



HHS Public Access

Author manuscript

Nat Biotechnol. Author manuscript; available in PMC 2019 September 12.

Published in final edited form as:

Nat Biotechnol. 2017 April 11; 35(4): 319–321. doi:10.1038/nbt.3838.

Reproducible RNA-seq analysis using *recount2*

Leonardo Collado-Torres^{1,2,3,11}, **Abhinav Nellore**^{4,5,6,11}, **Kai Kammers**^{1,2,7}, **Shannon E Ellis**^{1,2}, **Margaret A Taub**^{1,2}, **Kasper D Hansen**^{1,2,8}, **Andrew E Jaffe**^{1,2,3,9}, **Ben Langmead**^{1,2,10}, **Jeffrey T Leek**^{1,2}

¹Department of Biostatistics, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland, USA.

²Center for Computational Biology, Johns Hopkins University, Baltimore, Maryland, USA.

³Lieber Institute for Brain Development, Johns Hopkins Medical Campus, Baltimore, Maryland, USA.

⁴Department of Biomedical Engineering, Oregon Health & Science University, Portland, Oregon, USA.

⁵Department of Surgery, Oregon Health & Science University, Portland, Oregon, USA.

⁶Computational Biology Program, Oregon Health & Science University, Portland, Oregon, USA.

⁷Division of Biostatistics and Bioinformatics, Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

⁸McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University, Baltimore, Maryland, USA.

⁹Department of Mental Health, Johns Hopkins University, Baltimore, Maryland, USA.

¹⁰Department of Computer Science, Johns Hopkins University, Baltimore, Maryland, USA.

¹¹These authors contributed equally to this work.

To the Editor:

RNA sequencing (RNA-seq) is used to measure gene expression levels across the transcriptome for a huge variety of samples. For example, RNA-seq has been applied to study gene expression in individuals with rare diseases¹, in hard-to-obtain tissues² or for rare forms of cancer³. Recently, enormous RNA-seq datasets have been produced in the GTEx (Genotype-Tissue Expression) study⁴, which comprises 9,662 samples from 551 individuals and 54 body sites, and in the Cancer Genome Atlas (TCGA) study, which comprises 11,350 samples from 10,340 individuals and 33 cancer types. Public data repositories, such as the

A.E.J. (andrew.jaffe@libd.org) or B.L. (langmea@cs.jhu.edu) or J.T.L. (jtleek@gmail.com).

Editor's note: This article has been peer-reviewed.

Note: Any Supplementary Information and Source Data files are available in the [online version of the paper](#).

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Sequence Read Archive (SRA), host >50,000 human RNA-seq samples. It is estimated that these repositories are likely to double in size every 18 months⁵. Deposited data are provided as raw sequencing reads, which are costly for standard academic labs researchers to analyze. Efforts have been made to standardize and publish ready-to-analyze summaries of both DNA sequencing⁶ and exome-sequencing⁷ data. Adopting a similar approach for archived RNA-seq data, we have developed *recount2*, which comprises >4.4 trillion uniformly processed and quantified RNA-seq reads.

Many researchers rely on processed forms of publicly available data, such as gene counts, for statistical methods development and re-analysis of candidate genes. Although these quantified data are sometimes available through the Gene Expression Omnibus⁸, there are no requirements to deposit these data, nor are data always processed with standard or complete pipelines^{9–13}. Five years ago, we began to address this problem by summarizing RNA-seq data into concise gene count tables and making these processed data and metadata available as Bioconductor¹⁴ ExpressionSet objects with one documented processing pipeline. Together this formed an RNA-seq resource named ReCount¹⁵ that contained 8 billion reads from 18 studies. ReCount was used in the development of the DESeq2 (ref. 16), voom¹⁷ and metagenomeSeq¹⁸ methods for differential expression and normalization, compilation of co-expression networks¹⁹ and to study the effect of ribosomal DNA dosage on gene expression²⁰. The amount of archived RNA-seq data has massively increased over the past five years. To meet the needs of researchers, we have produced *recount2*, which contains >4.4 trillion uniformly processed and quantified RNA-seq reads that are derived from in excess of 70,603 human RNA-seq samples deposited in the SRA, GTEx and TCGA projects aligned with Rail-RNA^{21,22}.

