

# 1 **Dual RNA-Seq analysis of *in vitro* infection multiplicity in *Chlamydia-*** 2 **infected epithelial cells**

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## 11 **Keywords**

12 *Chlamydia* (*Chlamydia trachomatis*), infection, dual RNA-seq, multiplicity of infection,  
13 transcriptomics  
14

## 15 **Abstract**

16 Dual RNA-seq experiments examining viral and bacterial pathogens are increasing, but vary  
17 considerably in their experimental designs, such as infection rates and RNA depletion methods.  
18 Here, we have applied dual RNA-seq to *Chlamydia trachomatis* infected epithelial cells to  
19 examine transcriptomic responses from both organisms. We compared two time points post  
20 infection (1 and 24 hours), three multiplicity of infection (MOI) ratios (0.1, 1 and 10) and two  
21 RNA depletion methods (rRNA and polyA). Capture of bacterial-specific RNA were greatest  
22 when combining rRNA and polyA depletion, and when using a higher MOI. However, under  
23 these conditions, host RNA capture was negatively impacted. Although it is tempting to use  
24 high infection rates, the implications on host cell survival, the potential reduced length of

25 infection cycles and real world applicability should be considered. This data highlights the  
26 delicate nature of balancing host-pathogen RNA capture and will assist future transcriptomic-  
27 based studies to achieve more specific and relevant infection-related biological insights.

## 28 **Introduction**

29 Dual species transcriptomic experiments (dual RNA-seq) allow multiple organisms to be  
30 simultaneously analysed from within the same sample, such as host and bacterial transcripts  
31 during an infection [1]. The number of dual RNA-seq experiments are increasing, with the  
32 underlying experimental designs and infection conditions often varying significantly,  
33 particularly in infection rates and RNA depletion methods [2-7].

34 Here, we have applied dual RNA-seq to human epithelial cells subjected to the bacteria  
35 *Chlamydia trachomatis*, which is an obligate intracellular, human-specific bacterial pathogen  
36 that causes trachoma and urogenital infections [8-10]. Ocular infections cause trachoma  
37 (infectious blindness), typically in disadvantaged communities, and is the leading cause of  
38 preventable blindness worldwide [9, 10]; while genital infections are the most prevalent  
39 sexually transmitted infection (STI) worldwide [9]. If infections are left untreated they can  
40 become problematic leading to more complex disease outcomes including ectopic pregnancy  
41 and infertility [11, 12]. Diagnosed chlamydial infections can successfully be treated with  
42 antibiotic therapy, however asymptomatic infections are common [13, 14] and thus challenging  
43 to treat. Genome-wide transcriptomic studies have explored gene expression from infected host  
44 cells and chlamydial-specific expression, either separately or simultaneously [15-23]. The  
45 previous chlamydial-based dual RNA-seq experiment encompassed an experimental design  
46 that used a multiplicity of infection (MOI) of 1, while their depletion technique removed  
47 rRNAs in all samples, followed by subjecting half of these libraries to polyA depletion to  
48 further enrich chlamydial transcripts. Although two depletion methods were used, it is

49 uncertain if this increased the abundance of chlamydial transcripts. Additionally, an MOI of 1  
50 at an early time point highlighted low capture rates of chlamydial transcripts [15].

51 In general, host-based RNA-seq experiments in an infection setting will typically try and  
52 achieve a ratio of 1 infectious entity per host cell. This ratio is referred to as the MOI, with an  
53 MOI of 1 indicating a 1:1 ratio, and is frequently used to assess baseline changes in both  
54 organisms without any directional bias. RNA-seq and microarray experiments that have  
55 focused on chlamydial infection have utilised a range of MOIs ranging from 1 [24] to 100 [16,  
56 20]; with higher ratios helping to exaggerate and highlight the chlamydial impact. However,  
57 too high an MOI and the whole monolayer of cells dies before the infection can proceed. In  
58 addition, a higher MOI will likely affect the developmental cycle due to the underlying stress  
59 this places on host cells [25, 26].

