Dual RNA sequencing (dRNA-Seq) of bacteria and their host cells

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Abstract

Bacterial pathogens subvert host cells by manipulating cellular pathways for survival and replication; in turn, host cells respond to the invading pathogen through cascading changes in gene expression. Deciphering these complex temporal and spatial dynamics to identify novel bacterial virulence factors or host response pathways is crucial for improved diagnostics and therapeutics. Dual RNA sequencing (dRNA-Seq) has recently been developed to simultaneously capture host and bacterial transcriptomes from an infected cell. This approach builds on the high sensitivity and resolution of RNA-Seq technology and is applicable to any bacteria that interact with eukaryotic cells, encompassing parasitic, commensal or mutualistic lifestyles. We pioneered dRNA-Seq to simultaneously capture prokaryotic and eukaryotic expression profiles of cells infected with bacteria, using *in vitro Chlamydia*-infected epithelial cells as proof of principle. Here we provide a detailed laboratory and bioinformatics protocol for dRNA-seq that is readily adaptable to any host-bacteria system of interest.

Introduction

Background

Upon infection or other interactions, bacteria and their host eukaryotic cells engage in a complex interplay as they negotiate their respective survival and defense strategies. Unraveling these coordinated regulatory interactions, virulence mechanisms, and innate responses is key for our understanding of pathogenesis, disease and the development of therapeutics [1]. Traditional transcriptomic approaches such as microarrays have typically focused on *either* the prokaryotic or eukaryotic organism to investigate the host-bacteria interaction network [2]. However, this approach cannot decipher reciprocal changes in gene expression that contribute to the global infection system. Instead, an integrated approach is required that acknowledges both interaction partners, i.e. both bacteria and host, from the same biological sample. Due to the increasing affordability and resolution of next-generation sequencing, this is now achievable via dual RNA sequencing (dRNA-Seq) [1].

RNA-Seq was developed for the study of transcriptomes based on the massively parallel sequencing of RNA [3]. In a typical experiment, total mRNA from a sample is subjected to high-throughput next-generation sequencing and mapped to a reference genome to deduce the structure and/or expression state of each transcript [4]. Gene expression changes can be accurately measured between samples with high coverage and sensitivity, while alternative splicing analyses can be applied to identify novel isoforms and transcripts, RNA editing, and allele-specific expression [5]. The high sensitivity and dynamic range of RNA-Seq has expanded our capability for whole transcriptome analysis and enabled new insight into the functional elements of the genome [6].

dRNA-Seq extends these capabilities to two (or potentially more) interacting organisms, allowing the simultaneous monitoring of gene expression changes without disturbing the complex interactions that define host-bacteria infection dynamics. We applied dRNA-Seq to map host and bacteria transcriptomes from *Chlamydia*-infected host epithelial cells, which highlighted a dramatic early response to infection and numerous altered pathways within the host cell [1]. dRNA-Seq has since been successfully used to study host-bacteria interactions for *Salmonella enterica* [7], *Azospirillum brasilense* [8], *Mycobacterium tuberculosis* [9], *Haemophilus influenzae* [10], *Yersinia pseudotuberculosis* [11] and *Actinobacillus pleuropnemoniae* [12].

Advantages and limitations

cDNA microarrays first enabled large-scale transcriptome analyses, allowing the expression pattern of tens of thousands of known genes to be measured. Drawbacks include (1) a high background signal [13]; (2) cross-hybridization between genes of similar sequence; (3) the limit of expression level detection to the 1,000-fold range, compared to the actual cellular 1,000,000-fold range [14] (4) restriction of analysis to known or predicted mRNAs [15]; and (5) the inability to detect novel transcripts [14]. Some of these were overcome with tiling arrays to measure antisense RNA expression and other non-coding RNA (ncRNA) transcripts, but the large size of eukaryotic genomes make this inordinately costly [16]. Tag-based sequencing does enable the enumeration of individual transcripts, but this method requires existing gene structure information, can only sample a small region of a transcript, and is incapable of capturing diverse classes of RNA and its isoforms.

RNA-Seq provides a wider dynamic range, higher technical reproducibility, and a better estimate of absolute expression levels with lower background noise [17-19], and has become the primary method to examine transcriptomes. By allowing an unbiased determination of gene expression, high resolution data on potentially transcribed regions upstream and downstream of the annotated coding region, and post-translational rearrangements such as splicing and different RNA isoforms can be reported [20]. As a result, RNA-Seq improves genome annotation, and identifies new ORFs, transcription start sites (TSSs), the 5' and 3' UTRs of known genes, non-coding RNAs such as microRNA, promoter-associated RNA, and antisense 3' termini-associated RNA [21]. dRNA-Seq can report these data for two (or potentially more) organisms from the same sample, while providing powerful insight into novel interaction dynamics. For example, gene expression changes in one organism can be correlated with the responses of the other to capture crucial events that signify the dynamic mechanisms of host adaption and the progression of infection [1,4,7,10,22].

Despite these advantages, dRNA-Seq remains technically challenging. Up to 98% of total RNA is ribosomal (rRNA) [23], while bacterial mRNA levels are typically low compared to the host, especially during early infection periods, requiring mRNA depletion and/or enrichment approaches for cost-effective sequencing. Additionally, the quantity of mRNA detected by RNA-Seq is often a poor indicator for protein abundance due to mRNA instability and turnover [24,25]. The wide range of expression levels can result in non-uniform coverage where only a few reads can be captured for genes subject to lower expression levels, while short isoforms and repeat sequences derived from the same gene may result in assembly ambiguities. These ambiguities are compounded when using *de novo* methods for genomes that are partially or fully un-sequenced [17], but can be avoided when assembling reads to a reference genome. Transcript length bias can distort the identification of differentially expressed genes in favor of longer transcripts [26], but can be standardized with appropriate normalization techniques. Despite these challenges, dRNA-Seq is a powerful, economical, sensitive, and species-independent platform for investigating the gene expression dynamics of host-bacteria interactions [4].

Overview of the technique

This protocol provides a clear and practical laboratory and a detailed bioinformatics analysis pipeline for a typical dRNA-Seq host-bacteria analysis. We describe an experiment based on human epithelial carcinoma (HeLa) cells (host) infected with *Chlamydia trachomatis* serovar E (bacteria), which is readily adaptable to any host-bacteria system of interest.

C. trachomatis is an obligate intracellular bacterial bacteria that is reliant on its host cell for survival. It has been otherwise recalcitrant to standard genetic analyses, rendering it ideal for the development and application of dRNA-Seq. The protocol includes all steps for the bacterial infection of host cells, total RNA extraction, and rRNA depletion in preparation for sequencing (Figure 1). Bioinformatic steps are then included for total RNA sequence quality control and trimming, the *in silico* segregation of host and bacteria reads, distinct sequence alignment and sorting techniques for host and bacteria data, alignment visualization, read quantification and normalization, the separate statistical analysis of host and bacteria data, and the final integration of host and bacteria transcriptomic responses (Figure 2).

From here, the user may diverge toward a number of possible downstream applications, including time-series analysis, host alternative splicing in response to the bacteria, gene set enrichment and ontology analysis, and elucidation of interaction and regulatory networks [27]. dRNA-Seq data may be integrated with other data sources to establish a more complete picture of gene regulation, including genotyping data to identify genetic loci responsible for gene expression variation, epigenetic information to highlight the influence of transcription factor binding, histone modification, and methylation, and miRNA-Seq data to identify the regulatory mechanisms of gene expression changes via non-coding RNA [28,29].

Experimental design

For RNA-Seq, a typical workflow includes experimental design, RNA extraction, library preparation, sequencing, and data analysis. The experiment should be designed to address the biological question(s) of interest and a key starting point is to determine the RNA species to be investigated (i.e. mRNA, miRNA, snRNA etc). This will influence the quantity of input RNA and sequencing depth required, which are both crucial factors for successful dRNA-Seq experiments. To capture sufficient RNA from both organisms, the ratio of bacteria to host genome size is a useful starting point followed by an estimation of the desired fold coverage. This can be determined by considering the number of replicates, the expected influence of housekeeping and structural RNA (rRNA and tRNA), the possibility of host:bacteria sequence overlap, the number of time-points, and the multiplicity of infection (MOI). We suggest at least three biological replicates for each sample rather than technical replicates taken from the same sample to minimize Type I and II errors and ensure an adequate estimation of within and between sample variation. As ~95% of total RNA will be ribosomal, a method of rRNA depletion and/or mRNA enrichment is recommended and several options are discussed below. Sequence overlap between host and bacteria can be predicted by mapping the bacterial sequence reads to the host genome and vice versa, which is also discussed below. The time-points of interest should be carefully considered as the initiation and period of transcriptional response can differ between host and bacteria [30]. Ideally, multiple time-points should be collected to suitably capture the dynamic host-bacteria transcriptional landscape. Finally, a suitable bacteria MOI should be selected to maximize the transcriptional signal from both host and bacteria, while reducing bias towards the uninfected cells that will flourish at the later time-points. Importantly, a high(er) MOI may also lead to a heightened and/or distorted host response with decreased biological relevance, depending on the system under investigation. Deeper sequencing may be necessary for the detection of low copy number transcripts or alternate isoforms, however increased sequencing depth can also increase the detection of transcriptional noise, spurious cDNA transcripts, or genomic DNA contamination so careful consideration is required [31,32]. Optionally, the addition of RNA spike-ins and unique molecular identifiers (UMI) can be useful for the quantitative calibration of RNA levels [33,34].

This protocol describes the collection and analysis of protein-coding mRNA from *C. trachomatis* and Hela cells at 1 and 24 hours post-infection (hpi) time-points as they are physiologically relevant to the early and mid-stages of the chlamydial development cycle. An MOI of 1.0 is used to maximize chlamydial RNA recovery, however a limited quantity of bacterial RNA will be present at 1 hpi as replication would not yet have commenced. This caveat should be considered when estimating RNA quantities required. It is recommended that ~ 5×10^8 host reads and $> 1 \times 10^6$ bacterial reads are required for adequate coverage [4,35]. The *Chlamydia* to Hela genome size is ~1:3200 MB, indicating that *Chlamydia*'s RNA accounts for ~0.03% of total host-bacteria RNA. As ~95% of this will be uninformative rRNA and tRNA [4], 0.0015% and 4.9985% of total RNA will represent informative bacteria and host mRNA, respectively. Given this ratio, 1 × 10^{10} host reads and ~3.33 × 10^{9} bacterial reads would be required to capture sufficient RNA from both organisms. Thus, to achieve sufficient coverage overall, > 1 × 10^{10} reads would be required for dRNA-Seq of *Chlamydia* and host.

Infection

Host cells are cultured in Dulbecco's modified Eagle's medium (DMEM) (see Materials section) and are infected with *C. trachomatis* at a multiplicity of infection (MOI) of 1 to ensure that 100% of host cells are infected. As cycloheximide (an inhibitor of protein synthesis used to maximize chlamydial yields *in vitro*), is not used throughout this experiment, so the host cells are seeded at ~60% confluency at the time of infection to ensure continued host cell viability throughout the time-course of the study. Given the time-sensitive nature of the experiment, it is critical to synchronize the initial infection by centrifugation, followed by the removal of dead or non-viable bacterial cells by washing twice with DPBS. HeLa and *Chlamydia* cells are harvested into sucrose phosphate glutamate (SPG) media, and frozen at -80°C prior to RNA extraction.

RNA Preparation

To ensure high-quality data, dRNA-Seq typically requires a relatively large amount of input RNA. Extreme care must be taken to prevent DNA contamination or RNA degradation, which can be minimized by adhering to the protocol time and temperature requirements, purchasing highly pure and RNase-free reagents, and using RNA-free equipment and consumables. Always conduct RNA work in a clean environment that is partitioned from non-RNA work. Wherever possible we routinely use commercially available kits due to their reliability and reproducibility, however we have carefully optimized the manufacturer's instructions to suit this protocol.

Total nucleic acid is extracted using a MasterPure™ RNA Purification Kit (Epicenter) according to the manufacturer's instructions. At this stage it is critical to minimize the delay between host cell lysis and RNA extraction to avoid unwanted degradation. HeLa cells are lysed, host proteins digested, and total nucleic acid precipitated with isopropanol. It is critical to ensure the complete removal of contaminating DNA before proceeding. We have found that two treatments with TURBO DNA-*free*™ DNase (Thermo Fisher) is most effective. We perform three real-time qPCR assays for human targets and one endpoint PCR assay for *C. trachomatis* to confirm DNA removal. The qPCR assays are based on TaqMan® Gene Expression assays (Applied Biosystems) with primer and probe sets targeting beta actin, mitochondrially encoded ATP synthase 6, and eukaryotic 18S rRNA [1]. The endpoint PCR is based on custom-designed primer sets that are specific for *C. trachomatis*, which were designed using PrimerExpress software (Applied Biosystems).

