++                  | N/A         | +++   | [https://github.com/ratschlab/mmr]                                     |
| ContextMap | 2015 | Allows parallel mapping against several reference genomes                    | Java                  | N/A         | +++   | [http://www.bio.ifi.lmu.de/ContextMap]                                 |
| HISAT  | 2015 | Aligning reads using an indexing scheme based on the Burrows-Wheeler transform and the Ferragina-Manzini (FM) index | C++                  | Anaconda    | ++    | [http://www.ccb.jhu.edu/software/hisat/index.shtml]                    |
| Tool     | Year | Description                                                                 | Programming Languages | Environment | Website                                      | Notes |
|----------|------|-----------------------------------------------------------------------------|------------------------|-------------|----------------------------------------------|-------|
| Segemehl | 2014 | Multi-split mapping for circular RNA, trans-splicing, and fusion events in addition to performing splice alignment | C, C++, Perl, Python, Shell (Bash) | Anaconda | [http://www.bioinf.uni-leipzig.de/Software/segemehl/](http://www.bioinf.uni-leipzig.de/Software/segemehl/) | 2     |
| JAGuAR   | 2014 | Uses a modified GTF (Gene Transfer Format) of known splice sites to build the complete sequence from all reads mapped to the transcript. | Python | N/A | [https://www.bcgsc.ca/resources/software/jaguar](https://www.bcgsc.ca/resources/software/jaguar) | 2     |
| CRAC     | 2013 | Uses double K-mer indexing and profiling approach to map reads, predict SNPs, gene fusions, repeat borders. | C++ | Anaconda | [http://crac.gforge.inria.fr/](http://crac.gforge.inria.fr/) | 2     |
| STAR     | 2013 | Aligns long reads against genome reference database | C++ | Anaconda | [https://github.com/alexdobin/STAR](https://github.com/alexdobin/STAR) | 1     |
| Tool         | Year | Description                                                                 | Language   | Platform  | Rating | Website                                                                 |
|-------------|------|------------------------------------------------------------------------------|------------|-----------|--------|------------------------------------------------------------------------|
| Subread28   | 2013 | Mapping reads to a reference genome using multi-seed strategy, called seed-and-vote | C, R       | Anaconda, Bioconductor | ++     | https://bioconductor.org/packages/release/bioc/html/Rsubread.html      |
| TopHat29    | 2013 | Alignment of transcriptomes in the presence of insertions, deletions and gene fusions | C++, Python | Anaconda | ++     | http://ccb.jhu.edu/software/tophat/index.shtml                          |
| OSA30       | 2012 | K-mer profiling approach to map reads                                        | C#         | N/A       | +++    | http://www.arrayserver.com/wiki/index.php?title=OSA                    |
| PASSion31   | 2012 | Pattern growth pipeline for splice junction detection                        | C++, Perl, Shell (Bash) | N/A       | +++    | https://trac.nbic.nl/passion/                                           |
| RUM32       | 2011 | Comparative analysis of RNA-seq alignment algorithms and the RNA-seq unified mapper | Perl, Python | N/A       | +++    | http://www.cbil.upenn.edu/RUM/                                           |
| SOAPSplice33 | 2011 | Ab initio detection of splice junctions                                       | Perl       | Anaconda  | ++     | http://soap.genomics.org.cn/soapssplice.html                            |
| Package       | Year | Function                                                                 | Language | Distribution | Support | URL                                                                 |
|--------------|------|--------------------------------------------------------------------------|----------|--------------|---------|----------------------------------------------------------------------|
| MapSplice     | 2010 | De novo detection of splice junctions                                    | C++      | Anaconda     | ++      | https://github.com/LiuBioinfo/MapSplice                                |
| SpliceMap     | 2010 | De novo detection of splice junctions and RNA-seq alignment              | C++      | Anaconda     | ++      | http://web.stanford.edu/group/wonglab/SpliceMap/                      |
| Supersplat    | 2010 | De novo detection of splice junctions                                    | C++      | N/A          | +++     | http://mocklerlab.org/tools/1/manual                                  |
| HMMsparser    | 2010 | Detection of splice junctions of short sequence reads                    | Python   | N/A          | +++     | http://derisilab.ucsf.edu/software/hmmsplicer                          |
| QPALMA        | 2008 | Spliced alignments of short sequence reads                              | C++, Python | N/A   | +++     | http://www.raetschlab.org/suppl/qpalma                                |
| SQANTI        | 2018 | Analyses quality of long reads transcriptomes and removes artefacts.     | Python   | Anaconda     | ++      | https://github.com/ConesaLab/SQANTI                                  |

c. Gene annotations

| SQANTI | 2018 | Analyses quality of long reads transcriptomes and removes artefacts.     | Python   | Anaconda     | ++      | https://github.com/ConesaLab/SQANTI                                  |
| Tool     | Year | Description                                                                 | Programming Languages | OS | Quality | Website                                                                 | Version |
|----------|------|------------------------------------------------------------------------------|-----------------------|----|---------|-------------------------------------------------------------------------|---------|
| Annocript| 2015 | Databases are downloaded to annotate protein coding transcripts with the prediction of putative long non-coding RNAs in whole transcriptomes. | Perl, Python, R       | N/A | +++     | https://github.com/frankMusacchia/Annocript                              | 1       |
| CIRI     | 2015 | De novo circular RNA identification                                           | Perl                  | N/A | +++     | https://sourceforge.net/projects/ciri/                                  | 1       |
| TSSAR    | 2014 | Automated de novo TSS annotation from differential RNA-seq data              | Java, Perl, R         | Anaconda | ++ | http://rna.tbi.univie.ac.at/TSSAR                                       | 2       |
| **d. Transcriptome assembly**                                                                                                                                          |
| FLAIR    | 2020 | Full-length alternative isoform analysis of RNA                              | Python                | Anaconda | ++ | https://github.com/BrooksLabUCSC/FLAIR                                   | 1       |
| Scallop  | 2017 | Splice-graph-decomposition algorithm which optimizes two competing objectives while | C++                   | Anaconda | +++ | https://github.com/Kingsford-Group/scallop                               | 1       |
| Tool            | Year | Description                                                                 | Language(s) | Optional Assembler | Website                                                                 | Score |
|-----------------|------|------------------------------------------------------------------------------|--------------|--------------------|-------------------------------------------------------------------------|-------|
| CLASS2          | 2016 | Satisfying all phasing constraints posed by reads spanning multiple vertices | C++, Perl, Shell | Anaconda           | [https://sourceforge.net/projects/splicebox/](https://sourceforge.net/projects/splicebox/) | 1     |
| StringTie       | 2015 | Applies a network flow algorithm originally developed in optimization theory, together with optional de novo assembly, to assemble transcripts | C++          | N/A                | [http://ccb.jhu.edu/software/stringtie](http://ccb.jhu.edu/software/stringtie) | 2     |
| Bridger         | 2015 | De novo transcript assembler using a mathematical model, called the minimum path cover | C++, Perl    | N/A                | [https://sourceforge.net/projects/rnaseqassembly/files/?source=navbar](https://sourceforge.net/projects/rnaseqassembly/files/?source=navbar) | 1     |
| Bayesembler     | 2014 | Reference genome guided transcriptome                                         | C++          | N/A                | [https://github.com/bioinformatics-centre/bayesembler](https://github.com/bioinformatics-centre/bayesembler) | 1     |
| Program     | Year | Description                                                                 | Language | Last Updated | Score | Website                                      | Score |
|-------------|------|------------------------------------------------------------------------------|----------|--------------|-------|----------------------------------------------|-------|
| SEECER⁴⁹    | 2013 | De novo transcriptome assembly using hidden Markov Model (HMM) based method | C++      | N/A          | +++   | http://sb.cs.cmu.edu/seeacer/                | 2     |
| BRANCH⁵⁰   | 2013 | De novo transcriptome assemblies by using genomic information that can be partial or complete genome sequences from the same or a related organism. | C++      | N/A          | +++   | https://github.com/baoe/BRANCH               | 1     |
| EBARDenovo⁵¹ | 2013 | De novo transcriptome assembly uses an efficient chimera-detection function | C#       | N/A          | +++   | https://sourceforge.net/projects/ebardenovo/ | 1     |
| Oases⁵²     | 2012 | De novo transcriptome assembly using k-mer profiling and                       | C        | N/A          | +++   | https://github.com/dzerbino/oases/tree/master | 1     |
| Program   | Year | Description                                                                 | Language | Platform  | Complexity | Website                                                                 |
|-----------|------|------------------------------------------------------------------------------|----------|-----------|------------|------------------------------------------------------------------------|
| Cufflinks | 2012 | Ab initio transcript assembly, estimates their abundances, and tests for differential expression | C++      | N/A       | +++        | https://github.com/coll-trapnell-lab/cufflinks                       |
| IsoInfer  | 2011 | Infer isoforms from short reads                                              | C/C++    | N/A       | +++        | http://www.cs.ucr.edu/~jianxing/IsoInfer.html                        |
| IsoLasso  | 2011 | Reference genome guided using LASSO regression approach                      | C++      | N/A       | +++        | http://alumni.cs.ucr.edu/~liw/isolasso.html                          |
| Trinity   | 2011 | De novo transcriptome assembly                                               | C++, Java, Perl, R, Shell (Bash) | Anaconda | ++         | https://github.com/trinityrnaseq/trinityrnaseq/wiki                   |
| Trans-ABySS | 2010 | De novo short-read transcriptome assembly and can also be used for fusion detection | Python   | N/A       | +++        | https://github.com/bcgsc/transabyss                                 |
Ab initio reconstruction of transcriptomes of pluripotent and lineage committed cells

Java
N/A
+++
www.broadinstitute.org/software/Scripture/

Long-read transcriptome discovery and quantification

Python
N/A
+++
https://github.com/dewyman/TALON

Composed of: lightweight-mapping model, an online phase that estimates initial expression levels and model parameters, and an offline phase that refines expression estimates models, and measures sequence-specific, fragment GC, and positional biases