The *recount2* resource summarizes expression data for genes, exons, exon–exon splice junctions and base-level coverage (Supplementary Methods), which enables multiple downstream analyses, including testing for differential expression of potentially unannotated transcribed sequence²³. A searchable interface is available at this site (<https://jhubiostatistics.shinyapps.io/recount/>) and via the accompanying Bioconductor package (<http://bioconductor.org/packages/recount>).

We first compared *recount2*-processed data with the publicly available data from the GTEx project, which comprises 9,662 samples from >250 individuals²⁴ to demonstrate that our processing pipeline produced gene counts similar to the published counts (Supplementary Methods). We downloaded the official release of the gene counts from the GTEx portal and compared them with the *recount2* gene counts (Supplementary Note 1, Section 4). For protein coding genes, the gene expression levels that we estimated using the *recount2* pipeline had a median (IQR) correlation of 0.987 (0.971, 0.993) with the v6 release from GTEx (Fig. 1a and Supplementary Note 1, Section 4). A differential expression analysis comparing colon and whole blood samples using the gene expression measurements from *recount2* matched the results obtained using the v6 release from the GTEx portal ($r^2 = 0.92$ between fold changes for *recount2* and GTEx v6 counts for protein coding genes; Fig. 1b and Supplementary Note 1, Section 5). These results suggest that *recount2* produces directly comparable gene counts to one of the largest published studies.

The advantage of using the *recount2* version of GTEx data is that all data are identically processed, therefore enabling integrated analyses of multiple datasets. To illustrate how *recount2* can be used to investigate or validate cross-tissue differences using publicly available data, we computed expression differences comparing samples from healthy colon tissue and whole blood from healthy individuals (Supplementary Methods, Supplementary Note 2, Section 1.8 and Supplementary Code 1). Control samples were used to limit differences observed to those due to tissue type and not disease status. Colon control samples were used from studies [SRP029880](#) (a study of colorectal cancer²⁵, $n = 19$) and [SRP042228](#) (a study of Crohn's disease²⁶, $n = 41$). Whole blood control samples were used from [SRP059039](#) (virus-induced diarrhea, unpublished, $n = 24$), [SRP059172](#) (a study of blood biomarkers for brucellosis, unpublished, $n = 47$) and [SRP062966](#) (a study of lupus, unpublished, $n = 18$). After filtering genes to include only those with an average normalized count of at least 5 across samples (to restrict to genes that were expressed well above the limit of detection), we carried out gene-level differential expression analysis using *limma*²⁷ and *voom*¹⁷ (Supplementary Note 2, Section 1.8).

To validate the meta-analysis results, we evaluated whether we had found similar patterns of differential expression between the same tissues collected as part of a single project. We selected all of the colon and whole blood samples from the GTEx project ($n = 376$ and 456 , respectively) and performed the same analysis, adjusting for batch effects by including the reported batch from GTEx as a covariate in the linear model. We then computed rank-based concordance, examining the fraction of the top differentially expressed genes that were included in both analyses. Approximately 20% of the top 100 genes from the two analyses were concordant (Fig. 1c and Supplementary Note 2, Section 1.9).

As a comparison and to provide context for this result, we performed two additional comparisons. First, we used GTEx lung data ($n = 374$) in place of the colon data and computed differentially expressed genes compared with whole blood. In this case, only ~5% of the top 100 differentially expressed genes were shared in the top 100 genes from our multi-study analysis (Supplementary Note 2, Section 1.9). Second, to represent concordance results expected for a comparison of unrelated things, we used ranked coefficients for batch instead of for tissue and saw very little concordance. These comparisons show that we can use the resources found in *recount2* to perform a valid tissue-specific meta-analysis without generating the necessary data in-house, which would add considerable time and expense, provided that samples were even available to analyze.