As rRNA constitutes >95% of total RNA, a method of rRNA depletion should be considered to maximize the recovery of mRNA and reduce the sequencing depth required. There are a number of commercial kits available for nuclease digestion and size-selection; this protocol utilizes both a hybridization-based rRNA depletion and poly(A)-depletion step. For hybridization, cDNA oligonucleotides attach to complementary rRNA that is immobilized on magnetic beads; always ensure that the oligonucleotides are compatible with your organism(s) of interest. For this, we combine an equivalent volume of Ribo-Zero beads from both a Human/Mouse/Rat-specific and Gram-negative bacteria-specific Ribo-Zero™ rRNA Removal Kit (Epicenter), enabling simultaneous elimination of both host and bacterial rRNA. It is important to note that this method will not enrich immature mRNAs and non-coding RNAs; specific target enrichment techniques that are outside the scope of this protocol should be considered if these are of experimental interest. Aliquots of rRNA-reduced samples may be then subjected to poly(A) depletion to further enrich host mRNA transcripts and separate mRNA from rRNA contaminants. Poly(A)-depleted and rRNA-depleted eluates can also be further purified before being combined for library construction. The remaining RNA is concentrated and purified with a RNA Clean & Concentrator™-5 kit (Zymo Research). A Bioanalyzer (Agilent) is used to examine the concentration and quality of purified RNA via a capillary electrophoresis-based system.

Library Preparation and Sequencing

Prior to sequencing, total RNA is converted to cDNA [36]. There are a number of sequencing platforms currently available, including Illumina, SOLID, Ion Torrent, Roche 454, Nanopore, and Pacific Biosciences, and each are suited to specific purposes and should be investigated by the user according to their desired outcome. This stage of the (d)RNA-Seq protocol (library preparation and sequencing) is often outsourced to a commercial enterprise or central sequencing facility so the steps involved are outside the scope of this protocol. Each facility will provide detailed instructions on the quality and quantity of RNA required and the sample preparation guidelines. Nevertheless, here we provide some general guidelines based on our experience.

We generally use the TruSeq Sample Prep Kit for library preparation and sequencing by the Illumina platform. For this, the mRNA is chemically fragmented and primed with random hexamer primers. First-strand cDNA synthesis occurs using reverse transcriptase, followed by second strand cDNA synthesis using DNA polymerase I and RNase H. The cDNA is purified and end-repaired and 3' adenylated. Adapters containing six nucleotide indexes are ligated to the double-stranded cDNA, which is purified with AMPure XT beads (Beckman Coulter) and enriched via polymerase chain reaction (PCR) amplification. While we suggest that paired-end reads > 50 nucleotides will promote increased fragment randomization and is a good guideline, longer reads will enable greater coverage, reduced multi-mapping, and improved transcript identification [37].

Data preparation

Sequence data from dRNA-seq comprises cDNA as input from the experiment, with the majority derived from the eukaryotic host (depending on the experimental conditions and system under study). Thus, careful attention is required to accurately segregate the reads from each organism. For paired-end sequencing, host and bacteria read data is generally provided as two FASTQ format files, which are comprised of a unique read identifier, the sequence read, an optional alternate identifier, and the quality scores for each read position. These are examined for possible sample contamination by screening total reads against a sequence database with FastQ Screen (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/) (Figure 3). The reads are then checked for quality using FASTQC, a Java-based software that reports several quality control statistics and a judgment on each metric (pass, warn, fail) (Figure 4) [36]. Short reads, low quality reads, and adapters are then removed with Trimmomatic [38]. Other available QC tools available include PRINSEQ [39] and FastX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/).

The organisms of interest and experimental question will dictate which mapping software is most appropriate; we currently use HISAT2 [40], a powerful yet efficient program capable of identifying the splice junctions between exons that are characteristic of eukaryotic data, while the short-read aligner, Bowtie2 [41], is sufficient for bacterial read mapping. Other non-splice-aware aligners for bacterial reads include SEAL [42] and SOAP2 [43], and alternative splice-aware aligners for host reads include MapSplice [44], STAR [45], and Tophat2 [46]. It is important to note that read aligners are an active area of research, with new tools and updates frequently appearing [47].

The combined host and bacteria reads are mapped to the host reference genome with specific settings to preserve unmapped (bacterial) reads, which are then mapped to the bacteria reference genome. Assembly to a reference transcriptome is also possible, but this relies on the accuracy of annotated gene models which may restrict the discovery of novel genes and isoforms; this approach is more suitable when there is no reference genome available. If available for the organisms of interest, reference genomes and the annotation file can be obtained from either NCBI [48], UCSC [49], or Ensembl [50]. Each repository formats these files slightly differently so it is important to obtain both files from the same source. This protocol utilizes the GRCh37 release of the *Homo sapiens* genome and annotation file from Ensembl and the *Chlamydia trachomatis* serovar D genome from NCBI (NC_000117.1).

The resulting alignment files for both host and bacteria are sorted by position (i.e. chromosomal location) with Samtools [51] to produce alignment quality statistics, including the number of mapped reads, the number of mapped first mates and second mates (for reads from paired-end sequencing), reads with multiple hits in the genome, and host reads mapping to exonic, intronic, and intergenic regions. Ideally, > 70% of host reads should map to exonic regions of the genome while less than 5% of reads mapping to intronic regions and less than 1% of reads mapping to intergenic regions [52]. The alignment file is further converted to a BigWig format for the visualization of the number of reads aligned to every single base position in the genome using Integrated Genome Viewer (IGV) (Figure 5) [53], or other visualization tools such as UCSC Genome Browser or JBrowse [54]. Using IGV, the coverage of aligned reads across the genome for both host and bacteria can be visualized to identify genomic regions of high/low coverage that could indicate technical or biological errors, as well as host exon-intron boundaries, splice sites, exon junction read counts, and read strand [55]. The alignment files are then sorted by read name to facilitate feature counting.

Feature counting and normalization

Both host and bacteria counts are generated from their respective alignment files using the python wrapper script *htseq-count* from the HTSeq package [56]. This process quantitates the number of reads that align to a biologically meaningful feature such as exons, transcripts, or genes [56], and is guided by the reference annotation file. This protocol describes the quantification of reads on a gene level, where a gene is considered the union of its exons. Any reads that map to several genomic locations are automatically discarded by HTSeq and we generally take a conservative approach to also discard reads that overlap with more than one gene.

Sample read counts are collapsed into a single file containing a matrix of genes (rows) and samples (columns), with one file each for host and bacteria (Figure 6). To minimize statistical noise and enable better adjusted *p*-values, the matrix is pre-filtered so that greater than three counts remain in more than two of the samples [36]. Additionally, the last five lines of the matrix containing statistics for ambiguous counts from *htseq-count* are removed. Raw counts are normalized to minimize technical bias due to transcript length and sequencing depth; there are several normalization methods available, including Reads Per Kilobase Per Million (RPKM) [20], EDASeq [57], Conditional Quartile Normalization (CQN) [58], Upper Quartile (UQ) [59], and Transcripts Per Million (TPM) [60], and each have their benefits. For the host counts we use Trimmed Mean of M-Values (TMM) which corrects for differences in RNA composition and sample outliers, while providing better across-sample comparability [61].

Data analysis

Prior to differential expression analysis, both a Multi-Dimension Scaling (MDS) plot and hierarchical cluster plot is constructed to visualize the distances between samples and help identify problematic and outlying samples (Figures 7 and 8). A metadata table is generated to list the experimental variables, which in turn guides the construction of design and contrast matrices, which are mathematical representations of the experimental design and a description of the relevant treatment comparisons, respectively. This protocol describes a simple design and contrast matrix that allows differential expression comparisons to be made between infected and uninfected cells within each time-point. Additional time-points and other experimental factors would lend themselves to more complex design matrices and may be added by the user if required.

Due to the specific nature of the host and bacteria count data, distinct statistical analyses are required for each. For bacterial transcripts, TPM is the most appropriate measure of relative transcript abundance, but this approach can suffer from biases where the calculated abundance of one transcript can affected other transcripts in the sample. Alternatively, absolute abundance may be calculated with the use of spike-in controls. Whichever method is chosen, these abundances represent a descriptive characterization of the *Chlamydia* transcriptome at the 1- and 24hpi time-points, which is moderately informative on its own, but when analyzed in combination with the interacting host response can allow deeper insight into the host-bacteria interactome.

For the host, differential expression analysis is performed to identify genes that have changed significantly in transcript abundance between infected and time-matched non-infected samples [5]. There are multiple differential expression packages available, each with advantages, including BaySeq [62], Cufflinks [63], DESeq [64], edgeR [65], Salmon [66], and Kallisto [67]. Protocols for the Cufflinks suite and edgeR/DESeq packages have been described previously [5,68]. The majority of these packages model RNA-Seq counts via a negative binomial (NB) distribution and apply various approaches to calculate reliable dispersion estimates. Alternatively, this protocol describes the use of the Limma package which uses linear modeling to describe the expression data for each gene (Limma stands for "Linear Modeling for Microarray Data") [69]. In contrast to these other RNA-Seq packages, Limma attempts to correctly model the mean-variance relationship between samples to achieve a more probabilistic distribution of the counts (Figure 9). This has proven to be the best method for analyzing both simple and complex experimental designs of dRNA-seq experiments that incorporate different sample types and time-points [70].

The output for the host is a set of differentially expressed genes, applying a False Discovery Rate (FDR) cutoff ≤ 0.05 (i.e. 5% false positives), and at least two-fold up/down-regulation and expression levels greater than 1 percentile in either condition (Table 1). These lists can then be used as input for downstream analysis of the enrichment of gene ontology and metabolic pathways using several tools, including GOSeq [71], DAVID [72], and Ingenuity Pathway Analysis (IPA) Toolkit (QIAGEN Redwood City, www.qiagen.com/ingenuity).

Integration of host and bacterial transcriptomic data

The final and most powerful stage of a dRNA-seq experiment is the integration of host and bacterial data to draw biological conclusions that could not have been obtained by investigating each organism in isolation. Following Gene Ontology and pathway analyses, interacting host-bacteria responses can be identified, within and between time-points, to highlight pathogenic mechanisms in the bacterium and the reciprocal regulatory patterns, responses, and transcriptional reprogramming of the host (depending on the system under investigation).

Application

dRNA-Seq can be used to address a number of experimental questions. Host differential mRNA and miRNA expression, differential exon usage, alternative splicing, and novel transcript and isoform discovery in response to the bacteria can be determined [5], which may be correlated with the transcriptomic response of the bacteria to determine interaction dynamics. These results can be further integrated with other sources of biological input, including genotyping data and epigenetic information (transcription factor binding, histone modification, methylation etc.) to contribute to a systems level definition of host regulatory mechanisms.

Procedure: Laboratory

Materials

Reagents

HeLa 229 epithelial cells (ATCC® CCL-2.1™) **CRITICAL** All experiments that use human or animal tissues must comply with governmental and institutional guidelines and regulations.

Chlamydia trachomatis serovar E **CAUTION** *Chlamydia* is a human bacteria that poses a risk of infection. All work with this organism should be conducted in a class II biosafety cabinet while wearing appropriate personal protective equipment (PPE).

Dulbecco's modified Eagle's medium, high glucose, pyruvate (Thermo Fisher, cat. no. 11995-065)

Heat-inactivated fetal bovine serum (Thermo Fisher, cat. no. 10082-147)

Sucrose (Sigma-Aldrich, cat. no. S7903)

Glutamic acid (Sigma-Aldrich, cat. no. 49449)

Di-sodium hydrogen phosphate (Scharlau, cat. no. SO03390500)

Sodium di-hydrogen phosphate (Scharlau, cat. no. SO03310500)

Dulbecco's phosphate buffered saline, no calcium, no magnesium (Thermo Fisher, cat. no. 14190-144)

Gentamicin (50 mg/mL) (Thermo Fisher, cat. no. 15750-060) **CAUTION** Harmful if swallowed or inhaled. Causes irritation to the skin. Wear suitable protective clothing when handling.

Streptomycin (Sigma-Aldrich, cat. no. S9137) **CAUTION** Harmful if swallowed. Suspected of damaging fertility or the unborn child. Wear suitable protective clothing when handling.

Ethanol (Sigma-Aldrich, cat. no. E7023) **CAUTION** Highly flammable. Causes skin and serious eye irritation. Handle using appropriate safety equipment.

Isopropanol (Sigma-Aldrich, cat. no. I9516)

TruSeq RNA Sample Prep Kit (Illumina, cat. no. RS-122-2101)

AMPure XT beads (Beckman Coulter, cat. no. A63880)

Poly(A)Purist Mag Purification Kit (Thermo Fisher, cat. no. AM1922)

TURBO DNA-*free*™ kit (Thermo Fisher, cat. no. AM1907)

DEPC-treated water (Thermo Fisher, cat. no. AM9915G)

MasterPure™ RNA Purification Kit (Epicenter, cat. no. MCR85102)

RNA Clean & Concentrator™-5 (Zymo Research, cat. no. R1015)

Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat) (Epicenter, cat. no. RZH1046)

Ribo-Zero™ rRNA Removal Kit (Gram-negative bacteria) (Epicenter, cat. no. RZNB1056)

TaqMan® Gene Expression Assay (Thermo Fisher, cat. no. 4453320)

TaqMan® Universal Master Mix (Thermo Fisher, cat. no. 4352042)

Equipment

MicroAmp® optical 96-well reaction plates (Thermo Fisher, cat. no. 4306737)

MicroAmp® optical adhesive film (Thermo Fisher, cat. no. 4311971)

MicroAmp® optical film compression pad (Thermo Fisher, cat. no. 4312639)

Flask rocker (Grant Instruments, PS-M3D)

DNase and RNase-free 1.5 mL microcentrifuge tubes (Sarstedt, cat. no. 72.692.210)

Refrigerated microcentrifuge (Beckman Coulter, 20R)

Centrifuge (Beckman Coulter, X-12R)

Vortex (Scientific Industries, G-560E)

Heating block (x2) (Thermo Fisher, cat. no. 2001Q)

Ice

Ice bucket

Timer

Real-time qPCR machine (Applied Biosystems, 7900HT)

Temperature cycler (Bio-Rad, C1000)

Incubator, 37°C, 5% CO_2 (Sanyo, MCO-19AIC)

Bioanalyzer

6-well plates (Thermo Scientific, cat. no. 140675)

Cell scrapers (Sarstedt, cat. no. 83.1830)

15 mL centrifuge tubes (Thermo Scientific, cat. no. 339652)

Reagent Setup

SPG media 10 mM sodium phosphate, 250 mM sucrose, 5 mM glutamic acid. Make solution with nuclease-free water at pH 7.4. Store at 4°C for up to six months.