C++
Anaconda
++
https://github.com/COMBINE-lab/Salmon

| Tool | Year | Description | Language | Environment | Rating | Website | Score |
|------|------|-------------|----------|-------------|--------|---------|-------|
| Scripture | 2010 | Ab initio reconstruction of transcriptomes of pluripotent and lineage committed cells | Java | N/A | +++ | www.broadinstitute.org/software/Scripture/ | 2 |
| TALON | 2019 | Long-read transcriptome discovery and quantification | Python | N/A | +++ | https://github.com/dewyman/TALON | 1 |
| Salmon | 2017 | Composed of: lightweight-mapping model, an online phase that estimates initial expression levels and model parameters, and an offline phase that refines expression estimates models, and measures sequence-specific, fragment GC, and positional biases | C++ | Anaconda | ++ | https://github.com/COMBINE-lab/Salmon | 1 |
| Tool      | Year | Description                                                                 | Programming Languages | Installation | Website                                      | Rating |
|-----------|------|------------------------------------------------------------------------------|-----------------------|--------------|----------------------------------------------|--------|
| Kallisto  | 2016 | K-mer based pseudoalignment for alignment-free transcript and gene expression quantification | C, C++, Perl          | Anaconda     | [https://github.com/pachterlab/kallisto](https://github.com/pachterlab/kallisto) | ++     |
| Wub       | 2016 | Sequence and error simulation tool to calculate read and genome assembly accuracy. | Python                | Anaconda     | [https://github.com/nanoporetech/wub](https://github.com/nanoporetech/wub) | ++     |
| Rcount    | 2015 | GUI based tool used for quantification using counts per feature              | Web based tool        | N/A          | [https://github.com/MWSchmid/Rcount](https://github.com/MWSchmid/Rcount) | +      |
| Ht-seq    | 2015 | Calculates gene counts by counting number of reads overlapping genes         | Python                | pip          | [https://htseq.readthedocs.io/en/release_0.11.1/overview.html](https://htseq.readthedocs.io/en/release_0.11.1/overview.html) | ++     |
| EMSAR     | 2015 | Estimation by mappability-based segmentation and reclustering using          | C                     | N/A          | [https://github.com/parklab/emsar](https://github.com/parklab/emsar) | +++    |
| Package       | Year | Description                                                                 | Language | Environment | Support | Website                                                                 | Notes |
|---------------|------|------------------------------------------------------------------------------|----------|-------------|---------|-------------------------------------------------------------------------|-------|
| Maxcounts     | 2014 | a joint Poisson model                                                        | C++      | N/A         | +++     | [http://sysbiobig.dei.uniPD.it/?q=Software#MAXCOUNTS](http://sysbiobig.dei.uniPD.it/?q=Software#MAXCOUNTS) | 2     |
| FIXSEQ        | 2014 | Quantify the expression assigned to an exon as the maximum of its per-base counts | R        | N/A         | ++      | [https://bitbucket.org/thashim/fixseq/src/master/](https://bitbucket.org/thashim/fixseq/src/master/) | 1     |
| Sailfish      | 2014 | A nonparametric and universal method for processing per-base sequencing read count data. | C, C++   | Anaconda    | ++      | [https://github.com/kingfordgroup/sailfish](https://github.com/kingfordgroup/sailfish) | 1     |
| Casper        | 2014 | EM based quantification using statistical coupling between k-mers.            | R        | Anaconda, Bioconductor | ++ | [http://www.bioconductor.org/packages/release/bioc/html/casper.html](http://www.bioconductor.org/packages/release/bioc/html/casper.html) | 1     |
| MaLTA         | 2014 | Bayesian modeling framework to quantify alternative splicing.              | C++      | N/A         | +++     | [http://alan.cs.gsu.edu/NGS/?q=malta](http://alan.cs.gsu.edu/NGS/?q=malta) | 2     |
| Software   | Year | Description                                                                 | Language(s)          | Platform(s) | Rating | Website                                      | Notes |
|------------|------|------------------------------------------------------------------------------|----------------------|-------------|--------|----------------------------------------------|-------|
| Featurecounts<sup>7</sup> | 2014 | Read summarization program for counting reads generated.                      | R, N/A               | ++         | http://subread.sourceforge.net/               | 1     |
| MITIE<sup>72</sup> | 2013 | Transcript reconstruction and assembly from RNA-Seq data using mixed integer optimisation. | MATLAB, C++          | +++        | https://github.com/ratschlab/MiTie            | 1     |
| iReckon<sup>73</sup>  | 2013 | EM-based method to accurately estimate the abundances of known and novel isoforms. | Java, N/A            | +++        | http://compbio.cs.toronto.edu/ireckon/       | 2     |
| eXpress<sup>74</sup>  | 2013 | Online EM based algorithm for quantification which considers one read at a time. | C++, Shell (Bash), Anaconda | ++         | https://pachterlab.github.io/eXpress/manual.html | 1     |
| Tool       | Year | Approach                                                                 | Language | Additional Features | Website                                      | Score |
|------------|------|--------------------------------------------------------------------------|----------|---------------------|----------------------------------------------|-------|
| BitSeq\textsuperscript{75} | 2012 | Bayesian transcript expression quantification and differential expression. | C++, R   | Anaconda, Bioconductor | http://bitseq.github.io/                    | 1     |
| IQSeq\textsuperscript{76}   | 2012 | Integrated isoform quantification analysis.                              | C++      | N/A                 | http://archive.gersteinlab.org/proj/rnaseq/IQSeq/ | 2     |
| CEM\textsuperscript{77}     | 2012 | Statistical framework for both transcriptome assembly and isoform expression level estimation. | Python   | N/A                 | http://alumni.cs.ucr.edu/~liw/cem.html       | 2     |
| SAMMate\textsuperscript{78} | 2011 | Analysis of differential gene                                           | Java     | N/A                 | http://sammate.sourceforge.net/             | 1     |
| Method       | Year | Description                                                                 | Languages          | Implementation | Website                                      | Score |
|--------------|------|------------------------------------------------------------------------------|--------------------|----------------|----------------------------------------------|-------|
| Isoformex\(^{79}\) | 2011 | Estimation method to estimate the expression levels of transcript isoforms. | N/A                | N/A            | http://bioinformatics.wistar.upenn.edu/isoformex | 2     |
| IsoEM\(^{80}\)   | 2011 | EM based method for inference of isoform and gene-specific expression levels | Java               | N/A            | http://dna.engr.uconn.edu/software/IsoEM/     | 2     |
| RSEM\(^{81}\)     | 2011 | Ab initio EM based method for inference of isoform and gene-specific expression levels | C++, Perl, Python, R | Anaconda       | https://github.com/deweylab/RSEM             | 1     |
| EDASeq\(^{82}\)   | 2011 | Within-lane GC-content normalization,                                        | R                  | Anaconda, Bioconductor                      | https://bioconductor.org/packages/devel/bioc/html/EDASeq.html | 1     |
|   |   | between-sample normalization, visualization. |   |   |   |   |
|---|---|---------------------------------------------|---|---|---|---|
| MMSEQ\textsuperscript{83} | 2011 | Haplotype and isoform specific expression estimation | C++, R, Ruby, Shell (Bash) | N/A | ++ | https://github.com/eturro/mmseq |
| MISO\textsuperscript{84} | 2010 | Statistical model that estimates expression of alternatively spliced exons and isoforms | C, Python | N/A | +++ | https://miso.readthedocs.io/en/fastmiso/#latest-version-from-github |
| SOLAS\textsuperscript{85} | 2010 | Prediction of alternative isoforms from exon expression levels | R | N/A | ++ | http://cmb.molgen.mpg.de/2ndGenerationSequencing/Solas/ |
| Rseq\textsuperscript{86} | 2009 | Statistical inferences for isoform expression | C++ | N/A | +++ | http://www-personal.umich.edu/~jia nghui/rseq/#download |
| rQuant\textsuperscript{87} | 2009 | Estimating density biases and | Matlab, Shell | N/A | +++ | https://galaxy.inf.ethz.ch/?tool_id=rquantweb& |
| Name       | Year | Techniques                                                                 | Languages     | Packages/Tools                                                                 |
|------------|------|----------------------------------------------------------------------------|---------------|-------------------------------------------------------------------------------|
| ERANGE     | 2008 | Mapping and quantifying mammalian transcripts                            | Python        | [http://woldlab.caltech.edu/rnaseq](http://woldlab.caltech.edu/rnaseq)        |
| f. Differential expression                      |      |                                                                            |               |                                                                                |
| Swish      | 2019 | Non-parametric model for differential expression analysis using inferential replicate counts | R, Bioconductor | [https://bioconductor.org/packages/release/bioc/html/fishpond.html](https://bioconductor.org/packages/release/bioc/html/fishpond.html) |
| Yanagi     | 2019 | Transcriptome segment analysis                                            | Python/C      | [https://github.com/HCBravoLab/yanagi](https://github.com/HCBravoLab/yanagi) |
| Whippet    | 2018 | Quantification of transcriptome structure and gene expression             | Julia         | [https://github.com/timbetz/Whippet.jl](https://github.com/timbetz/Whippet.jl) |
| Tool               | Year | Description                                                                 | Programming Languages | Version | Website                                                                 | Score |
|--------------------|------|-----------------------------------------------------------------------------|------------------------|---------|-------------------------------------------------------------------------|-------|
| ReQTL²²             | 2018 | Identifies correlations between SNVs and gene expression from RNA-seq data   | R, N/A                  | ++      | https://github.com/HorvathLab/ReQTL                                    | 1     |
| vast-tools³³        | 2017 | Profiling and comparing alternative splicing events in RNA-Seq data and for downstream analysis | R, Perl                | +++     | https://github.com/vastgroup/vast-tools                                 | 1     |
| Software   | Year | Description                                                                 | Language | Platform | Version | Website                                                                 | Notes |
|------------|------|-----------------------------------------------------------------------------|----------|----------|---------|-------------------------------------------------------------------------|-------|
| Ballgown   | 2015 | Linear model-based differential expression analyses                          | R        | Anaconda, Bioconductor | ++      | [https://github.com/alyssafrazee/ballgown](https://github.com/alyssafrazee/ballgown) | 1     |
| Limma/Voom | 2014 | Linear model-based differential expression and differential splicing analyses | R        | Anaconda, Bioconductor | ++      | [https://bioconductor.org/packages/release/bioc/html/lm.html](https://bioconductor.org/packages/release/bioc/html/lm.html) | 1     |
| rMATS      | 2014 | Detect major differential alternative splicing types in RNA-seq data with replicates. | Python, C++ | Anaconda | ++      | [http://rnaseq-mats.sourceforge.net/rmats3.2.5/](http://rnaseq-mats.sourceforge.net/rmats3.2.5/) | 1     |
| DESeq2     | 2014 | Differential analysis of count data, using shrinkage estimation for dispersions and fold changes | R        | Bioconductor, CRAN     | ++      | [https://bioconductor.org/packages/release/bioc/html/DESeq2.html](https://bioconductor.org/packages/release/bioc/html/DESeq2.html) | 1     |
| Package | Year | Description | Programming Languages | Other Tools | Additional Notes | Version | Weight |
|---------|------|-------------|-----------------------|-------------|-----------------|---------|--------|
| Corset | 2014 | Differential gene expression analysis for de novo assembled transcriptomes | C++ | Anaconda | ++ | https://github.com/Oshlack/Corset/wiki | 1 |
| BADGE | 2014 | Bayesian model for accurate abundance quantification and differential analysis | Matlab | N/A | +++ | http://www.cbil.ece.vt.edu/software.htm | 2 |
| compcdeR | 2014 | Benchmarking of differential expression analysis methods | R | Anaconda, Bioconductor | ++ | https://www.bioconductor.org/packages/compcodeR/ | 1 |
| metaRNASeq | 2014 | Differential metaanalyses of RNA-seq data | R | Anaconda, CRAN | ++ | http://cran.r-project.org/web/packages/metaRNASeq | 1 |
| Characteristic Direction | 2014 | Geometrical multivariate approach to identify differentially expressed genes | R, Python, MATLAB | N/A | ++ | http://www.maayanlab.net/CD | 2 |
| HTSFilter | 2013 | Filter-replicated high-throughput | R | Anaconda, Bioconductor | ++ | http://www.bioconductor.org/packages/release | 1 |
| Package          | Year | Description                                                                 | Language(s) | Version | Availability                      |
|------------------|------|------------------------------------------------------------------------------|--------------|---------|-----------------------------------|
| NPEBSeq[^104]    | 2013 | Nonparametric empirical bayesian-based procedure for differential expression analysis | R, N/A       | ++      | [http://bioinformatics.wistar.upenn.edu/NPEBSeq.html](http://bioinformatics.wistar.upenn.edu/NPEBSeq.html) |
| EBSeq[^105]      | 2013 | Identifying differentially expressed isoforms.                              | R, Anaconda, Bioconductor | ++      | [http://bioconductor.org/packages/release/bioc/html/EBSeq.html](http://bioconductor.org/packages/release/bioc/html/EBSeq.html) |
| sSeq[^106]       | 2013 | Shrinkage estimation of dispersion in Negative Binomial models               | R, Anaconda, Bioconductor | ++      | [http://bioconductor.org/packages/release/bioc/html/sSeq.html](http://bioconductor.org/packages/release/bioc/html/sSeq.html) |
| Cuffdiff2[^107]  | 2013 | Differential analysis at transcript resolution                              | C++, Python, N/A | +++     | [http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/](http://cole-trapnell-lab.github.io/cufflinks/cuffdiff/) |
| SAMseq[^108]     | 2013 | Nonparametric method with resampling to account for the different           | R, CRAN      | ++      | [https://rdrr.io/cran/samr/man/SAMseq.html](https://rdrr.io/cran/samr/man/SAMseq.html) |
| Software | Year | Methodology | Requirements | Websites |
|----------|------|-------------|--------------|---------|
| DSGseq\textsuperscript{109} | 2013 | NB-statistic method that can detect differentially spliced genes between two groups of samples without using a prior knowledge on the annotation of alternative splicing. | R | http://bioinfo.au.tsinghua.edu.cn/software/DSGseq/ |
| NOISeq\textsuperscript{110} | 2011 | Uses a non-parametric approach for differential expression analysis and can work in absence of replicates | R | https://bioconductor.org/packages/release/bioc/html/NOISeq.html |
| EdgeR\textsuperscript{111} | 2010 | Examining differential expression of replicated count data and differential exon usage | R | https://bioconductor.org/packages/release/bioc/html/edgeR.html |
| Tool   | Year | Description                                                                 | Language | Environment   | Website                                      | Notes |
|--------|------|-----------------------------------------------------------------------------|----------|---------------|----------------------------------------------|-------|
| DEGseq | 2010 | Identify differentially expressed genes or isoforms for RNA-seq data from different samples. | R        | Bioconductor  | [http://bioconductor.org/packages/release/bioc/html/DEGseq.html](http://bioconductor.org/packages/release/bioc/html/DEGseq.html) | 1     |
| LeafCutter | 2018 | Detects differential splicing and maps quantitative trait loci (sQTLs). | R        | Anaconda      | [https://github.com/daviddknowles/leafcutter.git](https://github.com/daviddknowles/leafcutter.git) | 1     |
| MAJIQ-SPEL | 2018 | Visualization, interpretation, and experimental validation of both classical and complex splicing variation and automated RT-PCR primer design. | C++, Python | N/A           | [https://galaxy.biociphers.org/galaxy/root?tol_id=majiq_spel](https://galaxy.biociphers.org/galaxy/root?tol_id=majiq_spel) | 2     |
| MAJIQ  | 2016 | Web-tool that takes as input local splicing variations (LSVs) quantified from RNA-seq data | C++, Python | N/A           | [https://majiq.biociphers.org/commercial.php](https://majiq.biociphers.org/commercial.php) | 2     |
and provides users with a visualization package (VOILA) and quantification of gene isoforms.