60 In this experiment, both host and chlamydial gene expression were examined applying dual-  
61 RNA-seq to *in vitro* *C. trachomatis*-infected HEp-2 epithelial cells. The first aim was to  
62 understand the influence different MOIs have on sequence capture rates, but also the  
63 transcriptional variation from *Chlamydia* and the host-cell. The second aim attempted to  
64 improve the enrichment of chlamydial reads by comparing different RNA depletion methods.  
65 To address these questions, two time points were chosen covering the chlamydial  
66 developmental cycle (1 and 24 hours), with each time point split into three MOIs (0.1, 1 and  
67 10), each in triplicate. Each of these biological replicates (16 samples) were split in half, where  
68 one library was prepared solely with rRNA depletion, while the second was prepared with  
69 rRNA depletion followed by polyA depletion (**Figure 1A**).

70

## 71 **Methods**

### 72 **Cell culture and infection**

73 Human epithelial type 2 (HEp-2) cells (American Type Culture Collection, ATCC No. CCL-  
74 23) were grown as monolayers in 6x 100 mm tissue culture dishes until cells were 90%  
75 confluent. To harvest EBs for the subsequent infections, additional monolayers were grown  
76 and infected with *C. trachomatis* serovar E in sucrose phosphate glutamate (SPG) as  
77 previously outlined [27]. The resulting EBs and cell lysates were then harvested and used to  
78 infect new HEp2 monolayers.

79 Infections for each dataset used the previously prepared HEp2 monolayers, infecting with *C.*  
80 *trachomatis* serovar E in 3.5 mL SPG buffer as previously outlined [27]; infections were  
81 synchronised using centrifugation. EBs were introduced into monolayers from three MOIs  
82 (0.1, 1 and 10) using 1:10 dilutions beginning from an MOI of 10. EBs were quantified as  
83 previously described [15]. To remove non-viable or dead EBs, each sample was incubated at  
84 25°C for 2 hours, and washed twice in SPG. Cell monolayers were incubated at 37°C with 5%  
85 CO<sub>2</sub>, including the addition of 10 mL fresh medium (DMEM+10% FBS, 25 µg/ml  
86 gentamycin, 1.25 µg/ml Fungizone). After each infection time point, the infected and  
87 uninfected dishes were harvested by scraping and resuspending in 150 µL sterile PBS. Any  
88 resuspended samples were stored at -80°C.

### 89 **Library preparation and sequencing**

90 Ribo-Zero rRNA Removal kits (Human/Mouse/Rat and Gram-negative) were used to deplete  
91 samples of both human and gram-negative bacterial rRNA. Equivalent volumes from each kit  
92 were combined, thereby allowing the removal of bacterial and human rRNA simultaneously  
93 within each sample. Each sample was equally separated, with one half subjected to polyA  
94 depletion by the Poly(A) Purist Mag purification kit (Ambion), whereby removing host-based

95 polyA transcripts to allow the enrichment of bacterial transcripts. Magnetic beads were used to  
96 bind to polyA mRNAs and were extracted from the solution with a magnet. Samples with  
97 combined depletion methods were further purified using Zymo-Spin IC columns (Zymo  
98 Research) before being re-combined for library construction.

99 The mRNA libraries were prepared from depleted samples as previously stated at 1 and 24  
100 hours post infection, using the TruSeq RNA Sample Prep kit (Illumina, San Diego, CA) per the  
101 manufacturer's protocol with IGS-specific optimisations. Adapters and indexes (6 bp) were  
102 ligated to the double-stranded cDNA, which was subsequently purified with AMPure XT beads  
103 (Beckman Coulter Genomics, Danvers, MA) between enzymatic reactions and size selection  
104 steps (~250 to 300 bp). The resulting libraries were sequenced on an Illumina HiSeq2000  
105 using the 100 bp paired-end protocol at the Genome Resource Centre, Institute for Genome  
106 Sciences, University of Maryland School of Medicine.