70% ethanol Combine 350 mL ethanol with 150 mL nuclease free water. Store at room temperature for up to six months.

Dulbecco's modified Eagle's medium (DMEM) Media is supplemented with 10% v/v fetal bovine serum, 100 µg/mL streptomycin, 50 µg/mL gentamicin. Store at 4°C for up to six months.

Streptomycin Make stock concentration to 100 mg/mL with nuclease-free water. Store as single-use aliquots at -20°C for up to 12 months.

Sodium phosphate buffer

Ribo-Zero™ rRNA Removal Kit The Ribo-Zero kits are composed of two parts: the magnetic core kit and the rRNA removal reagents. Store the magnetic core kits at 4°C and Mouse/Human/Rat and Gram-negative bacteria rRNA removal reagents at -80°C.

RNA Clean & Concentrator™-5 Add 48 mL of 100% ethanol to the 12 mL RNA Wash Buffer before use.

Procedure: Laboratory

Seeding and infection

1. Seed 8 x 10^5 HeLa cells per well in all wells of a six-well plate. Ensure there are three plates per time-point (one infected plate and two non-infected control plates). Incubate plates overnight at 37°C, 5% CO_2.

   Following seeding, sit plates on a bench at room temperature for 15 minutes to allow cells to settle, ensuring an even distribution of cells.

2. The next day, infect all plates (except mock-infected control plates) at an MOI of 1.0 to ensure that 100% of the host cells will be infected.

3. Centrifuge plates at 500 x *g* for 30 minutes at room temperature. Incubate plates at 37°C, 5% CO_2.

   Centrifugation is important to synchronize the infections and time-points.

4. Wash cells twice with DPBS and overlay with warm, fresh DMEM media (containing streptomycin, gentamicin, 10% FBS). Incubate at 37°C, 5% CO_2.

Harvesting cells

5. At each time-point, wash cells twice with DPBS and add 1mL DPBS to each well. Harvest cells with a cell scraper and dispense solution into a 15 mL centrifuge tube. Store tubes at -80°C until all time-points are complete. Cells can be stored for up to six weeks at -80°C.

Cell lysis

6. Remove centrifuge tubes from -80°C freezer and thaw at room temperature.

7. Pre-set heating block to 65°C.

8. Add 1 µL of 50 µg/mL Proteinase K (MasterPure™ RNA Purification Kit; Epicenter) to 300 µL of Tissue and Cell Lysis Buffer (MasterPure™ RNA Purification Kit; Epicenter) for each sample.

9. Pellet cells by centrifugation at 5,000 x *g* for 30 minutes. Discard the supernatant, leaving ~25 µL of liquid.

10. Vortex for 10 seconds to resuspend the pellet.

11. Add 300 µL of Tissue and Cell Lysis Solution (containing Proteinase K) to each 25 µL sample and mix thoroughly by vortexing.

12. Incubate tubes in a heating block at 65°C for 15 minutes, vortexing briefly every 5 minutes.
13. Place samples on ice for 3-5 minutes.

Total nucleic acid precipitation

14. Add 175 µL of MPC Protein Precipitation Reagent (MasterPure™ RNA Purification Kit; Epicenter) to each 300 µL of lysed sample and vortex vigorously for 10 seconds.

15. Pellet the debris by centrifugation at 10,000 x *g* for 10 minutes at 4°C.

16. Transfer the supernatant (containing total nucleic acid) to a clean 1.5 mL microcentrifuge tube and discard the pellet.

17. Add 500 µL of isopropanol to the recovered supernatant and invert the tube 30-40 times. Do not vortex.

18. Pellet the total nucleic acid by centrifugation at 10,000 x *g* for 10 minutes at 4°C.

19. Carefully pour off the isopropanol without dislodging the pellet.

20. Rinse the pellet twice with 70% ethanol, being careful not to dislodge the pellet. Centrifuge briefly if pellet is dislodged.

21. Remove residual ethanol with a pipette and resuspend the pellet in 35 µL of TE Buffer (MasterPure™ RNA Purification Kit; Epicenter). Samples can be stored in TE Buffer overnight at 4°C.

DNA digestion

22. Pre-set heating block to 37°C.

23. Add 4 μL of 10x TURBO Dnase Buffer (TURBO™ DNA-*free*™ Kit; Thermo Fisher) to each 35 μL sample.

24. Add 1 μL of TURBO™ DNase to each sample. Gently flick the tubes to mix and pulse-spin to distribute liquid to the bottom of the tube.

    Increase DNase volume to 2-3 μL if digesting a higher amount of DNA. Alternatively, add half the DNase to each reaction, incubate for 30 minutes, then add the remainder of the enzyme and incubate for another 30 minutes.

25. Incubate tubes in a heating block at 37°C for 30 minutes.

26. After incubation, add 8 μL of DNase Inactivation Solution (TURBO™ DNA-*free*™ Kit; Thermo Fisher) and incubate tubes at room temperature for 5 minutes, mixing occasionally.

    Environments colder than 22°C can reduce the inactivation of TURBO™ DNase. Move tubes to a heating block to control the temperature if necessary.

27. Centrifuge tubes at 10,000 x *g* for 1.5 minutes.

28. Transfer supernatant (containing the RNA) to a fresh 1.5 mL microcentrifuge tube.

Validation of DNA removal by RT qPCR

29. Remove 5 μL from each RNA sample and place in a clean 1.5 mL microcentrifuge tube.

30. Add 95 μL of nuclease-free water (1:20 dilution).

31. Prepare enough RT qPCR master mix to assay each sample in triplicate, as well as non-template controls:

20x TaqMan® Gene Expression Assay:	1 µL
2x TaqMan® Gene Expression Master Mix:	10 µL
RNase-free water:	5 µL

32. Cap the tube and invert the tube several times to mix the reaction components. Pulse vortex.

33. Aliquot 15 µL of master mix into individual wells of a 96-well reaction plate (Thermo Fisher). Include a triplicate reaction for each sample and non-template controls.

34. Add 5 µL of each diluted template to appropriate wells and gently tap plate on benchtop to distribute contents to the base of the well.

35. Place adhesive film (Thermo Fisher) over the plate and seal with compression pad (Thermo Fisher).

If any bubbles are visible in the wells or liquid is present on the sides of the wells, centrifuge plate at 500 x *g* for 2 minutes. Do not touch the film with bare hands at any point.

36. Place plate in RT-PCR machine and run assay according to the following cycling conditions:

Hold:	95°C, 10 minutes
Cycle (40x):	95°C, 15 seconds
	60°C, 1 minute.

rRNA depletion

37. Pre-set one heating block to 68°C and one at 50°C.

38. Remove Ribo-Zero™ rRNA Removal Magnetic Core Kit from 4°C and allow to warm to room temperature. Remove Human/Mouse/Rat and Gram-negative bacteria components of the Ribo-Zero™ rRNA Removal kits from -80°C and thaw on ice.

    Do not place the Ribo-Zero™ Magnetic Core Kit on ice.

39. Vigorously mix magnetic beads (Ribo-Zero™ rRNA Removal kit; Illumina) for 20 seconds by vortexing.

40. Carefully pipette 65 µL of magnetic beads into 2 mL microsphere wash tubes (Ribo-Zero™ rRNA Removal kit; Illumina); two tubes per sample.

    Store unused beads at 4°C. Do not place the magnetic beads on ice.

41. Centrifuge microspheres at 12,000 x *g* for 3 minutes. Carefully remove supernatant without dislodging the pellet.

    The supernatant contains sodium azide.

42. Wash the microsphere wash tubes by adding 130 µL of microsphere wash solution (Ribo-Zero™ rRNA Removal kit; Illumina) to each tube. Vortex vigorously.

43. Centrifuge microsphere wash tubes at 12,000 x *g* for 3 minutes. Carefully remove supernatant without dislodging the pellet.

44. Add 65 µL of microsphere resuspension solution (Ribo-Zero™ rRNA Removal kit; Illumina) to each tube and vortex vigorously until a homogenous suspension is produced.

45. Add 1 µL of RiboGuard RNase inhibitor (Ribo-Zero™ rRNA Removal kit; Illumina) to each tube. Mix by vortexing for 10 seconds and set aside (at room temperature).

Avoid creating air bubbles when adding RNase inhibitor.

46. Treat two aliquots of each sample with Ribo-Zero rRNA removal solution (Ribo-Zero™ rRNA Removal kit; Illumina) according to the following preparation (two removal preps per sample):

RNase-free water (Ribo-Zero™ rRNA Removal Kit):	1 µL
Ribo-Zero Reaction Buffer:	4 µL
RNA sample:	25 µL
Ribo-Zero rRNA Removal Solution (Gram negative bacteria kit):	5 µL
Ribo-Zero rRNA Removal Solution (Human/Mouse/Rat kit):	5 µL

Fully mix the samples by pipette-mixing 10-15 times.

47. Gently mix the reactions and incubate at 68°C for 10 minutes in heating block. Return the Ribo-Zero reaction buffer to -80°C.

48. Remove the microsphere wash tubes from the heating block and incubate at room temperature for 15 minutes.

49. Vortex the microsphere wash tubes at medium speed for 20 seconds to ensure a homogenous slurry.

50. Pipette hybridized RNA sample to the resuspended microsphere wash tubes, pipette-mixing 10-15 times to mix. Immediately vortex the microsphere wash tubes at medium speed for five seconds.

The washed magnetic beads must be at room temperature for use in this step. The order of the addition (hybridized RNA *to* the magnetic beads) is critical for rRNA removal efficiency.

51. Incubate microsphere wash tubes at room temperature for 10 minutes. Vortex at medium speed for 5 seconds, every 3-4 minutes.

52. Following incubation, mix samples again by vortexing at medium speed for 5 seconds.

53. Incubate samples in heating block at 50°C for 10 minutes.

54. Transfer the RNA-microsphere suspension to a Microsphere Removal Unit (Ribo-Zero™ rRNA Removal kit; Illumina) and centrifuge at 12,000 x *g* for 1 minute at room temperature. Save the eluate and discard the removal unit.

At this stage, the eluate should be ~100 µL.

Purification of rRNA-depleted samples

55. Add 2 volumes of RNA Binding Buffer (RNA Clean & Concentrator™-5; Zymo Research) to each volume of RNA sample and mix well.

The minimum recommended sample volume for use with this kit is 50 µL.

56. Add 1 volume of 100% ethanol to the mixture and mix well.

57. Transfer the mixture to a Zymo-Spin IC column (RNA Clean & Concentrator™-5; Zymo Research) in a collection tube and centrifuge at 12,000 x *g* for 1 minute. Discard the flow-through.

58. Combine two reactions of the same sample to one column and spin multiple times until the entire mixture passes through the column. The column capacity is 5 µg of RNA.

59. Add 400 µL of RNA Prep Buffer (RNA Clean & Concentrator™-5; Zymo Research) to the column and centrifuge at 12,000 x *g* for 1 minute. Discard the flow-through.

60. Add 800 µL of RNA Wash Buffer (RNA Clean & Concentrator™-5; Zymo Research) to the column and centrifuge at 12,000 x *g* for 1 minute. Discard the flow-through.

61. Add 400 µL of RNA wash buffer to the column and centrifuge at 12,000 x *g* for 1 minute. Discard the flow-through.

62. Centrifuge the column in an emptied collection tube at 12,000 x *g* for 2 minutes. Carefully remove the column from the collection tube and transfer to a new RNase-free microcentrifuge tube.

63. Add 20 µL of DNase/RNase-free water directly to one column matrix and let stand for 1 minute at room temperature. Centrifuge at 10,000 x *g* for 30 seconds.

The eluted RNA can be used immediately or stored at -80°C.

Library Preparation and Sequencing

The method of library preparation is dependent on the sequencing platform used and thus is outside the scope of this protocol. Commercial sequencing enterprises and sequencing centers will provide detailed guidelines on the library preparation and sample submission guidelines that begin with the isolation of pure mRNA as described above. Following sequencing, the user will be provided with a series of raw FASTQ sequence files that serve as the input for the following bioinformatics section.

Procedure: Bioinformatics

Materials

Hardware requirements

The analysis of dRNA-seq experiments is a computationally intensive process that requires the manipulation of gigabytes of data. Access to a computer cluster, core facility, or cloud service is recommended to expedite the analysis and free up resources on the local system.

Operating system

This protocol provides commands that are designed to run on a Unix-based operating system such as Linux or Mac OS. The protocol was specifically designed to run on the Ubuntu 16.04.1 operating system on a Linux machine. Please ensure you have administrative rights.