The *recount2* pipeline enables an in-depth characterization of transcriptional differences across biological conditions. To illustrate this using data from breast cancer subtypes, we first chose HER2-positive and triple-negative breast cancer (TNBC) samples from study [SRP032789](#) (TNBC, $n = 6$; HER2-positive, $n = 5$)²⁸, and extracted feature-level expression across genes, exons, junctions and expressed regions, finding widespread expression differences by subtype (Table 1, Supplementary Note 3 and Supplementary Methods). Of these significant differentially expressed regions (DERs) found with *derfinder*²³, 1,350 did not overlap any annotated exons (Fig. 2a and Supplementary Note 4, Section 4), demonstrating that 5% of DERs detected would not be reported using annotation-dependent methods of expression estimation. These DERs would only be identified when a

quantification method not reliant on annotation was used. Such quantifications are made readily available within *recount2* (Fig. 2b).

We further summarized junctions and exons at the gene level using the resulting differential expression *P*-values, and 73% of the top 100 genes were shared at the gene- and exon-level analyses. In comparison, expressed regions and exon–exon junction analyses shared only 58% and 4% of the top 100 features, respectively (Fig. 2c and Supplementary Note 3, Section 5). Furthermore, to validate the differential expression findings, we compared the gene-level results from study [SRP032789](#) with an independent study ([SRP019936](#) (ref. 29); TNBC, *n* = 8; HER2-positive, *n* = 7; Supplementary Note 5). Expression analysis was carried out as described above, identifying 3,434 genes as differentially expressed (*q* < 0.05, Supplementary Note 5, Section 4). Given the low concordance (8% among the top 1,000 genes, Supplementary Note 5, Section 5.1) between these results and those from study [SRP032789](#), we then applied independent hypothesis weighting (IHW)³⁰ across the two studies, which slightly improved replication rates, although sample size is limited in these two studies and thus likely thwarts our ability to see a huge increase in power using IHW (Supplementary Note 5, Section 5.2). As the data within *recount2* have all been processed with the same analytical pipeline (Supplementary Code 2 and 3), the analytical burden on the user is minimized when comparing across datasets.

The *recount2* pipeline can be used for querying, downloading and analyzing large-scale human RNA-seq datasets across more than 70,000 samples, including all of GTEx, TCGA and the SRA. We also allow users to process and upload their own experimental data to *recount2* (Supplementary Methods and Supplementary Code 4). Although all *recount2* samples have been processed and summarized with a single pipeline, so-called ‘batch’ effects could occur and should be considered in downstream analyses, particularly when comparing among studies. As an example, the type of library preparation is not accounted for in our processing, but we will continue to annotate these variables so they can be included in downstream analyses. By removing a large number of data processing and quantification choices potentially made by researchers, *recount2* reduces the number of ‘researcher degrees of freedom’³¹, which can improve replication and reduce the potential for false positives created by processing pipeline differences.

Other tools have been developed to summarize publically deposited gene expression data. For example, the Expression Atlas³² provides final results that can be queried only at the gene level, Toil focuses only on curated datasets³³ and other efforts focus primarily on cancer^{34,35}. Unlike these resources, *recount2* uses analysis pipelines that are annotation agnostic to process and summarize samples. For example, in junction and expressed region analyses, gene annotations are only used to label summarized data post-analysis and not to align reads or discover splice junctions—downstream analyses are therefore fully aware of unannotated splicing events³⁶.