| Tool       | Year | Description                                                                 | Language | Environment | Rating | Website                                                                 | Notes |
|------------|------|------------------------------------------------------------------------------|----------|-------------|--------|------------------------------------------------------------------------|-------|
| SplAdder116| 2016 | Identification, quantification, and testing of alternative splicing events   | Python   | N/A         | +++    | http://github.com/ratschlab/spladder                                  | 1     |
| SplicePie117| 2015 | Detection of alternative, non-sequential and recursive splicing             | Perl, R  | N/A         | +++    | https://github.com/pulyakhina/splicing_analysis_pipeline               | 1     |
| SUPPA118   | 2015 | Alternative splicing analysis                                                | Python, R| Anaconda    | ++     | https://github.com/comprna/SUPPA                                     | 1     |
| SNPlie119  | 2015 | Identifying variants that modulate Intron retention                          | Python   | N/A         | +++    | https://code.google.com/p/snplice/                                     | 1     |
| IUTA120    | 2014 | Detecting differential isoform usage                                         | R        | N/A         | ++     | http://www.niehs.nih.gov/research/resources/software/biostatistics/iuta/index.cfm | 1     |
| Tool     | Year | Description                                                                 | Language | Platform | Version | Website                                      | Notes |
|----------|------|-----------------------------------------------------------------------------|----------|----------|---------|----------------------------------------------|-------|
| SigFuge  | 2014 | Identifying genomic loci exhibiting differential transcription patterns     | R        | Anaconda, Bioconductor | ++      | [http://bioconductor.org/packages/release/bioc/html/SigFuge.html](http://bioconductor.org/packages/release/bioc/html/SigFuge.html) | 1     |
| FineSplice | 2014 | Splice junction detection and quantification                               | Python   | N/A      | +++     | [https://sourceforge.net/p/finesplice/](https://sourceforge.net/p/finesplice/) | 1     |
| PennSeq  | 2014 | Statistical method that allows each isoform to have its own non-uniform read distribution | Perl     | N/A      | +++     | [http://sourceforge.net/projects/pennseq](http://sourceforge.net/projects/pennseq) | 1     |
| FlipFlop | 2014 | RNA isoform identification and quantification with network flows           | R        | Anaconda, Bioconductor | ++      | [https://bioconductor.org/packages/release/bioc/html/flipflop.html](https://bioconductor.org/packages/release/bioc/html/flipflop.html) | 1     |
| GESS     | 2014 | Graph-based exon-skipping scanner for de novo detection of skipping event sites | N/A      | N/A      | N/A     | [http://jinlab.net/GESS_Web/](http://jinlab.net/GESS_Web/) | 2     |
| Software       | Year | Description                                                                 | Language | Platform          | Rating | Website                                                                 |
|---------------|------|------------------------------------------------------------------------------|----------|-------------------|--------|-------------------------------------------------------------------------|
| spliceR       | 2013 | Classification of alternative splicing and prediction of coding potential   | R        | Anaconda, Bioconductor | ++     | [http://www.bioconductor.org/packages/2.13/bioc/html/spliceR.html](http://www.bioconductor.org/packages/2.13/bioc/html/spliceR.html) |
| RNASeq-MATS   | 2013 | Detects and analyzes differential alternative splicing events                | C, Python| N/A               | +++    | [http://rnaseq-mats.sourceforge.net/](http://rnaseq-mats.sourceforge.net/) |
| SplicingCompass | 2013 | Differential splicing detection                                              | R        | N/A               | ++     | [http://www.ichip.de/software](http://www.ichip.de/software)             |
| DiffSplice    | 2013 | Genome-wide detection of differential splicing                              | C++      | N/A               | +++    | [http://www.netlab.uky.edu/p/bioinfo/DiffSplice](http://www.netlab.uky.edu/p/bioinfo/DiffSplice) |
| DEXSeq        | 2012 | Statistical method to test for differential exon usage.                     | R        | Anaconda, Bioconductor | ++     | [https://bioconductor.org/packages/release/bioc/html/DEXSeq.html](https://bioconductor.org/packages/release/bioc/html/DEXSeq.html) |
| SpliceSeq     | 2012 | Identifies differential splicing events                                      | Java     | N/A               | +++    | [http://bioinformatics.medanderson.org/main/SpliceSeq:Overview](http://bioinformatics.medanderson.org/main/SpliceSeq:Overview). |
between test and control groups.

| JuncBASE \(^{132}\) | 2011 | Identification and quantification of alternative splicing, including unannotated splicing | Python | N/A | +++ | https://github.com/anbr ooks/juncBASE |
|---------------------|------|--------------------------------------------------------------------------------------|--------|-----|-----|--------------------------------------|
| ALEXA-seq \(^{133}\) | 2010 | Alternative expression analysis. | Perl, R, Shell (Bash) | N/A | +++ | http://www.alexaplatform.org/alexa_seq/ |

**h. Cell deconvolution**

| TIMER2.0 \(^{134}\) | 2020 | Web server for comprehensive analysis of Tumor-Infiltrating Immune Cells. | Web-tool R, Javascript | N/A | + | https://github.com/taiw enli/TIMER |
|---------------------|------|---------------------------------------------------------------------------------|----------------------|-----|---|--------------------------------------|
| CIBERSORTx \(^{135}\) | 2019 | Impute gene expression profiles and provide an estimation of the abundances of | Web-tool Java, R | N/A | + | https://cibersortx.stanfo rd.edu/ |

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\(^{132}\) JuncBASE, \(^{133}\) ALEXA-seq, \(^{134}\) TIMER2.0, \(^{135}\) CIBERSORTx
| ID       | Year | Description                                                                 | Language | System  | Rating | Website                                                                 | Score |
|----------|------|------------------------------------------------------------------------------|----------|---------|--------|------------------------------------------------------------------------|-------|
| quantIseq<sup>136</sup> | 2019 | Quantify the fractions of ten immune cell types from bulk RNA-sequencing data. | R, Shell (Bash) | N/A    | +      | [https://icbi.i-med.ac.at/quantiseq](https://icbi.i-med.ac.at/quantiseq) | 2     |
| Immunedeconv<sup>137</sup> | 2019 | Benchmarking of transcriptome-based cell-type quantification methods for immuno-oncology | R        | Anaconda | ++     | [https://github.com/icbi-lab/immunedeconv](https://github.com/icbi-lab/immunedeconv) | 1     |
| Linseed<sup>138</sup> | 2019 | Deconvolution of cellular mixtures based on linearity of transcriptional signatures. | C++, R   | N/A    | ++     | [https://github.com/ctlab/LinSeed](https://github.com/ctlab/LinSeed)    | 1     |
| deconvSEQ<sup>139</sup> | 2019 | Deconvolution of cell mixture distribution based | R        | N/A    | ++     | [https://github.com/rose-du1/deconvSeq](https://github.com/rose-du1/deconvSeq) | 1     |
| Tool      | Year | Description                                                                 | Software | Manual | Website                                                                 | Score |
|-----------|------|------------------------------------------------------------------------------|----------|--------|--------------------------------------------------------------------------|-------|
| CDSeq     | 2019 | Simultaneously estimate both cell-type proportions and cell-type-specific expression profiles. | MATLAB, R | N/A    | ++ [https://github.com/kkang7/CDSeq_R_Package](https://github.com/kkang7/CDSeq_R_Package) | 1     |
| Dtangle   | 2019 | Estimates cell type proportions using publicly available, often cross-platform, reference data. | R, Anaconda, CRAN | ++ [https://github.com/gjhu/dtangle](https://github.com/gjhu/dtangle) | 1     |
| GEDIT     | 2019 | Estimate cell type abundances.                                               | Web based tool, Python, R | + [http://webtools.mcdb.ucla.edu/](http://webtools.mcdb.ucla.edu/) | 2     |
| SaVant    | 2017 | Web based tool for sample level visualization of molecular                   | Javascript, R | +++ [http://newpathways.mcdb.ucla.edu/savant](http://newpathways.mcdb.ucla.edu/savant) | 2     |
| **EPIC**<sup>144</sup> | 2017 | Simultaneously estimates the fraction of cancer and immune cell types. | R | N/A | ++ | [http://epic.gfellerlab.org](http://epic.gfellerlab.org) | 2 |
| **WSCUnmix**<sup>145</sup> | 2017 | Automated deconvolution of structured mixtures. | MATLAB | N/A | +++ | [https://github.com/tedroman/WSCUnmix](https://github.com/tedroman/WSCUnmix) | 1 |
| **Infino**<sup>146</sup> | 2017 | Deconvolves bulk RNA-seq into cell type abundances and captures gene expression variability in a Bayesian model to measure deconvolution uncertainty. | R, Python | N/A | ++ | [https://github.com/hammerlab/infino](https://github.com/hammerlab/infino) | 1 |
| **MCP-counter**<sup>147</sup> | 2016 | Estimating the population abundance of | R | N/A | + | [https://github.com/ebecht/MCPcounter](https://github.com/ebecht/MCPcounter) | 1 |
| Method          | Year | Description                                                                 | Language | Version | Score | Website                                      | References |
|-----------------|------|------------------------------------------------------------------------------|----------|---------|-------|----------------------------------------------|------------|
| CellCode        | 2015 | Latent variable approach to differential expression analysis for heterogeneous cell populations. | R        | N/A     | ++    | http://www.pitt.edu/~mchkina/CellCODE/       | 2          |
| PERT            | 2012 | Probabilistic expression deconvolution method.                              | MATLAB   | N/A     | +++   | https://github.com/gquon/PERT                 | 1          |

**i. Immune repertoire profiling**

| Method         | Year | Description                                                                 | Language | Version | Score | Website                                         | References |
|----------------|------|------------------------------------------------------------------------------|----------|---------|-------|------------------------------------------------|------------|
| ImReP          | 2018 | Profiling immunoglobulin repertoires across multiple human tissues.        | Python   | N/A     | +++   | https://github.com/mandricigor/imrep/wiki       | 1          |
| TRUST (T cell) | 2016 | Landscape of tumor-infiltrating T cell repertoire                           | Perl     | N/A     | +++   | https://github.com/liulab-dfci/TRUST4          | 1          |
| Method                  | Year | Description                                                                 | Language | Environment | Notes                          | Version |
|------------------------|------|-----------------------------------------------------------------------------|----------|-------------|--------------------------------|---------|
| V’DJer                 | 2016 | Assembly-based inference of B-cell receptor repertoires from short reads with V’DJer. | C, C++   | Anaconda    | [https://github.com/mozack/vdjer](https://github.com/mozack/vdjer) | ++      |
| IgBlast-based pipeline | 2016 | Statistical inference of a convergent antibody repertoire response.          | C++      | N/A         | [https://www.ncbi.nlm.nih.gov/igblast/](https://www.ncbi.nlm.nih.gov/igblast/) | +++     |
| MiXCR                  | 2015 | Processes big immunome data from raw sequences to quantitated clonotypes.    | Java     | Anaconda    | [https://github.com/milaboratory/mixcr](https://github.com/milaboratory/mixcr) | ++      |