Command line nomenclature

This protocol assumes a basic understanding of both the Linux command line interface and the R statistical computing environment. All Linux commands are shown following a dollar sign ($), while R commands are shown following a greater-than sign (>):

```
$       linux command
>       R command
```

Software requirements

- FastQ Screen: Contamination screening (http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/)
- FASTQC: Sequence quality control tool (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)
- Trimmomatic: FASTQ sequence file trimming (http://www.usadellab.org/cms/?page=trimmomatic)
- HISAT2: Graph-based alignment of sequences to genomes (https://ccb.jhu.edu/software/hisat2/index.shtml)
- Samtools: Manipulation of sequence alignments and mapped reads (http://www.htslib.org)
- Bedtools genomecov and bedGraphToBigWig: genomic analysis tools (http://bedtools.readthedocs.io/en/latest/)
- Integrated Genome Viewer (IGV): Alignment and visualization tool (https://www.broadinstitute.org/igv/)
- HTSeq: read counting (http://www-huber.embl.de/users/anders/HTSeq/doc/overview.html)
- R statistical computing environment (https://www.r-project.org)
- Bioconductor packages: edgeR, limma, org.Hs.eg.db, GenomicFeatures, and their dependencies (see below)
- Bowtie2: Short-read aligner (http://bowtie-bio.sourceforge.net/index.shtml).

Always check that you are downloading and installing the latest version of each piece of software and consult the official user guide for more in-depth guidelines and options for troubleshooting any errors that may arise.

Samples and filenames

The protocol is arranged so that identical naming conventions are used for each sample and condition. For example, “1hpi_Host_infected_rep1” indicates that the sample relates to the first replicate of host cells infected with *Chlamydia* at the 1 hpi time point and “1hpi_Host_uninfected_rep1” indicates the first replicate of host cells only (i.e. uninfected host cells) at the 1 hpi time point. Conversely, the samples relating to the bacteria are named, “1hpi_Bacteria_rep1”. While subsequent replicates for both host and bacteria would have names ending in “rep2” and “rep3”, for conciseness this protocol describes the commands using “1hpi_Host_infected_rep1” as an example and it is expected that the user will repeat the process for the remaining replicates and samples. The filenames associated with raw FASTQ sequence files will depend on the sequencing facility pipeline and in this protocol are named “fastq_file_1_R1.fq”, where “R1” indicates read number one of paired end reads (the corresponding read file would be “fastq_file_1_R2.fq”. In some cases an output directory is required, which is noted as “<output_directory>” for the user to input their working directory of choice (without the “<>” symbols). Finally, reference, annotation, and gene info files are prefixed with the relating organism, i.e. “host_reference.fa” indicates a FASTA file containing the host reference genome.

Equipment setup

Download and install the following software. Check the developer website to ensure you are installing the latest version and for further information about dependencies and prerequisites.

Create a directory to install program executables and add to PATH

```
$	mkdir $HOME/bin
$	export PATH=$HOME/bin:$PATH
$	echo "export PATH=$HOME/bin:$PATH" >> ~/.bashrc
```

FastQ-Screen installation

```
$	wget
	http://www.bioinformatics.babraham.ac.uk/projects/fastq_screen/fastq_screen_v0.9.3.tar.gz
$	tar –zxf fastq_screen_v0.9.3.tar.gz
$	cd fastq_screen_v0.9.3
$	cp fastq_screen $HOME/bin
```

FastQC installation

```
$    sudo apt-get install default-jre
$    wget http://www.bioinformatics.babraham.ac.uk/projects/fastqc/fastqc_v0.11.5.zip
$    unzip fastqc_v0.11.5.zip
$    cd FastQC/
$    chmod 755 fastqc
$    cp fastqc $HOME/bin
```

Trimmomatic installation

```
$    wget http://www.usadellab.org/cms/uploads/supplementary/Trimmomatic/Trimmomatic-
     0.36.zip
$    unzip Trimmomatic-0.36.zip
$    cd Trimmomatic-0.36
$    cp trimmomatic $HOME/bin
```

HISAT2 installation

```
$    wget ftp://ftp.ccb.jhu.edu/pub/infphilo/hisat2/downloads/hisat2-2.0.5-Linux_x86_64.zip
$    unzip hisat2-2.0.5-Linux_x86_64.zip
$    cd hisat2-2.0.5
$    cp hisat2 $HOME/bin
$    cp hisat2-build $HOME/bin
```

Samtools installation

```
$    sudo apt-get install samtools
```

Bedtools installation

```
$    sudo apt-get install bedtools
```

bedGraphToBigWig installation

```
$	mkdir bedGraphToBigWig
$	cd bedGraphToBigWig
$	wget -O bedGraphToBigWig https://github.com/ENCODE-DCC/kentUtils/blob/v302.1.0/bin/
	linux.x86_64/bedGraphToBigWig?raw=true
$	chmod 755 bedGraphToBigWig
$	cp bedGraphToBigWig $HOME/bin
```

IGV and IGVtools installation

```
$	wget http://data.broadinstitute.org/igv/projects/downloads/IGV_2.3.88.zip
$	unzip IGV_2.3.88.zip

$	wget http://data.broadinstitute.org/igv/projects/downloads/igvtools_2.3.88.zip
$	unzip igvtools_2.3.88.zip
$	cd igvtools_2.3.88
$	cp igvtools $HOME/bin
```

HTSeq installation

```
$	sudo apt-get install build-essential python2.7-dev python-numpy python-matplotlib
$	wget --no-check-certificate https://pypi.python.org/packages/source/H/HTSeq/HTSeq-
	0.6.1p1.tar.gz
$	tar –zxvf HTSeq-0.6.1p1.tar.gz
$	cd HTSeq-0.6.1p1
$	python setup.py build
$	sudo python setup.py install
$	cd scripts
$	cp htseq-count $HOME/bin
```

R and Bioconductor package installation

```
$	sudo apt-get install libcurl4-openssl-dev libxml2-dev
$	sudo apt-get update
$	echo "deb https://cran.rstudio.com/bin/linux/ubuntu xenial/" | sudo tee -a /etc/apt/sources.list
$	sudo apt-key adv --keyserver keyserver.ubuntu.com --recv-keys E084DAB9
$	sudo add-apt-repository ppa:marutter/rdev
$	sudo apt-get update
$	sudo apt-get upgrade
$	sudo apt-get install r-base
```

Open R and install Bioconductor packages using the BiocLite installation tool. All packages dependencies will automatically be installed.

```
$	R
>	source(“http://bioconductor.org/biocLite.R”)
>	biocLite(“BiocUpgrade”)
>	biocLite(c(“org.Hs.eg.db”, “edgeR”, “limma”, “GenomicFeatures”))
```

Bowtie2 installation

```
$	wget https://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.2.9/bowtie2-2.2.9-linux-
	x86_64.zip
$	unzip bowtie2-2.2.9-linux- x86_64.zip
$	cd bowtie2-2.2.9-linux-x86_64
$	cp bowtie2 $HOME/bin
$	cp bowtie2-build $HOME/bin
```

File preparation

Download reference genomes and gene model annotation files

For the eukaryotic host, download the *Homo sapiens* reference genome and GTF annotation file from Ensembl: http://asia.ensembl.org/info/data/ftp/index.html and rename files to "host_reference.fa" and "host_annotation.gtf", respectively. Download the *Homo sapiens* gene info file from: ftp://ftp.ncbi.nlm.nih.gov/gene/DATA/GENE_INFO/Mammalia/Homo_sapiens.gene_info.gz and rename file as "host_gene.info". For *C. trachomatis*, download the reference genome in FASTA format from NCBI: http://www.ncbi.nlm.nih.gov/nuccore/NC_000117 and rename the file to "bacteria_reference.fa". Download the *C. trachomatis* GTF (000590675) file from bacteria.ensembl.org/info/website/ftp/index.html and rename file to "bacteria_annotation.gtf". Save all files to your working directory. Reference genomes and annotation files should be obtained from the same repository to ensure consistent formatting and nomenclature.

Method: Host

1. Examine a subset of FASTQ sequence files for contamination using FastQ Screen:

```
$	fastq_screen –aligner bowtie2 fastq_file_1_R1.fq
```

This command will run FastQ Screen on the chosen FASTQ file, checking against locally pre-built databases for possible sources of contamination. "fastq_screen" runs the software, --aligner Bowtie2 specifies the aligner used to create the databases, and "fastq_file_1_R1.fq" is the input file. This step should be repeated for a random number of samples. To generate a database, the genomes of each species which to test against should be downloaded. Using the host and bacterial genomes already downloaded from the earlier steps, Bowtie2 (or other aligners) are used to build an index (See section 4 below). Once built, the location and index should be added to the FastQ Screen configuration file. See Figure 3.

2. Check the quality of FASTQ sequences using FASTQC:

```
$	fastqc –noextract –o <output_directory> fastq_file_1_R1.fq
```

In this command, "-noextract" tells FASTQC to not uncompress the output file, while "-o" defines the output directory. "fastq_file_1_R1.fq" is the FASTQ sequencing file. These commands produce a quality report with results saved to the directory defined by "<output_directory>". The results are reported in both illustrated form (the "fastqc_report.html" file) and text form (the "summary.txt" file). Repeat for all FASTQ files. As the FASTQ files are derived from total RNA sequencing, this step includes both host and bacterial sequences. See Figure 4.

3. Remove sequencing adapters and low quality reads using Trimmomatic:

```
$	java -jar trimmomatic-0.36.jar PE -threads 6 -phred33 fastq_file_1_R1.fq fastq_file_1_R2.fq
	fastq_file_1_R1_paired_trimmed.fq fastq_file_1_R1_unpaired_trimmed.fq
	fastq_file_1_R2_paired_trimmed.fq fastq_file_1_R2_unpaired_trimmed.fq
	ILLUMINACLIP:adapters.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15
	MINLEN:36
```

Run this command from the Trimmomatic installation directory. The command specifes PE as paired-end data, 6 threads and the FASTQ files are encoded with Phred + 33 quality scores. "fastq_file_1_R1.fastq.gz" and "fastq_file_1_R2.fastq.gz" specify the input FASTQ files to use. As paired-end data is inputted, four output files are needed to store the reads. Two 'paired' files from which both reads survived after processing, and two 'unpaired' files from which a single read survived, but the corresponding mate did not. "ILLUMINACLIP:adapters.fa" uses the "adapters.fa" file containing sequences and names of commonly used adapters to remove. "2:30:10" are three parameters used in the 'palindrome' mode of Trimmomatic to identify the supplied adapters, regardless of their location within a read. For a detailed description of the best use of these three parameters, consult the Trimmomatic manual. "LEADING:3" and "TRAILING:3" removes a base from either the start or end position if the quality is below "3". "SLIDINGWINDOW:4:15" performs trimming based on a sliding window method. "4" is the window size and "15" is the required average quality. By examining multiple bases, if a single low quality base is encountered, it will not cause high quality data later in the read to be removed. Finally, "MINLEN:36" removes any remaining reads that are less than 36 bases long. Repeat for all FASTQ files. As above, this step includes both host and bacterial sequences.

4. Build host transcriptome index and align host sequence reads to reference using HISAT2:

```
$	hisat2-build host_reference.fa host_reference.index
$	hisat2 –x host_reference.index –un-conc pair1_unmapped.fastq -1 fastq_file_1_.R1.trim.fq -2
	fastq_file_1_.R2.trim.fq | samtools view –bS -  > accepted_hits.bam
```

This "-x" specifies the path to the index previously built by Bowtie2: "host_reference.index". The "—un-conc" argument tells HISAT2 to write a fastq rile containing all unmapped reads ("pair1_unmapped.fastq"), and "-1" and "-2" specify the paired-end fastq file mates. The "samtools view –bS -" argument converts to the output file from SAM to BAM format. Ensure that the HISAT2 output files, "accepted_hits.bam" and "pair1_unmapped.fastq" are preserved in the working directory as these are required for bacterial read mapping.

5. Sort BAM files generated by HISAT2 by both name and position using Samtools:

```
$	samtools sort accepted_hits.bam –o 1hpi_Host_infected_rep1.sorted_position
$	samtools sort –n accepted_hits.bam –o 1hpi_Host_infected_rep1.sorted_name
```

The first command takes the "accepted_hits.bam" file and sorts it by position, with the output file called "1hpi_Host_infected_rep1.sorted_position". In the second command, "-n" tells Samtools to sort the "accepted_hits.bam" file by name, with the output file called "1hpi_Host_infected_rep1.sorted_name". Repeat for all BAM files.

6. Convert 'sorted by position' BAM file to BigWig format:

```
$	samtools faidx host_reference.fa
$	cut –f1,2 host_reference.fa.fai > host_reference.genome
$	bedtools genomecov –split –bg –ibam 1hpi_Host_infected_rep1.sorted_position.bam –g
	host_reference.genome > 1hpi_Host_infected_rep1.sorted_position.bedGraph
$	bedGraphToBigWig 1hpi_Host_infected_rep1.sorted_position.bedGraph
	host_reference.genome 1hpi_Host_infected_rep1.sorted_position.bigWig
```

The first command indexes the reference genome by creating a "host_reference.fa.fai" output file. The second command extracts the first two fields (sequence ID and sequence length) to generate the "host_reference.genome" file. The third command generates a histogram illustrating alignment coverage according to the reference genome. The "-split" argument tells *genomecov* to take into account spliced BAM alignments (as we used the splice-aware aligner HISAT2 for the host reads), while the "-bg" argument tells *genomecov* to report genome-wide coverage in bedGraph format. "ibam 1hpi_Host_infected_rep1.sorted_position.bam" is the input file in BAM format, "-g host_reference.genome" is the reference genome in FASTA format, and "1hpi_Host_infected_rep1.sorted_position.bedGraph" is the output file in bedGraph format. The fourth command converts this bedGraph file to BigWig format for use with IGV (below).