By providing an updateable resource of uniformly processed RNA-seq samples, together with R-based software for analysis, *recount2* will enable studies that individual laboratories would otherwise not have the resources to undertake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank C. Kingsford and D. Filippova for their assistance in adding SHARQ metadata to *recount2*. *recount2* data are hosted on SciServer, a collaborative research environment for large-scale data-driven science. It is being developed at, and administered by, the Institute for Data Intensive Engineering and Science at Johns Hopkins University. SciServer is funded by the National Science Foundation Award ACI-1261715. For more information about SciServer, visit <http://www.sciserver.org/>. We thank E. Lehnert and P. Radovic at Seven Bridges for their help accessing RNA-seq data from TCGA using the Cancer Genomics Cloud API and depositing results in a shared bucket on Amazon S3. We also thank other Seven Bridges team members for facilitating our TCGA reanalysis, including B. Dusenbery, N. Tijanac, M. Kovacevic, M. Sadoff and G. Kaushik. The Genotype-Tissue Expression (GTEx) Project was supported by the Common Fund of the Office of the Director of the National Institutes of Health. Additional funds were provided by the NCI, NHGRI, NHLBI, NIDA, NIMH and NINDS. Donors were enrolled at Biospecimen Source Sites funded by NCI/SAIC-Frederick, Inc. (SAIC-F) subcontracts to the National Disease Research Interchange (10XS170), Roswell Park Cancer Institute (10XS171), and Science Care, Inc. (X10S172). The Laboratory, Data Analysis, and Coordinating Center (LDACC) was funded through a contract (HHSN268201000029C) to The Broad Institute, Inc. Biorepository operations were funded through an SAIC-F subcontract to Van Andel Institute (10ST1035). Additional data repository and project management were provided by SAIC-F (HHSN261200800001E). The Brain Bank was supported by a supplement to University of Miami grants DA006227 and DA033684 and to contract N01MH000028. Statistical Methods development grants were made to the University of Geneva (MH090941 & MH101814), the University of Chicago (MH090951, MH090937, MH101820, MH101825), the University of North Carolina–Chapel Hill (MH090936 & MH101819), Harvard University (MH090948), Stanford University (MH101782), Washington University St. Louis (MH101810) and the University of Pennsylvania (MH101822). The data used for the analyses described in this manuscript were obtained from: the GTEx Portal on 11/21/15 and/or dbGaP accession number phs000424.v6.p1 on 11/30/15–12/04/15. Funding: B.L., J.T.L., L.C.T., S.E., A.N., M.T., K.H. and K.K. were supported by NIH R01 GM105705. L.C.T. was supported by Consejo Nacional de Ciencia y Tecnología México 351535. L.C.T. and A.E.J. were supported by IR21MH109956. Amazon Web Services experiments were supported by AWS in Education research grants. Storage costs on S3 for TCGA runs were partially covered by a grant from Seven Bridges Genomics for use of the Cancer Genomics Cloud.

References

1. Albers CA et al. *Nat. Genet* 44, 435–439, S431–432 (2012). [PubMed: 22366785]
2. Kohen R, Dobra A, Tracy JH & Haugen E *Transl. Psychiatry* 4, e366 (2014). [PubMed: 24594777]
3. Goh G et al. *Nat. Genet* 46, 613–617 (2014). [PubMed: 24747643]
4. Melé M et al. *Science* 348, 660–665 (2015). [PubMed: 25954002]
5. Kodama Y, Shumway M & Leinonen R *Nucleic Acids Res* 40, D54–D56 (2012). [PubMed: 22009675]
6. 1000 Genomes Project Consortium et al. *Nature* 467, 1061–1073 (2010). [PubMed: 20981092]
7. Lek M et al. *Nature* 536, 285–291 (2016). [PubMed: 27535533]
8. Barrett T et al. *Nucleic Acids Res* 39, D1005–D1010 (2011). [PubMed: 21097893]
9. Nookaew I et al. *Nucleic Acids Res* 40, 10084–10097 (2012). [PubMed: 22965124]
10. Dobin A et al. *Bioinformatics* 29, 15–21 (2013). [PubMed: 23104886]
11. Kim D et al. *Genome Biol* 14, R36 (2013). [PubMed: 23618408]
12. Engström PG et al. *Nat. Methods* 10, 1185–1191 (2013). [PubMed: 24185836]
13. Kumar PK, Hoang TV, Robinson ML, Tsonis PA & Liang C *Sci. Rep* 5, 13443 (2015). [PubMed: 26304587]
14. Gentleman RC et al. *Genome Biol* 5, R80 (2004). [PubMed: 15461798]
15. Frazee AC, Langmead B & Leek JT *BMC Bioinformatics* 12, 449 (2011). [PubMed: 22087737]
16. Love MI, Huber W & Anders S *Genome Biol* 15, 550 (2014). [PubMed: 25516281]
17. Law CW, Chen Y, Shi W & Smyth GK *Genome Biol* 15, R29 (2014). [PubMed: 24485249]
18. Paulson JN, Stine OC, Bravo HC & Pop M *Nat. Methods* 10, 1200–1202 (2013). [PubMed: 24076764]