**j. Allele specific expression**

| EAGLE                  | 2017 | Bayesian model for identifying GxE interactions                             | C++, R   | N/A         | [https://github.com/davidaknowles/eagle](https://github.com/davidaknowles/eagle) | ++      |
| Tool          | Year | Description                                                                 | Language | Version | Rating | Website                                                                 |
|--------------|------|------------------------------------------------------------------------------|----------|---------|--------|-------------------------------------------------------------------------|
| ANEVA-DOT/ANEVA 1 56 | 2019 | Identify ASE outlier genes / Quantify genetic variation in gene dosage from ASE data. | R        | N/A     | ++     | [https://github.com/PejLab/ANEVA-DOT](https://github.com/PejLab/ANEVA-DOT) |
| aFC 157       | 2017 | Quantifying the regulatory effect size of cis-acting genetic variation        | Python   | N/A     | +++    | [https://github.com/secastel/aFC](https://github.com/secastel/aFC)        |
| phASER 158    | 2016 | Uses readback phasing to produce haplotype level ASE data (as opposed to SNP level) | Python   | N/A     | +++    | [https://github.com/secastel/phaser](https://github.com/secastel/phaser)  |
| **RASQUAL**<sup>159</sup> | 2016 | Maps QTLs for sequenced based cellular traits by combining population and allele-specific signals. | C, R | N/A | +++ | [https://github.com/natsuhiko/rasqual](https://github.com/natsuhiko/rasqual) | 1 |
|-------------------------|------|-----------------------------------------------------------------|-----|-----|-----|--------------------------|---|
| **allelecounter**<sup>160</sup> | 2015 | Generate ASE data from RNAseq data and a genotype file. | Python | N/A | +++ | [https://github.com/secastel/allelecounter](https://github.com/secastel/allelecounter) | 1 |
| **WASP**<sup>161</sup> | 2015 | Unbiased allele-specific read mapping and discovery of molecular QTLs | C Python Anaconda | ++ | [https://github.com/bmvdgeijn/WASP/](https://github.com/bmvdgeijn/WASP/) | 1 |
| **Mamba**<sup>162</sup> | 2015 | Compares different patterns of ASE across tissues | R | N/A | ++ | [http://www.well.ox.ac.uk/~rivas/mamba/](http://www.well.ox.ac.uk/~rivas/mamba/) | 2 |
| **MBASED**<sup>163</sup> | 2014 | Allele-specific expression detection in cancer tissues and cell lines | R | Anaconda, Bioconductor | ++ | [https://bioconductor.org/packages/release/bioc/html/MBASED.html](https://bioconductor.org/packages/release/bioc/html/MBASED.html) | 1 |
| **Allim**<sup>164</sup> | 2013 | Estimates allele-specific gene expression. | Python, R | N/A | +++ | [https://sourceforge.net/projects/allim/](https://sourceforge.net/projects/allim/) | 1 |
| Project | Year | Description | Tools | Version | Website | Rating |
|---------|------|-------------|-------|---------|---------|--------|
| AlleleSeq\textsuperscript{165} | 2011 | Identifies allele-specific events in mapped reads between maternal and paternal alleles. | Python, Shell | N/A | +++ | [http://alleleseq.gersteinlab.org/](http://alleleseq.gersteinlab.org/) |
| k. Viral detection | | | | | |
| ROP\textsuperscript{166} | 2018 | Dumpster diving in RNA-sequencing to find the source of 1 trillion reads across diverse adult human tissues | Python, Shell (Bash) | Anaconda | ++ | [https://github.com/smanngull/rop](https://github.com/smanngull/rop) |
| RNA CoMPASS\textsuperscript{167} | 2014 | Simultaneous analysis of transcriptomes and metatranscriptomes from diverse biological specimens. | Perl, Shell, Java | N/A | ++ | [http://rnacompass.sourceforge.net/](http://rnacompass.sourceforge.net/) |
| VirusSeq\textsuperscript{168} | 2013 | Identify viruses and their integration sites using next- | Perl, Shell (Bash) | N/A | +++ | [http://odin.mdacc.tmc.edu/~xsu1/VirusSeq.html](http://odin.mdacc.tmc.edu/~xsu1/VirusSeq.html) |
| Tool                  | Year | Description                                                                                      | Language(s) | Note | Website                                                                 | Score |
|----------------------|------|--------------------------------------------------------------------------------------------------|--------------|------|-------------------------------------------------------------------------|-------|
| VirusFinder¹⁶⁹        | 2013 | Detection of Viruses and Their Integration Sites in Host Genomes through Next Generation Sequencing Data | Perl         | N/A  | http://bioinfo.mc.vanderbilt.edu/VirusFinder/                        | 2     |
| INTEGRATE-Vis¹⁷⁰      | 2017 | Generates plots focused on annotating each gene fusion at the transcript-and protein-level and assessing expression across samples. | Python       | N/A  | https://github.com/ChrisMaherLab/INTEGRATE-Vis                      | 1     |
| INTEGRATE-Neo¹⁷¹      | 2017 | Gene fusion neoantigen discovery tool, which uses RNA-Seq reads and is capable of                | Python, C++ | N/A  | https://github.com/ChrisMaherLab/INTEGRATE-Neo                      | 1     |
| Software | Year | Description | Programming Languages | Compatibility | Website | Notes |
|----------|------|-------------|------------------------|---------------|---------|-------|
| INTEGRATE | 2016 | Capable of integrating aligned RNA-seq and WGS reads and characterizes the quality of predictions. | C++ | N/A | +++ | https://sourceforge.net/projects/integrate-fusion/ | 1 |
| TRUP | 2015 | Combines split-read and read-pair analysis with de novo regional assembly for the identification of chimeric transcripts in cancer specimens. | C++, Perl, R | N/A | +++ | https://github.com/ruping/TRUP | 1 |
| PRADA | 2014 | Detect gene fusions but also performs alignments, | Python | Anaconda | ++ | http://sourceforge.net/projects/prada/ | 1 |
| Software | Year | Description | Programming Languages | Dependencies | Website | Rank |
|----------|------|-------------|-----------------------|--------------|---------|------|
| Pegasus[^75] | 2014 | Annotation and prediction of biologically functional gene fusion candidates. | Java, Perl, Python, Shell (Bash) | N/A | +++ | https://github.com/Raba
danLab/Pegasus |
| FusionCatcher[^76] | 2014 | Finding somatic fusion genes | Python | Anaconda | ++ | https://sourceforge.net/
projects/fusioncatcher/ |
| FusionQ[^77] | 2013 | Gene fusion detection and quantification from paired-end RNA-seq | C++, Perl, R | N/A | +++ | http://www.wakehealth.
edu/CTSB/Software/So
ftware.htm |
| Barnacle[^78] | 2013 | Detecting and characterizing tandem | Python, Perl | N/A | +++ | http://www.bcgsc.ca/pl
atform/bioinfo/software
/barnacle |
| Software | Year | Description | Programming Languages | Environment | Website | Version |
|----------|------|-------------|-----------------------|-------------|---------|---------|
| Dissect | 2012 | Detection and characterization of structural alterations in transcribed sequences | C | N/A | +++ | http://dissect-trans.sourceforge.net |
| BreakFusion | 2012 | Targeted assembly-based identification of gene fusions | C++, Perl | N/A | +++ | https://bioinformatics.mdanderson.org/public-software/breakfusion/ |
| EricScript | 2012 | Identification of gene fusion products in paired-end RNA-seq data. | Perl, R, Shell (Bash) | Anaconda | ++ | http://ericscript.sourceforge.net |
| Bellerophontes | 2012 | Chimeric transcripts discovery based on fusion model. | Java, Perl, Shell (Bash) | N/A | +++ | http://eda.polito.it/bellerophontes/ |
| GFML | 2012 | Standard format for organizing and representing | XML | N/A | + | http://code.google.com/p/gfml-prototype/ |
| Tool        | Year | Features                                                                 | Language(s) | Details                                                                 | Score | Notes |
|------------|------|---------------------------------------------------------------------------|-------------|--------------------------------------------------------------------------|-------|-------|
| FusionHunter\(^1\) | 2011 | Identifies fusion transcripts from transcriptional analysis.              | C++, N/A    | +++ [https://github.com/ma-compbio/FusionHunter](https://github.com/ma-compbio/FusionHunter) | 1     |       |
| ChimeraScan\(^5\) | 2011 | Identifying chimeric transcription.                                       | Python, Anaconda | ++ [https://code.google.com/archive/p/chimerascan/downloads](https://code.google.com/archive/p/chimerascan/downloads) | 1     |       |
| TopHat-fusion\(^6\) | 2011 | Discovery of novel fusion transcripts.                                   | C++, Python, N/A | +++ [http://ccb.jhu.edu/software/tophat/fusion_index.shtml](http://ccb.jhu.edu/software/tophat/fusion_index.shtml) | 2     |       |
| deFuse\(^7\)    | 2011 | Fusion discovery in tumor RNA-seq data.                                 | C++, Perl, R Anaconda | ++ [https://github.com/ampherson/defuse/blob/master/README.md](https://github.com/ampherson/defuse/blob/master/README.md) | 1     |       |