7. Index the 'sorted by position' BAM file for visualization in IGV:

```
$	samtools index 1hpi_Host_infected_rep1.sorted_position.bam
```

This command takes the "1hpi_Host_infected_rep1.sorted_position" bam file created above and creates an indexed "1hpi_Host_infected_rep1.sorted_position.bai" file for use with IGV.

8. Index the GTF file for visualization in IGV:

```
$	igvtools sort host_annotation.gtf host_annotation_sorted.gtf
```

```
$	igvtools index host_annotation_sorted.gtf
```

The first command sorts the GTF file, specifying an input file and output file. The second command creates an index of the sorted GTF file.

9. Visualize alignments with IGV:

```
$	java –jar igv.jar
```

Run this command from the IGV installation directory. Within the software, load the "host_reference.fa" reference genome by clicking on *Genomes* and then *Load genome from file*. Load the sample BAM files from step 9, the indexed GTF annotation file (step 10) and the bigwig file (step 8) by clicking on *File*, then *Open*. Inspect the mapped reads and visualize their alignment to the reference genome to ensure whole genome coverage and that they align with exons as defined by the GTF file. The sample BAM files and index files must be in the working directory. See Figure 5.

10. Create count matrix with HTSeq:

```
$	htseq-count –s no –a 10 –r name –f bam 1hpi_Host_infected_rep1.sorted_name.bam
	Host_annotation.gtf > 1hpi_Host_infected_rep1.sorted_name.count
```

This command calls the *htseq-count* python wrapper script which performs the gene-level counts. "-s no" indicates that the reads are unstranded and "-a 10" sets the minimum mapping quality for a read to be counted as 10. "-r name" indicates that the input file is sorted by name and "-f bam" indicates that this input file is in BAM format and named 1hpi_Host_infected_rep1.sorted_name.bam". "host_annotation.gtf" is the GTF annotation file from Ensembl and "1hpi_Host_infected_rep1.sorted_name.count" is the output file produced. Repeat command for each BAM file, which will produce a series of text files counting the gene-level reads for each sample. The last five lines of each file contain a list of reads that were not counting due to alignment ambiguities, multi-mapping, or low alignment quality.

11. Set working directory in R:

```
>	R
>	data <- setwd(…)
```

The first command opens R, and the second command sets the working directory to the folder location containing all the relevant files from step 12. Replace "…" with the complete path to this location, for example: setwd("/home/username/dRNA-Seq/HTSeq_counts").

12. Create data frame in R containing experiment metadata:

```
>	group <- factor(c(rep("1hpi_mock", 3), rep("1hpi_infected", 3), rep("24hpi_mock", 3),
	rep("24hpi_infected", 3)))
```

This creates the metadata table containing all the experimental variables, including sample name, treatment, and time point.

13. Combine count files into a DGEList in R:

```
>	library(edgeR)
>	counts.host <- readDGE(list.files(pattern = ".count"), data, columns = c(1,2))
```

A DGEList is an R object from the edgeR package that efficiently compiles the count dataset and experimental variables that is fed into subsequent downstream analyses. The first command loads edgeR into the current R workspace. The second command creates a variable called counts_host, which is a DGEList containing all data from columns 1 and 2 from all files in the current working directory ending in ".count". See Figure 6.

14. Remove the last five rows from the count matrix:

```
>	counts.host$counts <- counts.host$counts[1:(nrow(counts.host$counts)-5), ]
```

This command removes the last five rows of the count matrix, which contain a summary of the ambiguous and non-counted reads from htseq-count.

15. Filter counts to exclude low expressing genes:

```
>	counts.host$counts <- counts.host$counts[rowSums(counts.host$counts > 3) > 2, ]
```

This command returns the count matrix so that there are greater than three reads in at least two replicates across all of the samples. Any non-conforming samples are removed.

16. Inspect the count matrix:

```
>	head(counts.host$counts, 20)
>	dim(counts.host$counts)
```

It is often helpful to visualize the count matrix at this point to confirm that it is formatted correctly and that there are no errors. The second command provides the matrix dimensions, which is useful for determining the number of genes remaining following independent filtering.

17. Apply TMM normalization to the raw counts:

```
>	counts.host <- calcNormFactors(counts.host)
```

TMM normalization factors are calculated and incorporated into the DGEList object.

18. Create the design matrix and define the contrasts of interest:

```
>	design <- model.matrix(~0 + group)
>	rownames(design) <- colnames(counts.host$counts)
>	contrasts <- makeContrasts("Host_1hpi" = group1hpi_infected – group1hpi_mock,
	"Host_24hpi" = group24hpi_infected – group24hpi_mock, levels = design)
```

These commands define the design matrix and the contrasts of interest to enable differential expression to be calculated. In this case, the contrasts we are interested are the host genes differentially expressed when infected versus the host genes differentially expressed when uninfected at the 1 hpi time point and at the 24 hpi time point. More complex experimental designs that include multiple samples, time points, batch effects, and treatments are possible and are explained in detail in the Limma User's Guide [73].

19. Apply voom transformation to normalized counts:

```
>	library(limma)
>	png("host_voom.png")
>	y <- voomWithQualityWeights(counts = counts.host, design = design, plot = TRUE)
>	dev.off()
```

This command applies a voom transformation to the counts, by converting them to log-counts per million with associated precision weights [70]. We generally extend this by using the *voomWithQualityWeights* function, which applies sample-specific weights to down-weight any outlier samples. This can be especially useful if outliers were identified in the MDS plot constructed in step 21 (below). This function takes as input the normalized count matrix ("counts_host") and design matrix ("design") and outputs two quality control plots: an estimation of the mean variance relationship and the sample-specific weights that were applied. The output figure "host_voom.png" is generated containing the voom transformation plots. See Figure 9.

20. Construct an MDS plot to identify any outlier samples:

```
> plot.colors <- c(rep("blue", 3), rep("red", 3), rep("orange", 3), rep("black", 3))
> png("host_MDS.png")
> plotMDS(counts.host, main = "MDS Plot for Count Data", labels =
  colnames(counts.host$counts), col = plot.colors, cex = 0.9, xlim=c(-2,5))
> dev.off()
```

These commands generate a Multi-Dimensional Scaling (MDS) plot by taking the host DGEList as input ("counts_host"). The MDS plot allows the visual inspection of sample proximities to highlight possible batch effects and sample outliers that may need to be addressed. The MDS plot is saved to the working directory as "host_MDS.png". See Figure 7.

21. Construct a hierarchical clustering plot to visualize sample groupings:

```
> counts.host.mod <- t(cpm(counts.host))
> dist <- dist(counts.host.mod)
> png("host_HC.png")
> plot(hclust(dist), main="Hierarchical Clustering Dendrogram")
> dev.off()
```

Line 1 converts the counts into Counts Per Million (CPM) and then transposes the resulting matrix. The second command generates the distance matrix between each of the 12 samples, and the third command generates a hierarchical clustering dendrogram from the normalized counts in "counts_host" where the most similar samples occupy closer positions in the tree. The plot is saved to the working directory as "host_HC.png". See Figure 8.

22. Fit the model:

```
> fit <- lmFit(y, design)
>    fit <- contrasts.fit(fit, contrasts)
>    fit <- eBayes(fit)
```

The first two commands estimate expression fold changes and standard errors by fitting a linear model to each gene, using the comparisons defined by the contrast matrix (“contrasts”). The third command applies empirical Bayes smoothing to the standard errors to further weaken any outliers.

23. Print the differentially expressed transcripts for both the 1hpi and 24hpi time-points:

```
>    top_1hpi <- topTable(fit, coef = “Host_1hpi”, adjust = “fdr”, number = “Inf”, p.value = 0.05,
     sort.by = “P”)
>    top_24hpi <- topTable(fit, coef = “Host_24hpi”, adjust = “fdr”, number = “Inf”, p.value =
     0.05, sort.by = “P”)
```

This command prints all the Differentially Expressed Genes (DEG) with a *p*-value of 0.05 or less after correcting for multiple testing using the False Discovery Rate (Benjamini & Hochberg) method. Additionally, a log fold change (LFC) threshold may be included by adding an “lfc = 2” argument, which would return all DEG with a log fold change in expression greater than two. See Table 1.

24. Annotate the differentially expressed transcript tables with gene symbol, description, and type information:

```
>    library(org.Hs.eg.db)
>    gene.info <- select(org.Hs.eg.db, key = rownames(top_1hpi), keytype = “ENSEMBL”,
     columns = c(“ENSEMBL”, “SYMBOL”, “GENENAME”))
>    gene.info <- gene.info[!duplicated(gene.info$ENSEMBL), ]
>    rownames(gene.info) <- gene.info$ENSEMBL
>    identical(rownames(top_1hpi), rownames(gene.info))
>    gene.info <- gene.info[, -1]
>    host_DEG_table <- cbind(top_1hpi, gene.info)
```

Often for downstream applications it is necessary to have the gene name or identifier for each DEG. These commands are derived from the Limma documentation [73] and extract gene annotation information stored in both the org.Hs.eg.db R package and the "host_gene.info" that was previously downloaded to the working directory to annotate the DEG list with gene symbol and gene name. Repeat for the "Host_24hpi" DEG list.

25. Write the annotated differentially expressed transcript table to the local hard drive:

```
>	write.table(host_DEG_table, file = "Host_DEG_annotated.csv", sep = ",", col.names = NA)
```

This command writes the DEG list to a comma-separated file. Repeat for the "Ct_24hpi" DEG list.

Method: Bacteria

26. Build bacteria reference index file and map the unmapped reads (bacterial reads) from HISAT2 to the bacteria reference genome with Bowtie2:

```
$	bowtie2-build -f bacteria_reference.fa bacteria_reference_index
$	bowtie2 -q pair1_unmapped.fastq bacteria_reference_index
```

The first command indexes the bacteria reference genome "-f bacteria_reference.fa" and generates the "bacteria_reference_index" output file. The second command performs the read mapping using the unmapped reads from the Host mapping step ("pair1_unmapped.fastq").

27. Repeat steps 6-10 from the host-specific protocol above.

Sort the BAM files by both name and position, convert the "sorted by position" BAM files to BigWig format and visualize with IGV. Create count matrix with HTseq.

28. Repeat steps 13-16 from the host-specific protocol above.

Combine the count files into a DGEList, remove the last five rows from the counts, filter counts to remove low expression genes, and inspect the counts for errors.

29. Apply TMM normalization to counts

```
>	dge.bacteria <- calcNormFactors(dge_bacteria)
>	bacteria.cpm <- cpm(dge.bacteria, normalized.lib.sizes = TRUE)
```

The first command generates TMM normalization factors and the second command converts the raw counts to normalized counts.

30. Calculate gene lengths

```
>	library(GenomicFeatures)
>	txdb <- makeTxDbFromGFF(“bacteria_annotation.gtf”, format = “gtf”)
>	exons <- exonsBy(txdb, by = “gene”)
>	gene.length <- sum(width(reduce(exons)))
>	gene.length <- as.data.frame(gene.length)
```

These commands utilize the the GenomicFeatures R package to extract the gene lengths from the “bacteria_annotation.gtf” file, which are required for the calculation of TPM values (below).

31. Define a function to calculate TPM

```
>	TPM <- function(counts, lengths){
		rate <- counts / lengths
		rate / sum(rate) * 1e6
	}
```

32. Calculate TPM values

```
>	final.tpm <- apply(bacteria.cpm, 2, function(x) TPM(x, gene.length))
>	final.tpm <- as.data.frame(final.tpm)
>	colnames(final.tpm) <- colnames(bacteria.cpm)
```

This command converts the normalized counts in "bacteria.cpm" to TPM.

33. Write TPM values to file:

```
>	write.table(final.tpm, file = “Ct_relativeabundance.csv”, sep = “,”, col.names = NA)
```

Anticipated Results and Troubleshooting

RNA quality and quantity

The Bioanalyzer trace will provide an indication of sample quality, where large, well-defined and high molecular weight peaks are expected. Low molecular weight peaks with low definition are usually evidence of RNA degradation and the experiment should not proceed further if this is the case.

Raw sequence quality checks

Following the analysis of the FASTQ files with FASTQC (step 2), a HTML report is generated which provides a judgement on several sequence quality parameters. Of particular importance are the following: The per base sequence quality plot should indicate a lower quartile above 10 (corresponding to 90% accuracy), while the per sequence quality score should have a mean base quality of 25 or higher (for RNA-seq reads, it is expected that base quality will decrease in later sequencing cycles), with the histogram curved to the right. The per base sequence content plot should indicate an even proportion of each base, and the per sequence GC content plot should demonstrate a normal distribution of GC content; an abnormal distribution is likely evidence of contamination. In both the per base sequence content plot and per sequence GC content plot, a bias will be observed at the first bases which is specific to Illumina sequencing. The k-mer content plot will indicate the presence of k-mers at the early bases (also Illumina sequencing bias), but should otherwise not be at a higher than expected frequency. The read length distribution plot should indicate that the read length is at least 50 nucleotides, as represented by a single peak.

Visualize alignments in IGV

IGV allows the visualization of reads mapped to the reference genome and provides an efficient way of identifying problematic data. The alignments should agree with the known gene structures such as intron placements, and the reconstructed transcripts should sufficiently represent the alignments. Verify that genes are roughly evenly covered with reads and if there are known differentially expressed genes, confirm differential coverage between sample groups. Splice junctions can also be visualized for the host reads.