19. Iancu OD et al. *Bioinformatics* 28, 1592–1597 (2012). [PubMed: 22556371]
20. Gibbons JG, Branco AT, Yu S & Lemos B *Nat. Commun* 5, 4850 (2014). [PubMed: 25209200]
21. Nellore A et al. *Bioinformatics* 10.1093/bioinformatics/btw575 (2016).
22. Nellore A, Wilks C, Hansen KD, Leek JT & Langmead B *Bioinformatics* 32, 2551–2553 (2016). [PubMed: 27153614]
23. Collado-Torres L et al. *Nucleic Acids Res* 45, e9 (2017). [PubMed: 27694310]
24. GTEx Consortium G et al. *Science* 348, 648–660 (2015). [PubMed: 25954001]
25. Kim SK et al. *Mol. Oncol* 8, 1653–1666 (2014). [PubMed: 25049118]
26. Haberman Y et al. *J. Clin. Invest* 124, 3617–3633 (2014). [PubMed: 25003194]
27. Smyth GK in *Bioinformatics and Computational Biology Solutions using R and Bioconductor* 397–420 (Springer, 2005).
28. Eswaran J et al. *Sci. Rep* 3, 1689 (2013). [PubMed: 23604310]
29. Kalari KR et al. *PLoS One* 8, e79298 (2013). [PubMed: 24223926]
30. Ignatiadis N, Klaus B, Zaugg JB & Huber W *Nat. Methods* 13, 577–580 (2016). [PubMed: 27240256]
31. Simmons JP, Nelson LD & Simonsohn U *Psychol. Sci* 22, 1359–1366 (2011). [PubMed: 22006061]
32. Petryszak R et al. *Nucleic Acids Res* 44, D746–D752 (2016). [PubMed: 26481351]
33. Vivian J et al. *Nat. Biotechnol* 35, 314–316 (2017). [PubMed: 28398314]
34. Tatlow PJ & Piccolo SR *Sci. Rep* 6, 39259 (2016). [PubMed: 27982081]
35. Rahman M et al. *Bioinformatics* 31, 3666–3672 (2015). [PubMed: 26209429]
36. Nellore A et al. *Genome Biol* 17, 266 (2016). [PubMed: 28038678]

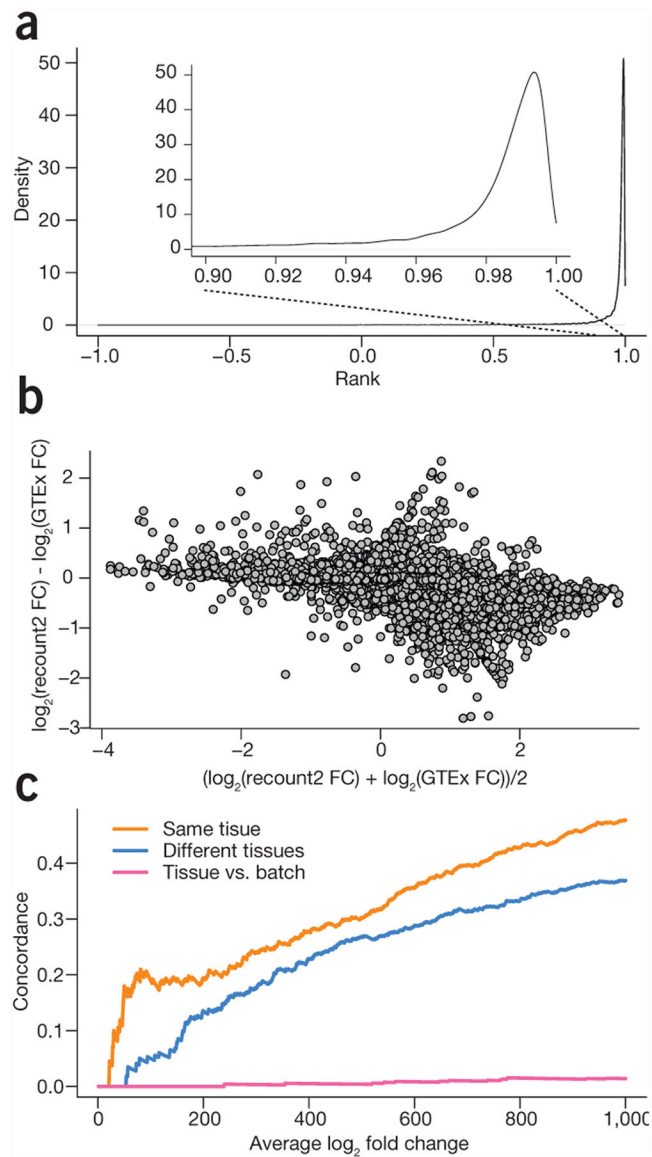


Figure 1.