**m. Detecting circRNA**

| Tool     | Year | Features                                                                 | Language(s) | Details                                                                 | Score | Notes |
|----------|------|---------------------------------------------------------------------------|-------------|--------------------------------------------------------------------------|-------|-------|
| CIRIquant\(^8\) | 2020 | Accurate quantification and differential expression analysis of circRNAs. | Python, N/A | ++ [https://sourceforge.net/projects/ciri/files/CIRIquant](https://sourceforge.net/projects/ciri/files/CIRIquant) | 1     |       |
| Name            | Year | Description                                                                 | Language | Environment | Rating | Website                                                                 | Notes |
|-----------------|------|------------------------------------------------------------------------------|----------|-------------|--------|--------------------------------------------------------------------------|-------|
| CIRI-vis         | 2020 | Visualization of circRNA structures.                                        | Java     | N/A         | +++    | https://sourceforge.net/projects/ciri/files/CIRI-vis                    |       |
| Ularcirc        | 2019 | Analysis and visualisation of canonical and back splice junctions.           | R        | Bioconductor| ++     | https://github.com/VCCRI/Ularcirc                                        |       |
| CLEAR           | 2019 | Circular and Linear RNA expression analysis.                                | Python   | N/A         | +++    | https://github.com/YangLab/CLEAR                                         |       |
| CIRI-full       | 2019 | Reconstruct and quantify full-length circular RNAs.                         | Java     | N/A         | +++    | https://sourceforge.net/projects/ciri/files/CIRI-full                    |       |
| circAST         | 2019 | Full-length assembly and quantification of alternatively spliced isoforms in Circular RNAs | Python   | N/A         | +++    | https://github.com/xiaofengsong/CircAST                                  |       |
| CIRI2           | 2018 | Denovo circRNA identification                                               | Pearl    | N/A         | +++    | https://sourceforge.net/projects/ciri/files/CIRI2                        |       |
| Sailfish-cir    | 2017 | Quantification of circRNAs using model-based framework                      | Python   | N/A         | +++    | https://github.com/zero/del/sailfish-cir                                 |       |
| Tool          | Year | Description                                                                 | Language | Platform | Score | Website                                                                 |
|--------------|------|------------------------------------------------------------------------------|----------|----------|-------|-------------------------------------------------------------------------|
| CircComPara  | 196  | Multi-method detection of circRNAs                                           | R, Python| N/A      | +++   | [http://github.com/egaffo/CirComPara](http://github.com/egaffo/CirComPara) |
| UROBORUS     | 197  | Computationally identifying circRNAs from RNA-seq data                       | Perl     | N/A      | +++   | [https://github.com/WGLab/UROBORUS/tree/master/bin](https://github.com/WGLab/UROBORUS/tree/master/bin) |
| PTESFinder   | 198  | Identification of non-co-linear transcripts                                 | Shell, Java | N/A      | +++   | [https://sourceforge.net/projects/ptesfinder-v1/](https://sourceforge.net/projects/ptesfinder-v1/) |
| NCLscan      | 199  | Identification of non-co-linear transcripts (fusion, trans-splicing and circular RNA) | Python   | N/A      | +++   | [https://github.com/TreesLab/NCLscan](https://github.com/TreesLab/NCLscan) |
| DCC          | 200  | Specific identification and quantification of circRNA                        | Python   | N/A      | +++   | [https://github.com/dietrich-lab/DCC](https://github.com/dietrich-lab/DCC) |
| CIRI-AS      | 201  | Identification of internal structure and alternative splicing events in circRNA | Perl     | N/A      | +++   | [https://sourceforge.net/projects/ciri/files/CIRI-AS](https://sourceforge.net/projects/ciri/files/CIRI-AS) |
| Tool                  | Year | Description                                                                 | Language | Dependencies | Quality | Website                                                                 | Version |
|-----------------------|------|-----------------------------------------------------------------------------|----------|--------------|---------|--------------------------------------------------------------------------|---------|
| circTest              | 2016 | Differential expression analysis and plotting of circRNAs                  | R        | N/A          | +++     | [https://github.com/diete rich-lab/CircTest](https://github.com/diete rich-lab/CircTest)  |         |
| CIRCexplorer          | 2016 | Annotation and de novo assembly of circRNAs                                 | Python   | Anaconda     | ++      | [https://github.com/YangLab/CIRCexplorer2](https://github.com/YangLab/CIRCexplorer2) |         |
| KNIFE                 | 2015 | Statistically based detection of circular and linear isoforms from RNA-seq data | Perl, Python, R | N/A          | +++     | [https://github.com/linda szabo/KNIFE](https://github.com/linda szabo/KNIFE) |         |
| circRNA_finder        | 2014 | Identification of circRNAs from RNA-seq data                               | Perl     | N/A          | +++     | [https://github.com/orze choj/circRNA_finder](https://github.com/orze choj/circRNA_finder) |         |
| find_circ             | 2013 | Identification of circRNAs based on head-to-tail spliced sequencing reads   | Python   | N/A          | ++      | [https://github.com/marvin-jens/find_circ](https://github.com/marvin-jens/find_circ) |         |
| **n. Small RNA detection** |      |                                                                              |          |              |         |                                                                         |         |
| miRTrace              | 2018 | Quality control of miRNA-seq data, identifies cross-species contamination. | Java, Anaconda | Anaconda     | +++     | [https://github.com/fried landerlab/mirtrace](https://github.com/fried landerlab/mirtrace) |         |
| Tool Name   | Year | Description                                                                 | Type            | Status | Website                                                                 | Score |
|------------|------|------------------------------------------------------------------------------|-----------------|--------|------------------------------------------------------------------------|-------|
| sRNAbench  | 2015 | Expression profiling of small RNAs, prediction of novel microRNAs, analysis of isomiRs, genome mapping and read length statistics. | Web based tool  | N/A    | +                                                                      | https://bioinfo5.ugr.es/srnatoolbox/srnabench/ | 2     |
| sRNAde     | 2015 | Detection of differentially expressed small RNAs based on three programs.   | Web based tool  | N/A    | +                                                                      | https://bioinfo5.ugr.es/srnatoolbox/srnade/   | 2     |
| sRNAblast  | 2015 | Aimed to determine the origin of unmapped or unassigned reads by means of a blast search against several remote databases. | Web based tool  | N/A    | +                                                                      | https://bioinfo5.ugr.es/srnatoolbox/srnablast/     | 2     |
| miRNAconsTarget | 2015 | Consensus target prediction on user provided input data.                   | Web based tool  | N/A    | +                                                                      | https://bioinfo5.ugr.es/srnatoolbox/amirconstarget/ | 2     |
| Tool          | Year | Description                                                                 | Platform | Environment | Accessibility | Notes |
|--------------|------|-----------------------------------------------------------------------------|----------|-------------|---------------|-------|
| sRNAjBrowser | 2015 | Visualization of sRNA expression data in a genome context.                  | Web based tool | N/A          | +             | https://bioinfo5.ugr.es/srnatoolbox/srnajbrowser/ | 2     |
| sRNAjBrowser DE | 2015 | Visualization of differential expression as a function of read length in a genome context. | Web based tool | N/A          | +             | https://bioinfo5.ugr.es/srnatoolbox/srnajbrowserde/ | 2     |
| ShortStack   | 2013 | Analyzes reference-aligned sRNA-seq data and performs comprehensive de novo annotation and quantification of the inferred sRNA genes. | Perl     | Anaconda    | +++           | https://github.com/MikeAxtell/ShortStack | 1     |
| mirTools 2.0 | 2013 | Detect, identify and profile various types, functional annotation and differentially expressed sRNAs. | Web based tool | N/A          | +             | http://www.wzgenomics.cn/mr2_dev/ | 2     |
| Tool Name          | Year | Description                                                                 | Programming Languages | C++ | Java | Web Link                                                                 | Score |
|-------------------|------|-----------------------------------------------------------------------------|-----------------------|-----|------|---------------------------------------------------------------------------|-------|
| UEA sRNA Workbench | 2012 | Complete analysis of single or multiple-sample small RNA datasets.          | Web based tool, C++, Java | N/A |      | [https://sourceforge.net/projects/srnaworkbench/](https://sourceforge.net/projects/srnaworkbench/) | 1     |
| miRDeep2          | 2011 | Discovers known and novel miRNAs, quantifies miRNA expression.              | Perl                  | Anaconda | +++ | [https://github.com/raje wsky-lab/mirdeep2](https://github.com/raje wsky-lab/mirdeep2) | 1     |
| miRanalyzer       | 2011 | Detection of known and prediction of new microRNAs in high-throughput sequencing experiments. | Web based tool        | N/A |      | [http://bioinfo2.ugr.es/miRanalyzer/miRanalyze r.php](http://bioinfo2.ugr.es/miRanalyzer/miRanalyze r.php) | 2     |
| SeqBuster         | 2010 | Provides an automatized pre-analysis for sequence annotation for analysing small RNA data from Illumina sequencing. | Web based tool        | Anaconda | +   | [http://estivill_lab.crg.es /seqbuster](http://estivill_lab.crg.es /seqbuster) | 2     |
| **Software** | **Year** | **Description** | **Language** | **License** | **Website** | **Score** |
|--------------|----------|-----------------|--------------|-------------|-------------|-----------|
| DARIO<sup>214</sup> | 2010 | Allows to study short read data and provides a wide range of analysis features, including quality control, read normalization, and quantification. | Web based tool | N/A | + | [http://dario.bioinf.uni-leipzig.de/index.py](http://dario.bioinf.uni-leipzig.de/index.py) | 2 |
| **o. Visualization tools** | | | | | | |
| BEAVR<sup>215</sup> | 2020 | Facilitates interactive analysis and exploration of RNA-seq data, allowing statistical testing and visualization of the table of differentially expressed genes obtained. | R | N/A | ++ | [https://github.com/developerpiru/BEAVR](https://github.com/developerpiru/BEAVR) | 1 |
| coseq<sup>216</sup> | 2018 | Co-expression analysis of sequencing data | R | Anaconda, Bioconductor | ++ | [https://bioconductor.org/packages/release/bioc/html/coseq.html](https://bioconductor.org/packages/release/bioc/html/coseq.html) | 1 |
| ReadXplorer<sup>217</sup> | 2016 | Read mapping analysis and visualization | Java | N/A | +++ | [https://www.uni-giessen.de/fbz/fb08/Inst](https://www.uni-giessen.de/fbz/fb08/Inst) | 2 |
| Name | Year | Description | Language | Available | URL |
|------|------|-------------|----------|-----------|-----|
| Integrated Genome Browser²¹⁸ | 2016 | An interactive tool for visually analyzing tiling array data and enables quantification of alternative splicing | Java | N/A | +++ | http://www.bioviz.org/ |
| Sashimi plots²¹⁹ | 2015 | Quantitative visualization comparison of exon usage | Python | N/A | ++ | http://miso.readthedocs.org/en/fastmiso/sashimi.html |
| ASTALAVISTA²²⁰ | 2015 | Reports all alternative splicing events reflected by transcript annotations | Java | Anaconda | ++ | http://astalavista.samme.th.net/ |
| RNASeqBrowser²²¹ | 2015 | Incorporates and extends the functionality of the UCSC genome browser | Java | N/A | +++ | http://www.australianprostatecentre.org/research/software/rnaseqbrowser |
| SplicePlot²²² | 2014 | Visualizing splicing quantitative trait loci | Python | N/A | +++ | http://montgomerylab.stanford.edu/spliceplot/index.html |
| Tool                  | Year | Description                                                                 | Language(s) | Dependencies | URL                                           | Notes |
|----------------------|------|------------------------------------------------------------------------------|--------------|--------------|-----------------------------------------------|-------|
| RNASeqVieweR         | 2014 | Compare gene expression and alternative splicing                           | Python       | N/A          | +++                                           | 1     |
|                       |      |                                                                              |              |              | [https://sourceforge.net/projects/rnaseqbrowser/](https://sourceforge.net/projects/rnaseqbrowser/) |       |
| PrimerSeq            | 2014 | Systematic design and visualization of RT-PCR primers using RNA seq data    | Java, C++,   | N/A          | +++                                           | 1     |
|                       |      |                                                                              | Python       |              | [http://primerseq.sourceforge.net/](http://primerseq.sourceforge.net/) |       |
| Epiviz               | 2014 | Combining algorithmic-statistical analysis and interactive visualization    | R            | Anaconda,   | ++                                            | 1     |
|                       |      |                                                                              |              | Bioconductor|                                               |       |
|                       |      |                                                                              |              |              | [https://epiviz.github.io/](https://epiviz.github.io/) |       |
| RNAbrowse            | 2014 | RNA-seq De Novo Assembly Results Browser                                    | N/A          | N/A          | N/A                                           | 2     |
|                       |      |                                                                              |              |              | [http://bioinfo.genotoul.fr/RNAbrowse](http://bioinfo.genotoul.fr/RNAbrowse) |       |
| ZENBU                | 2014 | Interactive visualization and analysis of large-scale sequencing datasets    | C++, Javascript | N/A            | +                                              | 2     |
|                       |      |                                                                              |              |              | [https://fantom.gsc.riken.jp/zenbu/](https://fantom.gsc.riken.jp/zenbu/) |       |
| CummeRbund           | 2012 | Navigate through data produced from a Cuffdiff RNA-seq differential expression analysis | R            | Anaconda,   | ++                                            | 1     |
|                       |      |                                                                              |              | Bioconductor|                                               |       |
|                       |      |                                                                              |              |              | [http://bioconductor.org/packages/devel/bioc/html/cummeRbund.html](http://bioconductor.org/packages/devel/bioc/html/cummeRbund.html) |       |
Table 1: Landscape of current computational methods for RNA-seq analysis. We categorized RNA-seq tools published from 2008 to 2020 based on processes in the RNA-seq pipeline and workflow; starting with data quality control, read alignment, gene annotations, transcriptome assembly, transcriptome quantification, differential expression, RNA splicing, cell deconvolution, immune repertoire profiling, allele specific expression, viral detection, fusion detection, detecting circRNA, small RNA detection, and visualization tools. The third column (“Notable Features”) presents key functionalities and methods used. The fourth column (“Programming Language”) presents the interface mode (e.g., GUI, web-based, programming language). The fifth column (“Package Manager”) highlights if a package manager such as Anaconda, Bioconductor, CRAN, Docker Hub, pip, or PyPI is available for the tool. We designated the assumed expertise level with a +, ++, or +++ in the sixth column (“Required Expertise”). A “+” represents little to no required expertise which would be assigned to a GUI based/web interface tool. “++” was assigned to tools that require R and/or multiple programming languages and whose software is located on Anaconda, Bioconductor, CRAN, Docker Hub, pip, or PyPI. “+++” was assigned to tools that require expertise in languages such as C, C++, Java, Python, Perl, or Shell (Bash) and may or may not have a package manager present. For each tool, we provide the links where the published tool software can be found and downloaded (“Software”). In the seventh column (“Type of URL”), each tool was assigned a “1” for web services designed to host source code or “2” for others (e.g., personal and/or university web services).
Supplementary Table - References

1. Kumar, G., Ertel, A., Feldman, G., Kupper, J. & Fortina, P. iSeqQC: a tool for expression-based quality control in RNA sequencing. *BMC Bioinformatics* 21, 56 (2020).

2. Hicks, S. C. *et al.* Smooth quantile normalization. *Biostatistics* 19, 185–198 (2018).

3. Babraham Bioinformatics - FastQC A Quality Control tool for High Throughput Sequence Data. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

4. Guo, Y. *et al.* Multi-perspective quality control of Illumina exome sequencing data using QC3. *Genomics* 103, 323–328 (2014).

5. Anvar, S. Y. *et al.* Determining the quality and complexity of next-generation sequencing data without a reference genome. *Genome Biol.* 15, 555 (2014).

6. Yang, X. *et al.* HTQC: a fast quality control toolkit for Illumina sequencing data. *BMC Bioinformatics* 14, 33 (2013).

7. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120 (2014).

8. Jiang, H., Lei, R., Ding, S.-W. & Zhu, S. Skewer: a fast and accurate adapter trimmer for next-generation sequencing paired-end reads. *BMC Bioinformatics* 15, 182 (2014).

9. Dodt, M., Roehr, J., Ahmed, R. & Dieterich, C. FLEXBAR—Flexible Barcode and Adapter Processing for Next-Generation Sequencing Platforms. *Biology* vol. 1 895–905 (2012).

10. Kroll, K. W. *et al.* Quality Control for RNA-Seq (QuaCRS): An Integrated Quality Control Pipeline. *Cancer Inform.* 13, 7–14
11. Cabanski, C. R. et al. BlackOPs: increasing confidence in variant detection through mappability filtering. *Nucleic Acids Res.* **41**, e178 (2013).

12. Wang, L., Wang, S. & Li, W. RSeQC: quality control of RNA-seq experiments. *Bioinformatics* **28**, 2184–2185 (2012).

13. DeLuca, D. S. et al. RNA-SeQC: RNA-seq metrics for quality control and process optimization. *Bioinformatics* **28**, 1530–1532 (2012).

14. Jones, D. C., Ruzzo, W. L., Peng, X. & Katze, M. G. A new approach to bias correction in RNA-Seq. *Bioinformatics* **28**, 921–928 (2012).

15. Lassmann, T., Hayashizaki, Y. & Daub, C. O. SAMStat: monitoring biases in next generation sequencing data. *Bioinformatics* **27**, 130–131 (2011).