Feature counting

HTseq will generate a separate tab-delimited file for all samples and replicates, containing the read count for every gene in the annotation file. This file is usually represented with two columns: the Ensembl gene identifier, and the read count: a number indicating how many reads overlap with the feature (gene) listed in the annotation file [5].

Multi-dimensional scaling plot and hierarchical clustering plot

In the MDS plot, replicate samples from each condition should generally cluster together, although some biological variation is expected. High sample variation may indicate noisy data and can be addressed by removing outlier samples, applying sample-specific quality weights as described in step 20, or applying additional normalization factors using specialized packages such as RUVseq [74]. Samples that cluster according to technical parameters, such as the time and date of sample collection, usually indicates evidence of batch effects which can be corrected by incorporating the effect into the design matrix (step 19). See the Limma user guide for more details on this [73].

Host differential expression analysis

Following gene annotation (step 25), the table of differentially expressed host genes compiles a number of columns containing significant information comprising the output of the host side of the experiment. The first column lists the Ensembl ID for each gene identified as being differentially expressed, while the remaining columns provide the statistical output for each gene, including (1) the $\log_2$ fold-change (log FC) measured between experimental conditions, (2) the average $\log_2$ expression (AveExp) for each gene, (3) the moderated *t*-statistic, which is the ratio of the $\log_2$ fold-change to its standard error, (4) the *p*-value, (5) the > 0.05 *p*-value adjusted for multiple testing (via the Benjamini & Hochberg method to control the false discovery rate), (6) the B-statistic representing the log-odds that the gene is differentially expressed, (7) and the gene annotation columns that were incorporated in step 25. This table may be sorted by any column, where the $\log_2$ fold-change and adjusted *p*-values are usually of most interest.

Bacterial transcript abundance analysis

The TPM values generated in step 35 are representative of the relative expression levels of the bacteria.

References

1. Humphrys MS, Creasy T, Sun Y, et al. Simultaneous transcriptional profiling of bacteria and their host cells. PLoS ONE 2013; 8:e80597
2. Oosthuizen JL, Gomez P, Ruan J, et al. Dual organism transcriptomics of airway epithelial cells interacting with conidia of *Aspergillus fumigatus*. PLoS ONE 2011; 6:e20527
3. Lister R, O'Malley RC, Tonti-Filippini J, et al. Highly integrated single-base resolution maps of the epigenome in *Arabidopsis*. Cell 2008; 133:523–536
4. Westermann AJ, Gorski SA, Vogel J. Dual RNA-seq of pathogen and host. Nature 2012; 10:618–630
5. Anders S, McCarthy DJ, Chen Y, et al. Count-based differential expression analysis of RNA sequencing data using R and Bioconductor. Nat Protoc 2013; 8:1765–1786
6. Nagalakshmi U, Wang Z, Waern K, et al. The transcriptional landscape of the yeast genome defined by RNA sequencing. Science 2008; 320:1344–1349
7. Westermann AJ, Förstner KU, Amman F, et al. Dual RNA-seq unveils noncoding RNA functions in host-pathogen interactions. Nature 2016; 529:496–501
8. Camilios-Neto D, Bonato P, Wassem R, et al. Dual RNA-seq transcriptional analysis of wheat roots colonized by *Azospirillum brasilense* reveals up-regulation of nutrient acquisition and cell cycle genes. BMC Genomics 2014; 15:378
9. Rienksma RA, Suarez-Diez M, Mollenkopf HJ. Comprehensive insights into transcriptional adaptation of intracellular *Mycobacteria* by microbe-enriched dual RNA sequencing. BMC Genomics 2015; 16:34
10. Baddal B, Muzzi A, Censini S, et al. Dual RNA-seq of nontypeable *Haemophilus influenzae* and host cell transcriptomes reveals novel insights into host-pathogen cross talk. mBio 2015; 6:e01765–15–e01765–15
11. Avican K, Fahlgren A, Huss M, et al. Reprogramming of *Yersinia* from virulent to persistent mode revealed by complex in vivo RNA-seq analysis. PLoS Pathog. 2015; 11:e1004600–28
12. Brogaard L, Klitgaard K, Heegaard PM, et al. Concurrent host-pathogen gene expression in the lungs of pigs challenged with *Actinobacillus pleuropneumoniae*. BMC Genomics 2015; 16:417

13. Richter A, Schwager C, Hentze S, et al. Comparison of fluorescent tag DNA labeling methods used for expression analysis by DNA microarrays. BioTechniques 2002; 33:620–630

14. Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet 2009; 10:57–63

15. Shendure J. The beginning of the end for microarrays? Nat. Methods 2008; 5:585–587

16. Bertone P, Stolc V, Royce TE, et al. Global identification of human transcribed sequences with genome tiling arrays. Science 2004; 306:2242–2246

17. Kawahara Y, Oono Y, Kanamori H, et al. Simultaneous RNA-seq analysis of a mixed transcriptome of rice and blast fungus interaction. PLoS ONE 2012; 7:e49423

18. Fu X, Fu N, Guo S, et al. Estimating accuracy of RNA-Seq and microarrays with proteomics. BMC Genomics 2009; 10:161

19. Marioni JC, Mason CE, Mane SM, et al. RNA-seq: an assessment of technical reproducibility and comparison with gene expression arrays. Genome Res 2008; 18:1509–1517

20. Mortazavi A, Williams BA, McCue K, et al. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nat. Methods 2008; 5:621–628

21. Tsuchihara K, Suzuki Y, Wakaguri H, et al. Massive transcriptional start site analysis of human genes in hypoxia cells. Nucleic Acids Res 2009; 37:2249–2263

22. Choi Y-J, Aliota MT, Mayhew GF, et al. Dual RNA-seq of parasite and host reveals gene expression dynamics during filarial worm-mosquito interactions. PLoS Negl Trop Dis 2014; 8:e2905

23. Giannoukos G, Ciulla DM, Huang K, et al. Efficient and robust RNA-seq process for cultured bacteria and complex community transcriptomes. Genome Biol 2012; 13:R23

24. Vogel C, Abreu R de S, Ko D, et al. Sequence signatures and mRNA concentration can explain two-thirds of protein abundance variation in a human cell line. Mol. Syst. Biol. 2010; 6:400

25. Wolf J. Principles of transcriptome analysis and gene expression quantification: an RNA-seq tutorial. Molecular Ecology Resources 2013; 13:559–572

26. Oshlack A, Wakefield MJ. Transcript length bias in RNA-seq data confounds systems biology. Biol Direct 2009; 4:14

27. Hecker M, Lambeck S, Toepfer S, et al. Gene regulatory network inference: data integration in dynamic models-a review. BioSystems 2009; 96:86–103

28. Oshlack A, Robinson MD, Young MD. From RNA-seq reads to differential expression results. Genome Biol 2010; 11:220

29. Thingholm LB, Andersen L, Makalic E, et al. Strategies for integrated analysis of genetic, epigenetic, and gene expression variation in cancer: addressing the challenges. Front Genet 2016; 7:2

30. Schulze S, Henkel SG, Driesch D, et al. Computational prediction of molecular pathogen-host interactions based on dual transcriptome data. Front Microbiol 2015;

31. Haas BJ, Chin M, Nusbaum C, et al. How deep is deep enough for RNA-Seq profiling of bacterial transcriptomes? BMC Genomics 2012; 13:734

32. Tarazona S, García-Alcalde F, Dopazo J, et al. Differential expression in RNA-seq: a matter of depth. Genome Res 2011; 21:2213–2223

33. Jiang L, Schlesinger F, Davis CA, et al. Synthetic spike-in standards for RNA-seq experiments. Genome Res 2011; 21:1543–1551

34. Parekh S, Ziegenhain C, Vieth B, et al. The impact of amplification on differential expression analyses by RNA-seq. Sci Rep 2016; 6:25533

35. Toung JM, Morley M, Li M, et al. RNA-sequence analysis of human B-cells. Genome Res 2011; 21:991–998

36. Korpelainen E, Tuimala J, Somervuo P, et al. RNA-seq Data Analysis: A Practical Approach. CRC Press. 2014.

37. Garber M, Grabherr MG, Guttman M, et al. Computational methods for transcriptome annotation and quantification using RNA-seq. Nat. Methods 2011; 8:469–477

38. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 2014; 30:2114–2120

39. Schmieder R, Edwards R. Quality control and preprocessing of metagenomic datasets. Bioinformatics 2011; 27:863–864

40. Kim D, Ben Langmead, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. Nat. Methods 2015; 12:357–360

41. Langmead B, Trapnell C, Pop M, et al. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009; 10:R25

42. Pireddu L, Leo S, Zanetti G. SEAL: a distributed short read mapping and duplicate removal

tool. Bioinformatics 2011; 27:2159–2160

43. Li R, Yu C, Li Y, et al. SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics 2009; 25:1966–1967

44. Wang K, Singh D, Zeng Z, et al. MapSplice: accurate mapping of RNA-seq reads for splice junction discovery. Nucleic Acids Res 2010; 38:e178–e178

45. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29:15–21

46. Trapnell C, Pachter L, Salzberg SL. TopHat: Discovering splice junctions with RNA-Seq. Bioinformatics 2009; 25:1105–1111

47. Baruzzo G, Hayer KE, Kim EJ, et al. Simulation-based comprehensive benchmarking of RNA-seq aligners. Nat. Methods 2016;

48. Pruitt KD, Brown GR, Hiatt SM, et al. RefSeq: an update on mammalian reference sequences. Nucleic Acids Res 2014; 42:D756–63

49. Kent WJ, Sugnet CW, Furey TS, et al. The Human Genome Browser at UCSC. Genome Res 2002; 12:996–1006

50. Flicek P, Amode MR, Barrell D, et al. Ensembl 2014. Nucleic Acids Res 2014; 42:D749–55

51. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics 2009; 25:2078–2079

52. Dobin A, Davis CA, Schlesinger F, et al. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 2013; 29:15–21

53. Robinson JT, Thorvaldsdóttir H, Winckler W, et al. Integrative genomics viewer. Nat Biotechnol 2011; 29:24–26

54. Skinner ME, Uzilov AV, Stein LD, et al. JBrowse: a next-generation genome browser. Genome Res 2009; 19:1630–1638

55. Griffith M, Walker JR, Spies NC, et al. Informatics for RNA sequencing: a web resource for analysis on the cloud. PLoS Comput Biol 2015; 11:e1004393

56. Anders S, Pyl PT, Huber W. HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics 2015; 31:166–169

57. Risso D, Schwartz K, Sherlock G, et al. GC-content normalization for RNA-Seq data. BMC Bioinformatics 2011; 12:480

58. Hansen KD, Irizarry RA, Wu Z. Removing technical variability in RNA-seq data using conditional quantile normalization. Biostatistics 2012; 13:204–216

59. Bullard JH, Purdom E, Hansen KD, et al. Evaluation of statistical methods for normalization and differential expression in mRNA-Seq experiments. BMC Bioinformatics 2010; 11:94

60. Wagner GP, Kin K, Lynch VJ. Measurement of mRNA abundance using RNA-seq data: RPKM measure is inconsistent among samples. Theory Biosci. 2012; 131:281–285

61. Robinson MD, Oshlack A. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol 2010; 11:R25

62. Hardcastle TJ, Kelly KA. baySeq: empirical Bayesian methods for identifying differential expression in sequence count data. BMC Bioinformatics 2010; 11:422

63. Trapnell C, Hendrickson DG, Sauvageau M, et al. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nat Biotechnol 2012; 31:46–53

64. Anders S, Huber W. Differential expression analysis for sequence count data. Genome Biol 2010; 11:R106

65. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 2010; 26:139–140

66. Patro R, Duggal G, Kingsford C. Accurate, fast, and model-aware transcript expression quantification with Salmon. bioRxiv 2015;

67. Bray NL, Pimentel H, Melsted P, et al. Near-optimal probabilistic RNA-seq quantification. Nat Biotechnol 2016; 34:525–527

68. Trapnell C, Roberts A, Goff L, et al. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 2012; 7:562–578

69. Smyth GK. Limma: Linear Models for Microarray Data. Bioinformatics and Computational Biology Solutions Using R and Bioconductor 2005; 397–420

70. Ritchie ME, Phipson B, Wu D, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res 2015; 43:e47–e47

71. Young MD, Wakefield MJ, Smyth GK, et al. Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol 2010; 11:R14

72. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009; 4:44–57

73. Smyth GK, Ritchie M, Thorne N, et al. Limma: Linear Models for Microarray Data user's guide. 2016;

74. Risso D, Ngai J, Speed TP, et al. Normalization of RNA-seq data using factor analysis of control genes or samples. Nat Biotechnol 2014; 32:896–902

Figures

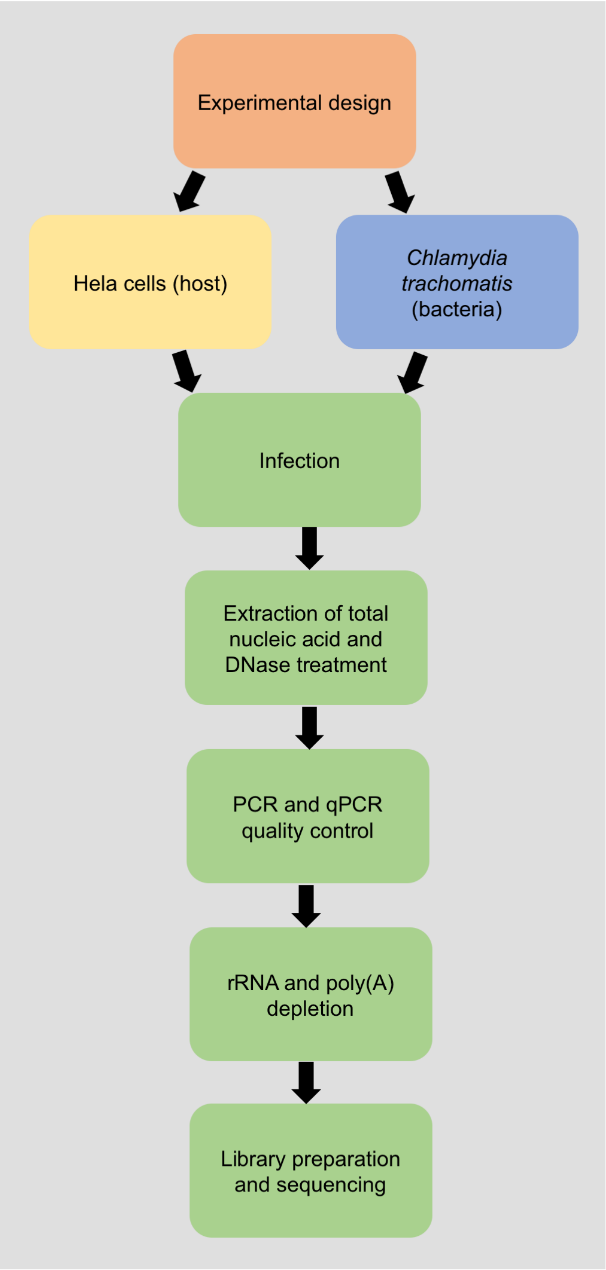

Experimental design
Hela cells (host)
Chlamydia trachomatis (bacteria)
Infection
Extraction of total nucleic acid and DNase treatment
PCR and qPCR quality control
rRNA and poly(A) depletion
Library preparation and sequencing

Figure 1. Flow-chart for the wet-lab protocol for dRNA-seq of bacteria and host.