Meta-analysis and study comparison facilitated by *recount2*. **(a)** The distribution of correlations between gene expression estimates for GTEx v6 from the GTEx portal and the counts calculated in *recount2* for protein coding genes. The gene expression counts are highly correlated between both quantifications for almost all genes. **(b)** A comparison of the fold changes for differential expression between colon and whole blood using the quantifications from GTEx and from *recount2* for protein coding genes. The majority of genes have a similar fold-change between the two analyses. **(c)** A concordance versus rank (i.e., ‘concordance at the top’, CAT) plot showing comparisons between a meta-analysis tissue comparison of whole blood and colorectal tissue in data from the sequence read archive and the GTEx project. When comparing the same tissues, there is a strong concordance between differential expression results on public data and GTEx (orange), less

concordance when different tissues are compared (blue) and almost none when comparing different analyses (pink).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

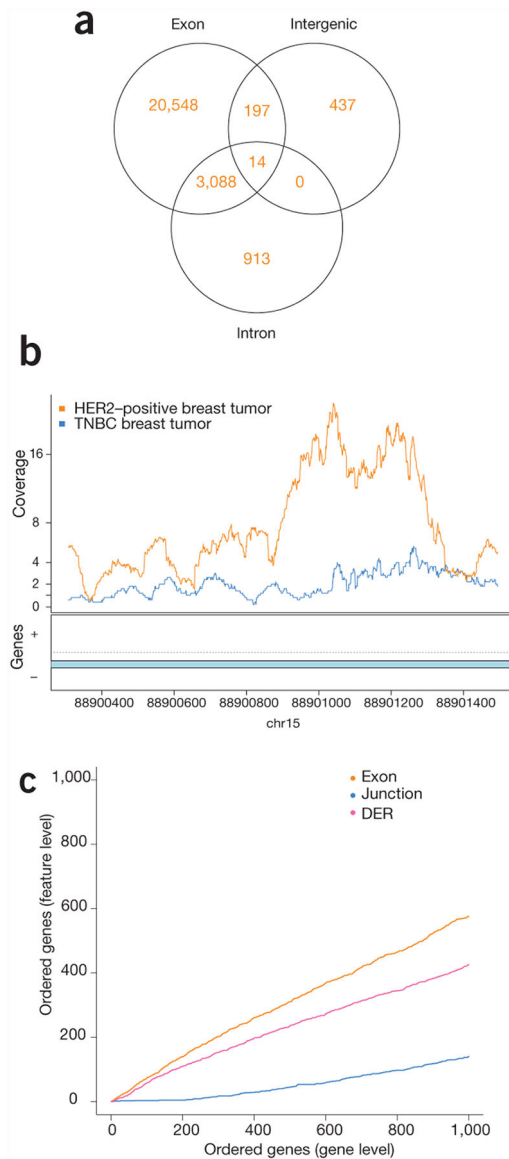


Figure 2. Multi-feature-level differential expression analysis is facilitated by *recount2*. **(a)** Venn diagram showing the number of expressed regions detected that overlap exons, intergenic regions and intronic regions, including expressed regions that overlap multiple annotation types. Differential expression occurs outside of previously annotated protein-coding regions. **(b)** An example of a region on chromosome 15 showing differential expression between breast cancer subtypes in an annotated intron. The lines show the average coverage in each group across samples. **(c)** CAT plot of concordance between gene-level analysis and then exon-, junction- and region-level (DER) analyses shows high concordance across the different feature levels.

Differentially expressed features comparing TNBC versus HER2-positive breast cancer samples at a false-discovery rate of 5%

Table 1

Feature level	Higher expression in TNBC	Higher expression in HER2-positive	Total differentially expressed features
Genes	1,362	1,612	2,974
Exons	14,546	13,159	27,705
Exon-exon junctions	1,732	18,073	19,805
Expressed regions	11,414	13,783	25,197