16. Li, H. et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).

17. Liu, B. et al. deSALT: fast and accurate long transcriptomic read alignment with de Bruijn graph-based index. doi:10.1101/612176.

18. Boratyn, G. M., Thierry-Mieg, J., Thierry-Mieg, D., Busby, B. & Madden, T. L. Magic-BLAST, an accurate DNA and RNA-seq aligner for long and short reads. doi:10.1101/390013.

19. Li, H. Minimap2: pairwise alignment for nucleotide sequences. *Bioinformatics* **34**, 3094–3100 (2018).

20. Lin, H.-N. & Hsu, W.-L. DART: a fast and accurate RNA-seq mapper with a partitioning strategy. *Bioinformatics* vol. 34 190–
21. Kahles, A., Behr, J. & Rätsch, G. MMR: a tool for read multi-mapper resolution. *Bioinformatics* **32**, 770–772 (2016).

22. Bonfert, T., Kirner, E., Csaba, G., Zimmer, R. & Friedel, C. C. ContextMap 2: fast and accurate context-based RNA-seq mapping. *BMC Bioinformatics* **16**, 122 (2015).

23. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory requirements. *Nat. Methods* **12**, 357–360 (2015).

24. Hoffmann, S. *et al.* A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection. *Genome Biol.* **15**, R34 (2014).

25. Butterfield, Y. S. *et al.* JAGuaR: junction alignments to genome for RNA-seq reads. *PLoS One* **9**, e102398 (2014).

26. Philippe, N., Salson, M., Commes, T. & Rivals, E. CRAC: an integrated approach to the analysis of RNA-seq reads. *Genome Biol.* **14**, R30 (2013).

27. Dobin, A. *et al.* STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15–21 (2013).

28. Liao, Y., Smyth, G. K. & Shi, W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Res.* **41**, e108 (2013).

29. Kim, D. *et al.* TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **14**, R36 (2013).

30. Hu, J., Ge, H., Newman, M. & Liu, K. OSA: a fast and accurate alignment tool for RNA-Seq. *Bioinformatics* vol. 28 1933–1934.
31. Zhang, Y. et al. PASSion: a pattern growth algorithm-based pipeline for splice junction detection in paired-end RNA-Seq data. *Bioinformatics* **28**, 479–486 (2012).

32. Grant, G. R. et al. Comparative analysis of RNA-Seq alignment algorithms and the RNA-Seq unified mapper (RUM). *Bioinformatics* **27**, 2518–2528 (2011).

33. Huang, S. et al. SOAPsplice: Genome-Wide ab initio Detection of Splice Junctions from RNA-Seq Data. *Front. Genet.* **2**, 46 (2011).

34. Wang, K. et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. *Nucleic Acids Res.* **38**, e178 (2010).

35. Au, K. F., Jiang, H., Lin, L., Xing, Y. & Wong, W. H. Detection of splice junctions from paired-end RNA-seq data by SpliceMap. *Nucleic Acids Res.* **38**, 4570–4578 (2010).

36. Bryant, D. W., Jr, Shen, R., Priest, H. D., Wong, W.-K. & Mockler, T. C. Supersplat--spliced RNA-seq alignment. *Bioinformatics* **26**, 1500–1505 (2010).

37. Dimon, M. T., Sorber, K. & DeRisi, J. L. HMMSplicer: a tool for efficient and sensitive discovery of known and novel splice junctions in RNA-Seq data. *PLoS One* **5**, e13875 (2010).

38. De Bona, F., Ossowski, S., Schneeberger, K. & Rätsch, G. Optimal spliced alignments of short sequence reads. *Bioinformatics* **24**, i174–80 (2008).
39. Tardaguila, M. et al. SQANTI: extensive characterization of long-read transcript sequences for quality control in full-length transcriptome identification and quantification. *Genome Res.* (2018) doi:10.1101/gr.222976.117.

40. Musacchia, F., Basu, S., Petrosino, G., Salvemini, M. & Sanges, R. Annocript: a flexible pipeline for the annotation of transcriptomes able to identify putative long noncoding RNAs. *Bioinformatics* **31**, 2199–2201 (2015).

41. Gao, Y., Wang, J. & Zhao, F. CIRI: an efficient and unbiased algorithm for de novo circular RNA identification. *Genome Biology* vol. 16 (2015).

42. Amman, F. et al. TSSAR: TSS annotation regime for dRNA-seq data. *BMC Bioinformatics* **15**, 89 (2014).

43. Tang, A. D. et al. Full-length transcript characterization of SF3B1 mutation in chronic lymphocytic leukemia reveals downregulation of retained introns. doi:10.1101/410183.

44. Shao, M. & Kingsford, C. Accurate assembly of transcripts through phase-preserving graph decomposition. *Nat. Biotechnol.* **35**, 1167–1169 (2017).

45. Song, L., Sabunciyan, S. & Florea, L. CLASS2: accurate and efficient splice variant annotation from RNA-seq reads. *Nucleic Acids Res.* **44**, e98 (2016).

46. Pertea, M. et al. StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. *Nat. Biotechnol.* **33**, 290–295 (2015).

47. Chang, Z. et al. Bridger: a new framework for de novo transcriptome assembly using RNA-seq data. *Genome Biology* vol. 16 (2015).
48. Maretty, L., Sibbesen, J. A. & Krogh, A. Bayesian transcriptome assembly. *Genome Biol.* **15**, 501 (2014).

49. Le, H.-S., Schulz, M. H., McCauley, B. M., Hinman, V. F. & Bar-Joseph, Z. Probabilistic error correction for RNA sequencing. *Nucleic Acids Res.* **41**, e109 (2013).

50. Bao, E., Jiang, T. & Girke, T. BRANCH: boosting RNA-Seq assemblies with partial or related genomic sequences. *Bioinformatics* vol. 29 1250–1259 (2013).

51. Chu, H.-T. *et al.* EBARDenovo: highly accurate de novo assembly of RNA-Seq with efficient chimera-detection. *Bioinformatics* **29**, 1004–1010 (2013).

52. Schulz, M. H., Zerbino, D. R., Vingron, M. & Birney, E. Oases: robust de novo RNA-seq assembly across the dynamic range of expression levels. *Bioinformatics* **28**, 1086–1092 (2012).

53. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578 (2012).

54. Feng, J., Li, W. & Jiang, T. Inference of isoforms from short sequence reads. *J. Comput. Biol.* **18**, 305–321 (2011).

55. Li, W., Feng, J. & Jiang, T. IsoLasso: a LASSO regression approach to RNA-Seq based transcriptome assembly. *J. Comput. Biol.* **18**, 1693–1707 (2011).

56. Grabherr, M. G. *et al.* Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol.* **29**, 644–652 (2011).

57. Robertson, G. *et al.* De novo assembly and analysis of RNA-seq data. *Nat. Methods* **7**, 909–912 (2010).
58. Guttman, M. et al. Ab initio reconstruction of cell type–specific transcriptomes in mouse reveals the conserved multi-exonic structure of lincRNAs. *Nature Biotechnology* vol. 28 503–510 (2010).

59. Wyman, D. et al. A technology-agnostic long-read analysis pipeline for transcriptome discovery and quantification. doi:10.1101/672931.

60. Patro, R., Duggal, G., Love, M. I., Irizarry, R. A. & Kingsford, C. Salmon provides fast and bias-aware quantification of transcript expression. *Nat. Methods* 14, 417–419 (2017).

61. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. *Nat. Biotechnol.* 34, 525–527 (2016).

62. nanoporetech. nanoporetech/wub. *GitHub* https://github.com/nanoporetech/wub.

63. Schmid, M. W. & Grossniklaus, U. Rcount: simple and flexible RNA-Seq read counting. *Bioinformatics* 31, 436–437 (2015).

64. Anders, S., Pyl, P. T. & Huber, W. HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31, 166–169 (2015).

65. Lee, S., Seo, C. H., Alver, B. H., Lee, S. & Park, P. J. EMSAR: estimation of transcript abundance from RNA-seq data by mappability-based segmentation and reclustering. *BMC Bioinformatics* 16, 278 (2015).

66. Finotello, F. et al. Reducing bias in RNA sequencing data: a novel approach to compute counts. *BMC Bioinformatics* vol. 15 S7 (2014).

67. Hashimoto, T. B., Edwards, M. D. & Gifford, D. K. Universal count correction for high-throughput sequencing. *PLoS Comput.*
68. Patro, R., Mount, S. M. & Kingsford, C. Sailfish enables alignment-free isoform quantification from RNA-seq reads using lightweight algorithms. *Nat. Biotechnol.* **32**, 462–464 (2014).

69. Rossell, D., Stephan-Otto Attolini, C., Kroiss, M. & Stöcker, A. QUANTIFYING ALTERNATIVE SPlicing FROM PAIRED-END RNA-SEQUENCING DATA. *Ann. Appl. Stat.* **8**, 309–330 (2014).

70. Mangul, S. *et al.* Transcriptome assembly and quantification from Ion Torrent RNA-Seq data. *BMC Genomics* **15 Suppl 5**, S7 (2014).

71. Liao, Y., Smyth, G. K. & Shi, W. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930 (2014).

72. Behr, J. *et al.* MITIE: Simultaneous RNA-Seq-based transcript identification and quantification in multiple samples. *Bioinformatics* **29**, 2529–2538 (2013).

73. Mezlini, A. M. *et al.* iReckon: simultaneous isoform discovery and abundance estimation from RNA-seq data. *Genome Res.* **23**, 519–529 (2013).

74. Roberts, A. & Pachter, L. Streaming fragment assignment for real-time analysis of sequencing experiments. *Nat. Methods* **10**, 71–73 (2013).

75. Glaus, P., Honkela, A. & Rattray, M. Identifying differentially expressed transcripts from RNA-seq data with biological variation. *Bioinformatics* **28**, 1721–1728 (2012).
76. Du, J. et al. IQSeq: integrated isoform quantification analysis based on next-generation sequencing. *PLoS One* 7, e29175 (2012).

77. Li, W. & Jiang, T. Transcriptome assembly and isoform expression level estimation from biased RNA-Seq reads. *Bioinformatics* 28, 2914–2921 (2012).

78. Xu, G. et al. SAMMate: a GUI tool for processing short read alignments in SAM/BAM format. *Source Code Biol. Med.* 6, 2 (2011).

79. Kim, H., Bi, Y., Pal, S., Gupta, R. & Davuluri, R. V. IsoformEx: isoform level gene expression estimation using weighted non-negative least squares from mRNA-Seq data. *BMC Bioinformatics* 12, 305 (2011).

80. Nicolae, M., Mangul, S., Măndoiu, I. I. & Zelikovsky, A. Estimation of alternative splicing isoform frequencies from RNA-Seq data. *Algorithm Mol. Biol.* 6, 9 (2011).

81. Li, B. & Dewey, C. N. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* vol. 12 (2011).

82. Risso, D., Schwartz, K., Sherlock, G. & Dudoit, S. GC-Content Normalization for RNA-Seq Data. *BMC Bioinformatics* 12, 1–17 (2011).

83. Turro, E. et al. Haplotype and isoform specific expression estimation using multi-mapping RNA-seq reads. *Genome Biol.* 12, R13 (2011).

84. Katz, Y., Wang, E. T., Airoldi, E. M. & Burge, C. B. Analysis and design of RNA sequencing experiments for identifying isoform regulation. *Nat. Methods* 7, 1009–1015 (2010).
85. Richard, H. et al. Prediction of alternative isoforms from exon expression levels in RNA-Seq experiments. *Nucleic Acids Res.* **38**, e112 (2010).

86. Jiang, H. & Wong, W. H. Statistical inferences for isoform expression in RNA-Seq. *Bioinformatics* **25**, 1026–1032 (2009).

87. Bohnert, R., Behr, J. & Rätsch, G. Transcript quantification with RNA-Seq data. *BMC Bioinformatics* vol. 10 (2009).

88. Mortazavi, A., Williams, B. A., McCue, K., Schaeffer, L. & Wold, B. Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nat. Methods* **5**, 621–628 (2008).

89. Zhu, A., Srivastava, A., Ibrahim, J. G., Patro, R. & Love, M. I. Nonparametric expression analysis using inferential replicate counts. *Nucleic Acids Res.* **47**, e105 (2019).

90. Gunady, M. K., Mount, S. M. & Corrada Bravo, H. Yanagi: Fast and interpretable segment-based alternative splicing and gene expression analysis. *BMC Bioinformatics* **20**, 421 (2019).

91. Sterne-Weiler, T., Weatheritt, R. J., Best, A. J., Ha, K. C. H. & Blencowe, B. J. Efficient and Accurate Quantitative Profiling of Alternative Splicing Patterns of Any Complexity on a Laptop. *Mol. Cell* **72**, 187–200.e6 (2018).