#### 107 **Bioinformatic analysis**

108 Sequencing reads were trimmed and quality checked using Trim Galore (0.45)  
109 ([https://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) and FastQC (0.11.5) [28].  
110 Host reads were aligned to the human genome (GRCh 38.87) using STAR (2.5.2b) [29], while  
111 chlamydial reads were aligned to the *Chlamydia trachomatis* (serovar E, Charm001) genome  
112 using Bowtie2 (2.3.2) [30] with additional parameters of '1 mismatch' and '-very-sensitive-  
113 local'. Samtools (1.6) [31] was used to remove duplicate reads in addition to only keeping  
114 mapped reads in both the host and chlamydial BAM files. To remove reads that mapped to  
115 both genomes, we first extracted the mapped reads back into paired-end fastq files using  
116 bedtools (2.26.0) [32]. Reads were then aligned using the initial mapping software to the  
117 reciprocal genomes. Any reads that mapped to both genomes were removed from the  
118 originating BAM files using the "FilterSamReads" command from Picard tools (2.10.4) [33].

119 Additional quality control metrics were examined using Bamtools (2.5.1) [34], MultiQC (1.2)  
120 [35] and various in-house scripts.

121 Features (genes) were counted using featureCounts (1.5.0-p1) [36] with additional parameters  
122 of “-Q 10 -p -C”. Genefilter (1.64.0) [37] was used to filter out genes with low counts, where  
123 host genes were retained if expression > 50 in at least three samples. To accommodate the vast  
124 differences in expression between host and chlamydial reads, a separate filter was used  
125 retaining chlamydial genes with expression > 10 in at least three samples. Chlamydial and host  
126 reads were further separated by time point due to the large amount of variability in expression  
127 between an MOI of 0.1 at 1 hour to an MOI of 10 at 24 hours. Once separated, library  
128 normalisation was performed using the trimmed mean of M-values (TMM) method [38].

129 To identify outliers, four PCA bi-plots were generated from library normalised counts using  
130 PCATools (0.99.13) [39], where eigenvalues from PC1 and PC2 for each replicate were  
131 calculated and used to highlight outlier samples if an eigenvalue was > |3| standard deviations  
132 from the mean within that group. If an outlier was removed, eigenvalues were recalculated and  
133 the process repeated until no further outliers were detected. To determine the underlying  
134 variation at each principal component, the “plotloadings” function within PCATools [39] was  
135 used.

136 Differential expression was performed with edgeR (3.24.3) [40], adding the difference between  
137 the depletion methods as a blocking factor, whereby allowing MOI and time point comparisons  
138 to utilise all six replicates to increase significance. Host DE genes were uploaded and enriched  
139 for KEGG pathways using the Enrichr database [41]. Relevant host pathways were determined  
140 using the combined score with a cutoff of > 50. Combined scores were calculated by adding  
141 together the combined scores comparing MOIs 0.1 vs 1, and 1 vs 10. Enrichment of chlamydial  
142 DE genes was performed using the ‘UniProt Keywords’ feature of STRING (11.0) [42].

## 143 **Data availability**

144 The data set supporting the results of this article is available in the GEO repository,  
145 GSE150039.

146

## 147 **Results**

### 148 **Quantifying expression differences between host and chlamydial reads**

149 Dual RNA-seq was applied to *C. trachomatis* serovar E-infected human HEp-2 epithelial cells  
150 in triplicate at 1 and 24 hours post-infection (hpi). Within each time point, three MOIs were  
151 used (0.1, 1 and 10), in addition to two depletion methods 1) rRNA depletion, 2) rRNA  
152 depletion and polyA depletion; totalling 36 samples across the experimental design.