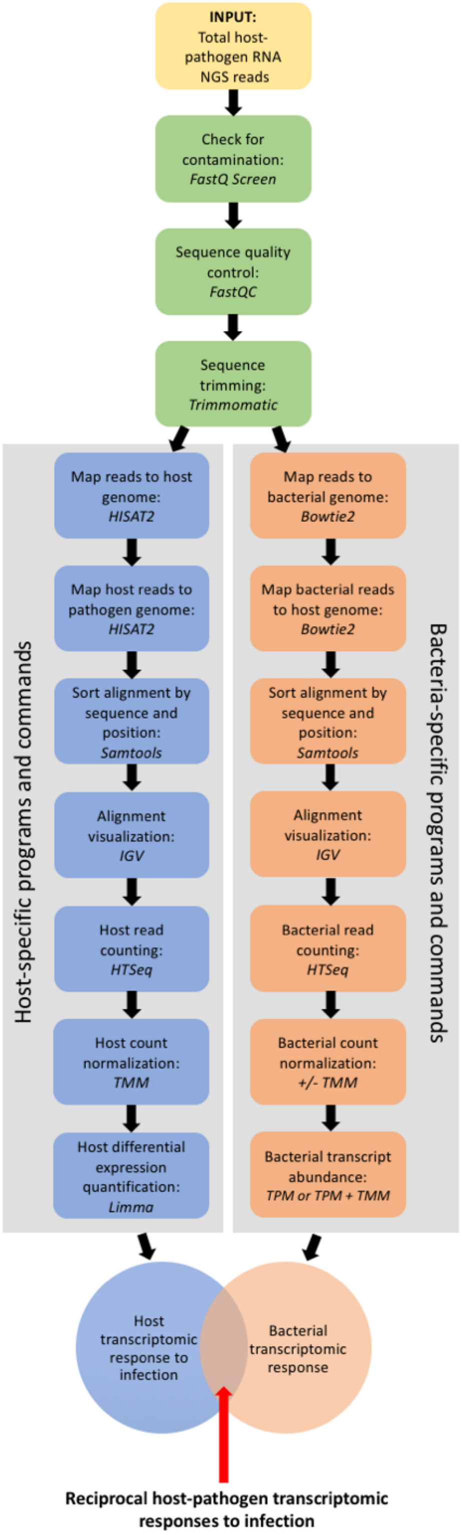
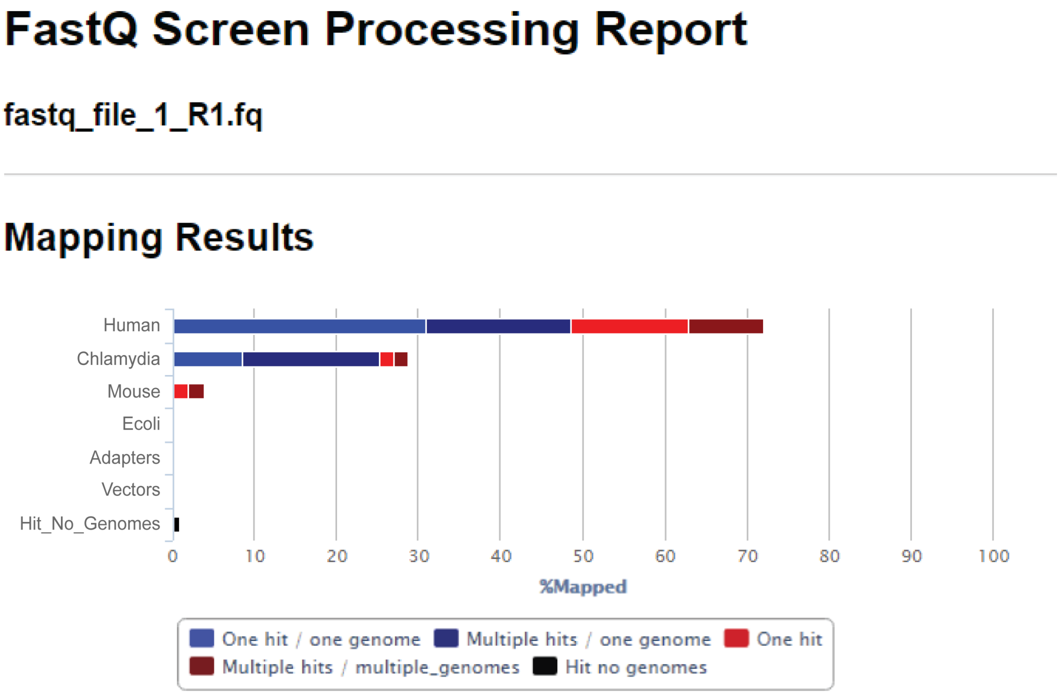

INPUT:
Total host-pathogen RNA NGS reads
Check for contamination: FastQ Screen
Sequence quality control: FastQC
Sequence trimming: Trimmomatic
Host-specific programs and commands
Map reads to host genome: HISAT2
Map host reads to pathogen genome: HISAT2
Sort alignment by sequence and position: Samtools
Alignment visualization: IGV
Host read counting: HTSeq
Host count normalization: TMM
Host differential expression quantification: Limma
Bacteria-specific programs and commands
Map reads to bacterial genome: Bowtie2
Map bacterial reads to host genome: Bowtie2
Sort alignment by sequence and position: Samtools
Alignment visualization: IGV
Bacterial read counting: HTSeq
Bacterial count normalization: +/- TMM
Bacterial transcript abundance: TPM or TPM + TMM
Host transcriptomic response to infection
Bacterial transcriptomic response
Reciprocal host-pathogen transcriptomic responses to infection

Figure 2. Flow-chart for the bioinformatic data analysis of dRNA-Seq of host and bacteria.



FastQ Screen Processing Report

fastq_file_1_R1.fq

Mapping Results

Figure 3. FastQ-Screen processing report of raw host and bacteria FASTQ sequencing reads. As expected, the majority of reads map to the human genome (70%), while 30% of reads map to the *Chlamydia* genome.

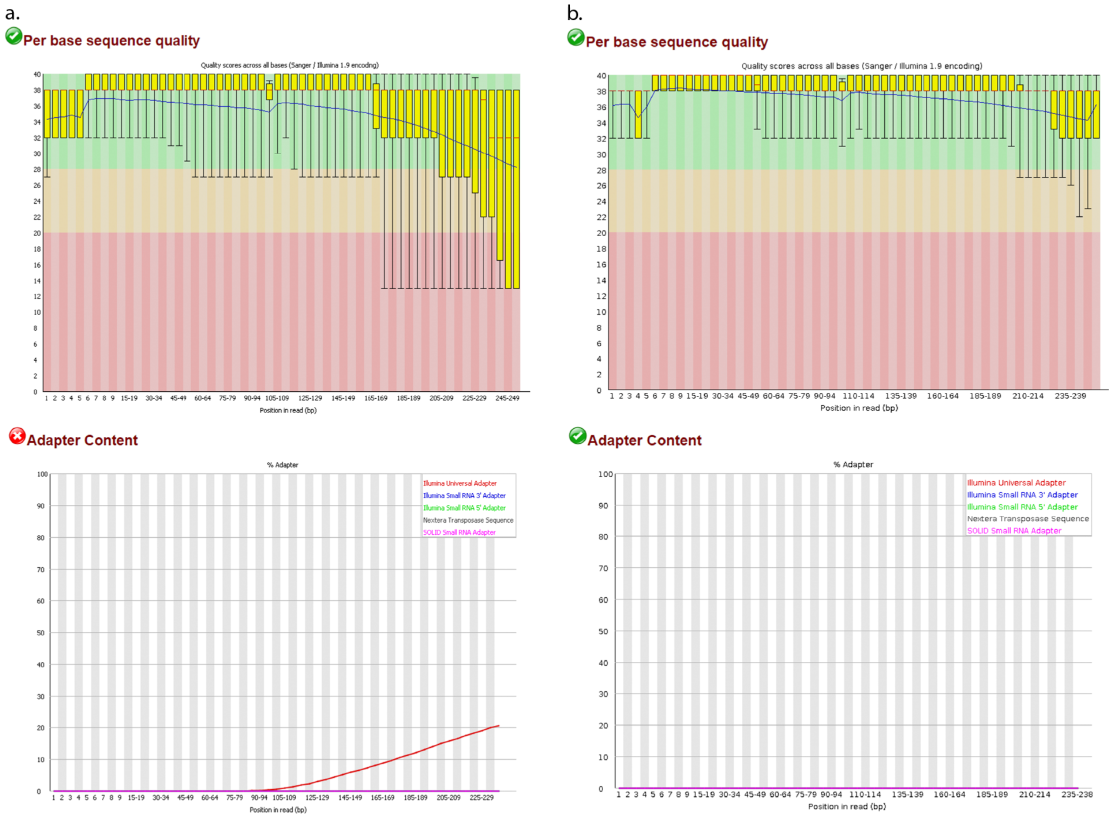


Figure 4. FASTQC report for per base sequence quality and adapter content. **a.** Sequence quality before removal of adapters with TRIMMOMATIC; **b.** Sequence quality after removal of adapters with TRIMMOMATIC.

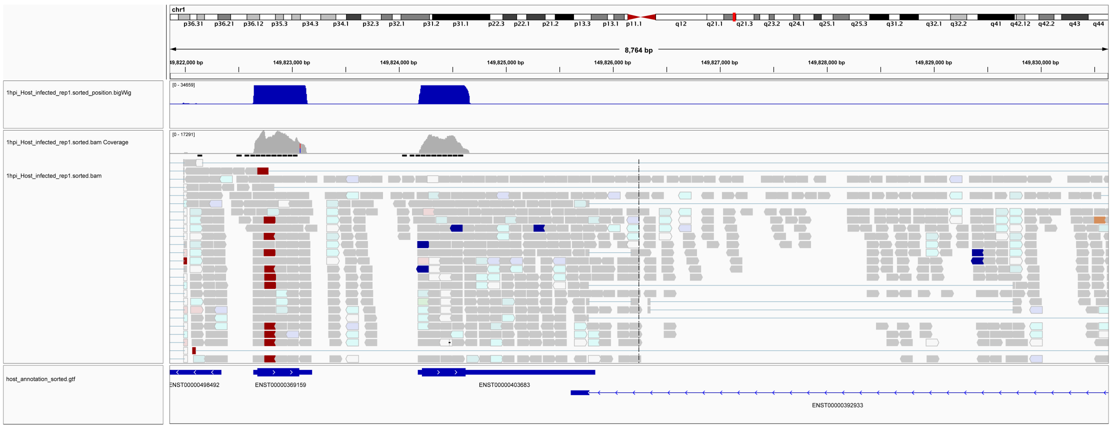
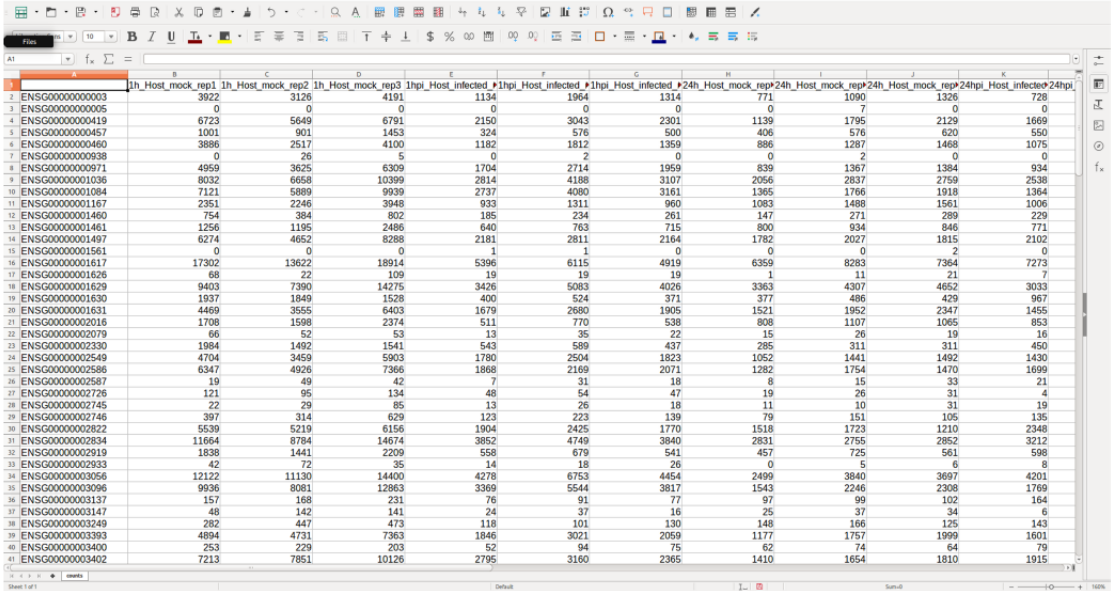