92. Spurr, L. et al. ReQTL – an allele-level measure of variation-expression genomic relationships. doi:10.1101/464206.

93. Tapial, J. et al. An atlas of alternative splicing profiles and functional associations reveals new regulatory programs and genes that simultaneously express multiple major isoforms. *Genome Res.* **27**, 1759–1768 (2017).

94. Frazee, A. C. et al. Ballgown bridges the gap between transcriptome assembly and expression analysis. *Nat. Biotechnol.* **33**, 243–246 (2015).
95. Law, C. W., Chen, Y., Shi, W. & Smyth, G. K. voom: Precision weights unlock linear model analysis tools for RNA-seq read counts. *Genome Biol.* **15**, R29 (2014).

96. Shen, S. *et al.* rMATS: robust and flexible detection of differential alternative splicing from replicate RNA-Seq data. *Proc. Natl. Acad. Sci. U. S. A.* **111**, E5593–601 (2014).

97. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* **15**, 550 (2014).

98. Davidson, N. M. & Oshlack, A. Corset: enabling differential gene expression analysis for de novo assembled transcriptomes. *Genome Biology* vol. 15 (2014).

99. Gu, J., Wang, X., Halakivi-Clarke, L., Clarke, R. & Xuan, J. BADGE: a novel Bayesian model for accurate abundance quantification and differential analysis of RNA-Seq data. *BMC Bioinformatics* **15** Suppl **9**, S6 (2014).

100. Soneson, C. compcodeR--an R package for benchmarking differential expression methods for RNA-seq data. *Bioinformatics* vol. 30 2517–2518 (2014).

101. Rau, A., Marot, G. & Jaffrézic, F. Differential meta-analysis of RNA-seq data from multiple studies. *BMC Bioinformatics* **15**, 91 (2014).

102. Clark, N. R. *et al.* The characteristic direction: a geometrical approach to identify differentially expressed genes. *BMC Bioinformatics* **15**, 79 (2014).

103. Rau, A., Galopin, M., Celeux, G. & Jaffrézic, F. Data-based filtering for replicated high-throughput transcriptome sequencing
experiments. *Bioinformatics* **29**, 2146–2152 (2013).

104. Bi, Y. & Davuluri, R. V. NPEBseq: nonparametric empirical bayesian-based procedure for differential expression analysis of RNA-seq data. *BMC Bioinformatics* **14**, 262 (2013).

105. Leng, N. *et al.* EBSeq: an empirical Bayes hierarchical model for inference in RNA-seq experiments. *Bioinformatics* **29**, 1035–1043 (2013).

106. Yu, D., Huber, W. & Vitek, O. Shrinkage estimation of dispersion in Negative Binomial models for RNA-seq experiments with small sample size. *Bioinformatics* **29**, 1275–1282 (2013).

107. Trapnell, C. *et al.* Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* **31**, 46–53 (2013).

108. Li, J. & Tibshirani, R. Finding consistent patterns: a nonparametric approach for identifying differential expression in RNA-Seq data. *Stat. Methods Med. Res.* **22**, 519–536 (2013).

109. Wang, W., Qin, Z., Feng, Z., Wang, X. & Zhang, X. Identifying differentially spliced genes from two groups of RNA-seq samples. *Gene* **518**, 164–170 (2013).

110. Tarazona, S., García-Alcalde, F., Dopazo, J., Ferrer, A. & Conesa, A. Differential expression in RNA-seq: a matter of depth. *Genome Res.* **21**, 2213–2223 (2011).

111. Robinson, M. D., McCarthy, D. J. & Smyth, G. K. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140 (2010).
112. Wang, L., Feng, Z., Wang, X., Wang, X. & Zhang, X. DEGseq: an R package for identifying differentially expressed genes from RNA-seq data. *Bioinformatics* vol. 26 136–138 (2010).

113. Li, Y. I. *et al.* Annotation-free quantification of RNA splicing using LeafCutter. *Nat. Genet.* 50, 151–158 (2018).

114. Green, C. J., Gazzara, M. R. & Barash, Y. MAJIQ-SPEL: web-tool to interrogate classical and complex splicing variations from RNA-Seq data. *Bioinformatics* 34, 300–302 (2018).

115. Vaquero-Garcia, J. *et al.* A new view of transcriptome complexity and regulation through the lens of local splicing variations. *Elife* 5, e11752 (2016).

116. Kahles, A., Ong, C. S., Zhong, Y. & Rätsch, G. SplAdder: identification, quantification and testing of alternative splicing events from RNA-Seq data. *Bioinformatics* 32, 1840–1847 (2016).

117. Pulyakhina, I. *et al.* SplicePie: a novel analytical approach for the detection of alternative, non-sequential and recursive splicing. *Nucleic Acids Res.* 43, 11068 (2015).

118. Alamancos, G. P., Pagès, A., Trincado, J. L., Bellora, N. & Eyras, E. Leveraging transcript quantification for fast computation of alternative splicing profiles. *RNA* 21, 1521–1531 (2015).

119. Mudvari, P. *et al.* SNPlice: variants that modulate Intron retention from RNA-sequencing data. *Bioinformatics* 31, 1191–1198 (2015).

120. Niu, L., Huang, W., Umbach, D. M. & Li, L. IUTA: a tool for effectively detecting differential isoform usage from RNA-Seq data. *BMC Genomics* 15, 862 (2014).
121. Kimes, P. K. et al. SigFuge: single gene clustering of RNA-seq reveals differential isoform usage among cancer samples. *Nucleic Acids Res.* 42, e113 (2014).

122. Gatto, A. et al. FineSplice, enhanced splice junction detection and quantification: a novel pipeline based on the assessment of diverse RNA-Seq alignment solutions. *Nucleic Acids Res.* 42, e71 (2014).

123. Hu, Y. et al. PennSeq: accurate isoform-specific gene expression quantification in RNA-Seq by modeling non-uniform read distribution. *Nucleic Acids Res.* 42, e20 (2014).

124. Bernard, E., Jacob, L., Mairal, J. & Vert, J.-P. Efficient RNA isoform identification and quantification from RNA-Seq data with network flows. *Bioinformatics* 30, 2447–2455 (2014).

125. Ye, Z. et al. Computational analysis reveals a correlation of exon-skipping events with splicing, transcription and epigenetic factors. *Nucleic Acids Res.* 42, 2856–2869 (2014).

126. Vitting-Seerup, K., Porse, B. T., Sandelin, A. & Waage, J. E. spliceR: An R package for classification of alternative splicing and prediction of coding potential from RNA-seq data. doi:10.7287/peerj.preprints.80.

127. Park, J. W., Tokheim, C., Shen, S. & Xing, Y. Identifying differential alternative splicing events from RNA sequencing data using RNASeq-MATS. *Methods Mol. Biol.* 1038, 171–179 (2013).

128. Aschoff, M. et al. SplicingCompass: differential splicing detection using RNA-seq data. *Bioinformatics* 29, 1141–1148 (2013).

129. Hu, Y. et al. DiffSplice: the genome-wide detection of differential splicing events with RNA-seq. *Nucleic Acids Res.* 41, e39 (2013).
130. Anders, S., Reyes, A. & Huber, W. Detecting differential usage of exons from RNA-seq data. *Genome Res.* **22**, 2008–2017 (2012).

131. Ryan, M. C., Cleland, J., Kim, R., Wong, W. C. & Weinstein, J. N. SpliceSeq: a resource for analysis and visualization of RNA-Seq data on alternative splicing and its functional impacts. *Bioinformatics* **28**, 2385–2387 (2012).

132. Brooks, A. N. *et al.* Conservation of an RNA regulatory map between Drosophila and mammals. *Genome Res.* **21**, 193–202 (2011).

133. Griffith, M. *et al.* Alternative expression analysis by RNA sequencing. *Nat. Methods* **7**, 843–847 (2010).

134. Li, T. *et al.* TIMER2.0 for analysis of tumor-infiltrating immune cells. *Nucleic Acids Res.* (2020) doi:10.1093/nar/gkaa407.

135. Newman, A. M. *et al.* Determining cell type abundance and expression from bulk tissues with digital cytometry. *Nat. Biotechnol.* **37**, 773–782 (2019).

136. Finotello, F. *et al.* Molecular and pharmacological modulators of the tumor immune contexture revealed by deconvolution of RNA-seq data. *Genome Med.* **11**, 34 (2019).

137. Sturm, G. *et al.* Comprehensive evaluation of transcriptome-based cell-type quantification methods for immuno-oncology. *Bioinformatics* **35**, i436–i445 (2019).

138. Zaitsev, K., Bambouskova, M., Swain, A. & Artyomov, M. N. Complete deconvolution of cellular mixtures based on linearity of transcriptional signatures. *Nat. Commun.* **10**, 2209 (2019).

139. Du, R., Carey, V. & Weiss, S. T. deconvSeq: deconvolution of cell mixture distribution in sequencing data. *Bioinformatics* **35**,
140. Kang, K. et al. CDSeq: A novel complete deconvolution method for dissecting heterogeneous samples using gene expression data. *PLoS Comput. Biol.* **15**, e1007510 (2019).

141. Hunt, G. J., Freytag, S., Bahlo, M. & Gagnon-Bartsch, J. A. dtangle: accurate and robust cell type deconvolution. *Bioinformatics* **35**, 2093–2099 (2019).

142. Nadel, B. et al. The Gene Expression Deconvolution Interactive Tool (GEDIT): Accurate Cell Type Quantification from Gene Expression Data. doi:10.1101/728493.

143. Lopez, D. et al. SaVanT: a web-based tool for the sample-level visualization of molecular signatures in gene expression profiles. *BMC Genomics* vol. 18 (2017).

144. Racle, J., de Jonge, K., Baumgaertner, P., Speiser, D. E. & Gfeller, D. Simultaneous enumeration of cancer and immune cell types from bulk tumor gene expression data. *Elife* **6**, (2017).

145. Roman, T., Xie, L. & Schwartz, R. Automated deconvolution of structured mixtures from heterogeneous tumor genomic data. *PLoS Comput. Biol.* **13**, e1005815 (2017).

146. Zaslavsky, M., Novik, J. B., Chang, E. & Hammerbacher, J. Infino: a Bayesian hierarchical model improves estimates of immune infiltration into tumor microenvironment. doi:10.1101/221671.

147. Becht, E. et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. *Genome Biol.* **17**, 218 (2016).
148. Chikina, M., Zaslavsky, E. & Sealfon, S. C. CellCODE: a robust latent variable approach to differential expression analysis for heterogeneous cell populations. *Bioinformatics* **31**, 1584–1591 (2015).

149. Qiao, W. *et al.* PERT: A Method for Expression Deconvolution of Human Blood Samples from Varied Microenvironmental and Developmental Conditions. *PLoS Computational Biology* vol. 8 e1002838 (2012).

150. Mangul, S. *et al.* Profiling immunoglobulin repertoires across multiple human tissues by RNA Sequencing. doi:10.1101/089235.

151. Li, B. *et al.* Landscape of tumor-infiltrating T cell repertoire of human cancers. *Nature Genetics* vol. 48 725–732 (2016).

152. Mose, L. E. *et al.* Assembly-based inference of B-cell receptor repertoires from short read RNA sequencing data with V’DJer. *Bioinformatics* vol. 32 3729–3734 (2016).

153. Strauli, N. B. & Hernandez, R. D. Statistical inference of a convergent antibody repertoire response to influenza vaccine. *Genome Med.* **8**, 60 (2016).

154. Bolotin, D. A. *et al.* MiXCR: software for comprehensive adaptive immunity profiling. *Nat. Methods* **12**, 380–381 (2015).

155. Knowles, D. A. *et al.* Allele-specific expression reveals interactions between genetic variation and environment. *Nat. Methods* **14**, 699–702 (2017).

156. Mohammadi, P. *et al.* Genetic regulatory variation in populations informs transcriptome analysis in rare disease. *Science* **366**, 351–356 (2019).

157. Mohammadi, P., Castel, S. E., Brown, A. A. & Lappalainen, T. Quantifying the regulatory effect size of cis-acting genetic variation using allelic fold change. doi:10.1101/078717.
158. Castel, S. E., Mohammadi, P., Chung, W. K., Shen, Y. & Lappalainen, T. Rare variant phasing and haplotypic expression from RNA sequencing with phASER. *Nat. Commun.* **7**, 12817 (2016).

159. Kumasaka, N., Knights, A. J. & Gaffney, D. J. Fine-mapping cellular QTLs with RASQUAL and ATAC-seq. *Nat. Genet.* **48**, 206–213 (2016).

160. Castel, S. E., Levy-Moonshine, A., Mohammadi, P., Banks, E. & Lappalainen, T. Tools and best practices for data processing in allelic expression analysis. *Genome Biol.* **16**, 195 (2015).

161. van de Geijn, B., McVicker, G., Gilad, Y. & Pritchard, J. K. WASP: allele-specific software for robust molecular quantitative trait locus discovery. *Nat. Methods* **12**, 1061–1063 (2015).

162. Pirinen, M. *et al.* Assessing allele-specific expression across multiple tissues from RNA-seq read data. *Bioinformatics* **31**, 2497–2504 (2015).

163. Mayba, O. *et al.* MBASED: allele-specific expression detection in cancer tissues and cell lines. *Genome Biol.* **15**, 405 (2014).

164. Pandey, R. V., Franssen, S. U., Futschik, A. & Schlötterer, C. Allelic imbalance metre (Allim), a new tool for measuring allele-specific gene expression with RNA-seq data. *Mol. Ecol. Resour.* **13**, 740–745 (2013).

165. Rozowsky, J. *et al.* AlleleSeq: analysis of allele-specific expression and binding in a network framework. *Molecular Systems Biology* vol. 7 522 (2011).

166. Mangul, S. *et al.* ROP: dumpster diving in RNA-sequencing to find the source of 1 trillion reads across diverse adult human tissues. *Genome Biol.* **19**, 36 (2018).
167. Xu, G. et al. RNA CoMPASS: a dual approach for pathogen and host transcriptome analysis of RNA-seq datasets. *PLoS One* **9**, e89445 (2014).

168. Chen, Y. et al. VirusSeq: software to identify viruses and their integration sites using next-generation sequencing of human cancer tissue. *Bioinformatics* **29**, 266–267 (2013).

169. Wang, Q., Jia, P. & Zhao, Z. VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. *PLoS One* **8**, e64465 (2013).

170. Zhang, J., Gao, T. & Maher, C. A. INTEGRATE-Vis: a tool for comprehensive gene fusion visualization. *Sci. Rep.* **7**, 17808 (2017).

171. Zhang, J., Mardis, E. R. & Maher, C. A. INTEGRATE-neo: a pipeline for personalized gene fusion neoantigen discovery. *Bioinformatics* **33**, 555–557 (2017).

172. Zhang, J. et al. INTEGRATE: gene fusion discovery using whole genome and transcriptome data. *Genome Res.* **26**, 108–118 (2016).

173. Fernandez-Cuesta, L. et al. Identification of novel fusion genes in lung cancer using breakpoint assembly of transcriptome sequencing data. * Genome Biol.* **16**, 7 (2015).

174. Torres-García, W. et al. PRADA: pipeline for RNA sequencing data analysis. *Bioinformatics* **30**, 2224–2226 (2014).

175. Abate, F. et al. Pegasus: a comprehensive annotation and prediction tool for detection of driver gene fusions in cancer. *BMC Syst. Biol.* **8**, 97 (2014).
176. Nicorici, D. *et al.* FusionCatcher - a tool for finding somatic fusion genes in paired-end RNA-sequencing data.

doi:10.1101/011650.

177. Liu, C., Ma, J., Chang, C. J. & Zhou, X. FusionQ: a novel approach for gene fusion detection and quantification from paired-end RNA-Seq. *BMC Bioinformatics* **14**, 193 (2013).

178. Swanson, L. *et al.* Barnacle: detecting and characterizing tandem duplications and fusions in transcriptome assemblies. *BMC Genomics* **14**, 550 (2013).

179. Yorukoglu, D. *et al.* Dissect: detection and characterization of novel structural alterations in transcribed sequences.

*Bioinformatics* **28**, i179–87 (2012).

180. Chen, K. *et al.* BreakFusion: targeted assembly-based identification of gene fusions in whole transcriptome paired-end sequencing data. *Bioinformatics* **28**, 1923–1924 (2012).

181. Benelli, M. *et al.* Discovering chimeric transcripts in paired-end RNA-seq data by using EricScript. *Bioinformatics* **28**, 3232–3239 (2012).

182. Abate, F. *et al.* Bellerophontes: an RNA-Seq data analysis framework for chimeric transcripts discovery based on accurate fusion model. *Bioinformatics* **28**, 2114–2121 (2012).

183. Kalyana-Sundaram, S., Shanmugam, A. & Chinnaiyan, A. M. Gene Fusion Markup Language: a prototype for exchanging gene fusion data. *BMC Bioinformatics* vol. 13 269 (2012).

184. Li, Y., Chien, J., Smith, D. I. & Ma, J. FusionHunter: identifying fusion transcripts in cancer using paired-end RNA-seq.
185. Iyer, M. K., Chinnaiyan, A. M. & Maher, C. A. ChimeraScan: a tool for identifying chimeric transcription in sequencing data. *Bioinformatics* **27**, 2903–2904 (2011).

186. Kim, D. & Salzberg, S. L. TopHat-Fusion: an algorithm for discovery of novel fusion transcripts. *Genome Biol.* **12**, R72 (2011).

187. McPherson, A. *et al.* deFuse: an algorithm for gene fusion discovery in tumor RNA-Seq data. *PLoS Comput. Biol.* **7**, e1001138 (2011).

188. Zhang, J., Chen, S., Yang, J. & Zhao, F. Accurate quantification of circular RNAs identifies extensive circular isoform switching events. *Nat. Commun.* **11**, 90 (2020).

189. Zheng, Y. & Zhao, F. Visualization of circular RNAs and their internal splicing events from transcriptomic data. *Bioinformatics* **36**, 2934–2935 (2020).

190. Humphreys, D. T., Fossat, N., Demuth, M., Tam, P. P. L. & Ho, J. W. K. Ularcirc: visualization and enhanced analysis of circular RNAs via back and canonical forward splicing. *Nucleic Acids Res.* **47**, e123 (2019).

191. Ma, X.-K. *et al.* A CLEAR pipeline for direct comparison of circular and linear RNA expression. doi:10.1101/668657.

192. Zheng, Y., Ji, P., Chen, S., Hou, L. & Zhao, F. Reconstruction of full-length circular RNAs enables isoform-level quantification. *Genome Med.* **11**, 2 (2019).

193. Wu, J. *et al.* CircAST: Full-length Assembly and Quantification of Alternatively Spliced Isoforms in Circular RNAs. *Genomics Proteomics Bioinformatics* **17**, 522–534 (2019).
194. Gao, Y., Zhang, J. & Zhao, F. Circular RNA identification based on multiple seed matching. *Brief. Bioinform.* **19**, 803–810 (2018).

195. Li, M. et al. Quantifying circular RNA expression from RNA-seq data using model-based framework. *Bioinformatics* **33**, 2131–2139 (2017).

196. Gaffo, E., Bonizzato, A., Kronnie, G. & Bortoluzzi, S. CirComPara: A Multi-Method Comparative Bioinformatics Pipeline to Detect and Study circRNAs from RNA-seq Data. *Non-Coding RNA* **vol. 3** 8 (2017).

197. Song, X. et al. Circular RNA profile in gliomas revealed by identification tool UROBORUS. *Nucleic Acids Res.* **44**, e87 (2016).

198. Izuogu, O. G. et al. PTESFinder: a computational method to identify post-transcriptional exon shuffling (PTES) events. *BMC Bioinformatics* **17**, 31 (2016).

199. Chuang, T.-J. et al. NCLscan: accurate identification of non-co-linear transcripts (fusion, trans-splicing and circular RNA) with a good balance between sensitivity and precision. *Nucleic Acids Res.* **44**, e29 (2016).

200. Cheng, J., Metge, F. & Dieterich, C. Specific identification and quantification of circular RNAs from sequencing data. *Bioinformatics* **32**, 1094–1096 (2016).

201. Gao, Y. et al. Comprehensive identification of internal structure and alternative splicing events in circular RNAs. *Nat. Commun.* **7**, 12060 (2016).

202. Zhang, X.-O. et al. Diverse alternative back-splicing and alternative splicing landscape of circular RNAs. *Genome Res.* **26**, 1277–1287 (2016).
203. Szabo, L. et al. Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development. *Genome Biol.* **16**, 126 (2015).

204. Westholm, J. O. et al. Genome-wide analysis of drosophila circular RNAs reveals their structural and sequence properties and age-dependent neural accumulation. *Cell Rep.* **9**, 1966–1980 (2014).

205. Memczak, S. et al. Circular RNAs are a large class of animal RNAs with regulatory potency. *Nature* **495**, 333–338 (2013).

206. Kang, W. et al. miRTrace reveals the organismal origins of microRNA sequencing data. *Genome Biol.* **19**, 213 (2018).

207. Rueda, A. et al. sRNAtoolbox: an integrated collection of small RNA research tools. *Nucleic Acids Res.* **43**, W467–73 (2015).

208. Axtell, M. J. ShortStack: comprehensive annotation and quantification of small RNA genes. *RNA* **19**, 740–751 (2013).

209. Wu, J. et al. mirTools 2.0 for non-coding RNA discovery, profiling, and functional annotation based on high-throughput sequencing. *RNA Biol.* **10**, 1087–1092 (2013).

210. Stocks, M. B. et al. The UEA sRNA workbench: a suite of tools for analysing and visualizing next generation sequencing microRNA and small RNA datasets. *Bioinformatics* **28**, 2059–2061 (2012).

211. Friedländer, M. R., Mackowiak, S. D., Li, N., Chen, W. & Rajewsky, N. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. *Nucleic Acids Res.* **40**, 37–52 (2012).

212. Hackenberg, M., Rodríguez-Ezpeleta, N. & Aransay, A. M. miRanalyzer: an update on the detection and analysis of microRNAs in high-throughput sequencing experiments. *Nucleic Acids Res.* **39**, W132–8 (2011).

213. Pantano, L., Estivill, X. & Martí, E. SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets,
reveals ubiquitous miRNA modifications in human embryonic cells. *Nucleic Acids Res.* 38, e34 (2010).

214. Fasold, M., Langenberger, D., Binder, H., Stadler, P. F. & Hoffmann, S. DARIO: a ncRNA detection and analysis tool for next-generation sequencing experiments. *Nucleic Acids Research* vol. 39 W112–W117 (2011).

215. Perampalam, P. & Dick, F. A. BEAVR: a browser-based tool for the exploration and visualization of RNA-seq data. *BMC Bioinformatics* 21, 221 (2020).

216. Rau, A. & Maugis-Rabusseau, C. Transformation and model choice for RNA-seq co-expression analysis. *Brief. Bioinform.* 19, 425–436 (2018).

217. Hilker, R. et al. ReadXplorer 2—detailed read mapping analysis and visualization from one single source. *Bioinformatics* vol. 32 3702–3708 (2016).

218. Freese, N. H., Norris, D. C. & Loraine, A. E. Integrated genome browser: visual analytics platform for genomics. *Bioinformatics* 32, 2089–2095 (2016).

219. Katz, Y. et al. Quantitative visualization of alternative exon expression from RNA-seq data. *Bioinformatics* 31, 2400–2402 (2015).

220. Foissac, S. & Sammeth, M. Analysis of alternative splicing events in custom gene datasets by AStalavista. *Methods Mol. Biol.* 1269, 379–392 (2015).

221. An, J. et al. RNASeqBrowser: a genome browser for simultaneous visualization of raw strand specific RNAseq reads and UCSC genome browser custom tracks. *BMC Genomics* 16, 145 (2015).
222. Wu, E., Nance, T. & Montgomery, S. B. SplicePlot: a utility for visualizing splicing quantitative trait loci. *Bioinformatics* **30**, 1025–1026 (2014).

223. Rogé, X. & Zhang, X. RNAseqViewer: visualization tool for RNA-Seq data. *Bioinformatics* **30**, 891–892 (2014).

224. Tokheim, C., Park, J. W. & Xing, Y. PrimerSeq: Design and visualization of RT-PCR primers for alternative splicing using RNA-seq data. *Genomics Proteomics Bioinformatics* **12**, 105–109 (2014).

225. Chelaru, F., Smith, L., Goldstein, N. & Bravo, H. C. Epiviz: interactive visual analytics for functional genomics data. *Nat. Methods* **11**, 938–940 (2014).

226. Mariette, J. *et al.* RNAbrowse: RNA-Seq de novo assembly results browser. *PLoS One* **9**, e96821 (2014).

227. Severin, J. *et al.* Interactive visualization and analysis of large-scale sequencing datasets using ZENBU. *Nat. Biotechnol.* **32**, 217–219 (2014).

228. Liu, Q. *et al.* Detection, annotation and visualization of alternative splicing from RNA-Seq data with SplicingViewer. *Genomics* **99**, 178–182 (2012).