chr1
p36.31 p36.21 p36.12 p35.3 p34.3 p34.1 p32.3 p32.1 p31.2 p31.1 p22.3 p22.1 p21.2 p13.3 p13.1 p11.1 q12 q21.1 q21.3 q23.2 q24.1 q25.1 q25.3 q31.2 q32.1 q32.2 q41 q42.12 q42.2 q43 q44
8,764 bp
49,822,000 bp
149,823,000 bp
149,824,000 bp
149,825,000 bp
149,826,000 bp
149,827,000 bp
149,828,000 bp
149,829,000 bp
149,830,000 bp
1hpi_Host_infected_rep1.sorted_position.bigWig
[0 - 34659]
1hpi_Host_infected_rep1.sorted.bam Coverage
[0 - 17291]
1hpi_Host_infected_rep1.sorted.bam
host_annotation_sorted.gtf
ENST00000498492
ENST00000369159
ENST00000403683
ENST00000392933

Figure 5. Screen shot of Integrated Genome Viewer (IGV) showing host mapped reads the associated gtf annotation file. The first bar labelled "chr1" indicates which portion of the human genome (or chromosome) is displayed, with the length (8,764 bp) and specific genomic region shown underneath. The graphs shown in blue and gray indicate read coverage and the sequence alignment tracks are shown below this. The bottom row is the gtf annotation file indicating which annotated transcripts the reads are aligning to.



	A	B	C	D	E	F	G	H	I	J	K	L
1		1h_Host_mock_rep1	1h_Host_mock_rep2	1h_Host_mock_rep3	1hpi_Host_infected_	1hpi_Host_infected_	1hpi_Host_infected_	24h_Host_mock_rep	24h_Host_mock_rep	24h_Host_mock_rep	24hpi_Host_infecte	24hpi_
2	ENSG00000000003	3922	3126	4191	1134	1964	1314	771	1090	1326	728	
3	ENSG00000000005	0	0	0	0	0	0	0	7	0	0	
4	ENSG00000000419	6723	5649	6791	2150	3043	2301	1139	1795	2129	1669	
5	ENSG00000000457	1001	901	1453	324	576	500	406	576	620	550	
6	ENSG00000000460	3886	2517	4100	1182	1812	1359	886	1287	1468	1075	
7	ENSG00000000938	0	26	5	0	2	0	0	2	0	0	
8	ENSG00000000971	4959	3625	6309	1704	2714	1959	839	1367	1384	934	
9	ENSG00000001036	8032	6658	10399	2814	4188	3107	2056	2837	2759	2538	
10	ENSG00000001084	7121	5889	9939	2737	4080	3161	1365	1766	1918	1364	
11	ENSG00000001167	2351	2246	3948	933	1311	960	1083	1488	1561	1006	
12	ENSG00000001460	754	384	802	185	234	261	147	271	289	229	
13	ENSG00000001461	1256	1195	2486	640	763	715	800	934	846	771	
14	ENSG00000001497	6274	4652	8288	2181	2811	2164	1782	2027	1815	2102	
15	ENSG00000001561	0	0	0	1	1	0	0	0	2	0	
16	ENSG00000001617	17302	13622	18914	5396	6115	4919	6359	8283	7364	7273	
17	ENSG00000001626	68	22	109	19	19	19	1	11	21	7	
18	ENSG00000001629	9403	7390	14275	3426	5083	4026	3363	4307	4652	3033	
19	ENSG00000001630	1937	1849	1528	400	524	371	377	486	429	967	
20	ENSG00000001631	4469	3555	6403	1679	2680	1905	1521	1952	2347	1455	
21	ENSG00000002016	1708	1598	2374	511	770	538	808	1107	1065	853	
22	ENSG00000002079	66	52	53	13	35	22	15	26	19	16	
23	ENSG00000002330	1984	1492	1541	543	589	437	285	311	311	450	
24	ENSG00000002549	4704	3459	5903	1780	2504	1823	1052	1441	1492	1430	
25	ENSG00000002586	6347	4926	7366	1868	2169	2071	1282	1754	1470	1699	
26	ENSG00000002587	19	49	42	7	31	18	8	15	33	21	
27	ENSG00000002726	121	95	134	48	54	47	19	26	31	4	
28	ENSG00000002745	22	29	85	13	26	18	11	10	31	19	
29	ENSG00000002746	397	314	629	123	223	139	79	151	105	135	
30	ENSG00000002822	5539	5219	6156	1904	2425	1770	1518	1723	1210	2348	
31	ENSG00000002834	11664	8784	14674	3852	4749	3840	2831	2755	2852	3212	
32	ENSG00000002919	1838	1441	2209	558	679	541	457	725	561	598	
33	ENSG00000002933	42	72	35	14	18	26	0	5	6	8	
34	ENSG00000003056	12122	11130	14400	4278	6753	4454	2499	3840	3697	4201	
35	ENSG00000003096	9936	8081	12863	3369	5544	3817	1543	2246	2308	1769	
36	ENSG00000003137	157	168	231	76	91	77	97	99	102	164	
37	ENSG00000003147	48	142	141	24	37	16	25	37	34	6	
38	ENSG00000003249	282	447	473	118	101	130	148	166	125	143	
39	ENSG00000003393	4894	4731	7363	1846	3021	2059	1177	1757	1999	1601	
40	ENSG00000003400	253	229	203	52	94	75	62	74	64	79	
41	ENSG00000003402	7213	7851	10126	2795	3160	2365	1410	1654	1810	1915	

Figure 6. The count matrix. Following read quantification with HTSeq the count files are combined to form the matrix of raw counts for each sample and replicate in the dataset.

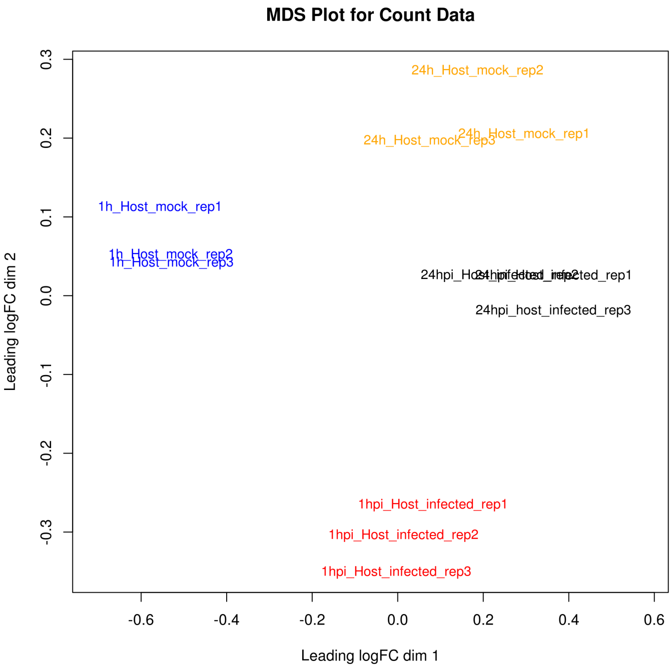


Figure 7. Multi-dimension Scaling Plot (MDS). This is a two-dimensional plot that visualizes the similarity between samples and replicates across conditions. It enables the identification of problematic samples that may obscure the subsequent statistical analysis. In this case, all replicates cluster together as expected.

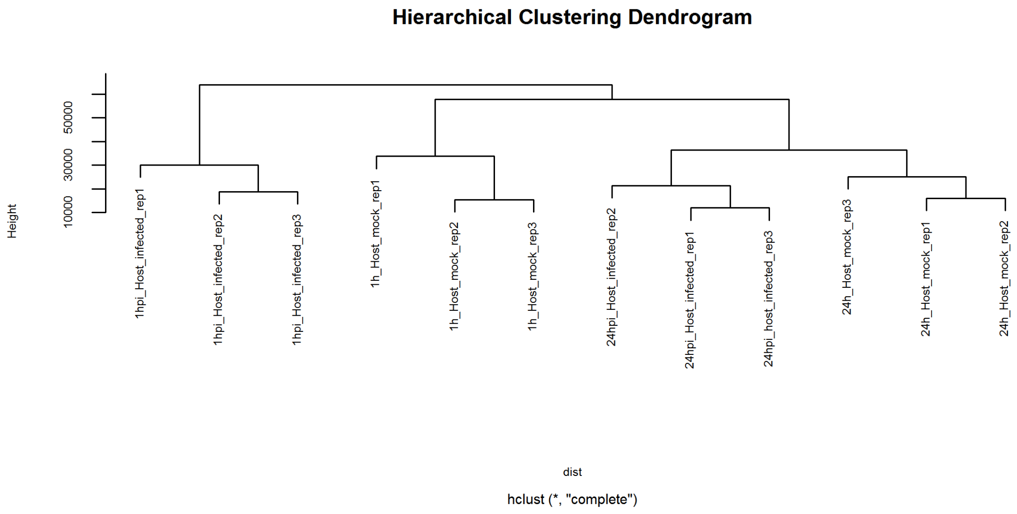


Figure 8. Hierarchical clustering dendrogram. An extension of the MDS plot, the Hierarchical clustering dendrogram illustrates sample similarity. As expected, all replicates for each condition cluster together.

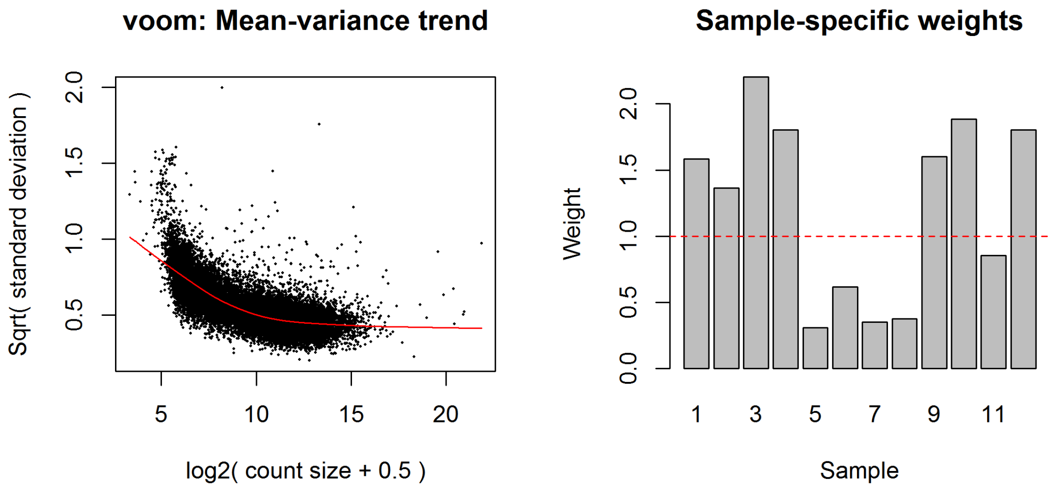


Figure 9. Limma voom plots. The mean variance trend plot displays the gene-wise square-root residual standard deviations plotted against average log-count, with the LOWESS fit represented by the red line. The sample-specific weights are the result of the "voomWithQualityWeights" function and represents the sample-specific quality weights that can be applied to down-weight outlier samples.

Table 1. Statistical output of the differential expression analysis of host reads in R. The first column contains the ENSEMBL ID for the genes, logFC indicates the log fold-change observed, AveExpr is the expression value for each gene, t is the moderated t-statistic, P.Value is the raw *p*-value, adj.P.Val is the false discovery rate-adjusted *p*-value, and B is the log odds that the gene is differentially expressed.

```
                     logFC    AveExpr         t      P.Value    adj.P.Val        B
ENSG00000003096  0.6525066  13.791554  28.40683 1.447731e-10 4.921022e-09 14.94091
ENSG00000005483  0.6818259  14.119036  28.15577 1.574679e-10 4.921022e-09 14.85045
ENSG00000003436  0.6141746  15.274943  28.06727 1.622315e-10 4.921022e-09 14.60957
ENSG00000004766  0.5506978  13.234187  27.08358 2.273831e-10 5.172965e-09 14.52388
ENSG00000003147  4.2364651   6.880526  22.53363 1.289647e-09 2.252721e-08 11.79033
ENSG00000001630 -1.8429486  11.760177 -22.19781 1.485311e-09 2.252721e-08 12.61091
```