153 Capture rates of chlamydial reads at early time points is challenging due to limited biological  
154 activity, where the majority of transcripts in a sample (>99%) will be associated with the host  
155 [15]. We increased the sequencing depth at 1 hour (> 6 fold) to try and capture more  
156 chlamydial reads; generating 391,847,337 mapped reads at 1 hour compared to 63,710,236  
157 mapped reads at 24 hours. Even with this greater depth of sequencing at 1 hour, the number of  
158 chlamydial reads was still quite low; especially at an MOI of 0.1 with an average of 1,407  
159 reads across the six replicates. However, as the MOI increases, we do see an increase in  
160 chlamydial transcripts, with average mapped reads of 10,392 (MOI 1), and 55,426 (MOI 10)  
161 (**Figure 2A**). At 24 hours we see an expected increase in the number of chlamydial reads,  
162 following a similar trend with 1 hour, where the number of mapped reads increases as the MOI  
163 increases (**Figure 2B**). The number of mapped host reads tends to vary more than the  
164 chlamydial reads, particularly between depletion methods of the same MOI (**Figure 2C** and  
165 **D**). This is likely due to the increased variety of host transcripts resulting from post-

166 transcriptional modifications, such as polyadenylation, which does not occur in bacterial  
167 systems.

168 When examining the proportions of host and chlamydial reads together across the experimental  
169 design, we see that 1 hour is dominated by host reads, while at 24 hours we see a gradual  
170 increase of chlamydial reads as the MOI increases. Surprisingly, at 24 hours with an MOI of  
171 10, the proportion of chlamydial reads across all replicates is over 60% (**Figure 1B**).

### 172 **Combining depletion methods increases yield of bacterial transcripts**

173 By combining two depletion methods (rRNA depletion and polyA depletion), we had  
174 anticipated capturing additional chlamydial reads. The addition of polyA depletion should  
175 theoretically remove any polyadenylated host transcripts, thereby increasing the number of  
176 chlamydial transcripts to be captured and sequenced.

177 Overall, we see an increase in chlamydial reads when combining depletion methods. Even at 1  
178 hour when there are limited transcripts circulating within the cell, we still see an average  
179 increase of 2.0x. At 24 hours when more chlamydial transcripts are being expressed, we see an  
180 average increase of 1.5x more reads. Interestingly, at 24 hours as the MOI increases, the  
181 capture efficiency begins to decline slightly from 1.7x to 1.2x (**Figure 1C**).

### 182 **Differences in chlamydial expression between depletion methods**

183 PCA bi-plots were created to compare the expression profiles across replicates from both  
184 depletion methods. At 1 hour, we see minimal separation at an MOI of 1 and 10 compared to  
185 0.1 where replicates appear separated and not grouped by depletion method as expected  
186 (**Figure 3A**). However, none of the replicates were considered outliers using a robust statistical  
187 approach as outlined in the methods. We therefore attribute this variability to the low number  
188 of chlamydial reads present at an MOI of 0.1 as identified earlier. At 24 hours a distinct  
189 separation between depletion methods within each MOI can be easily visualised (**Figure 3B**).



190 To understand if the variability between depletion methods is driven by a small subset of  
191 highly expressed genes, or an assortment of genes, we extracted the top 5% of genes driving  
192 the underlying variation at PC1 and PC2 for each MOI (**Figure 3C-D**). At both time points we  
193 see subsets of genes specific to each MOI, indicating that each MOI exhibits a slightly different  
194 chlamydial response. In addition, overlapping genes highlight that the variation between  
195 depletion methods was also captured and overlaps considerably. Therefore, the inclusion of  
196 polyA depletion increases bacterial reads and does not seem to be driven by small subsets of  
197 highly expressed transcripts, but allows for a wide array of transcripts to be captured.

### 198 **The removal of polyA transcripts increases non-protein coding host gene expression**

199 Examining PCA bi-plots for host reads show tight clustering between replicates, but also  
200 highlights the separation between depletion methods (**Figure 4A-B**). Extracting the underlying  
201 genes contributing the variation at PC1 and PC2, numerous non-coding genes were identified.  
202 To calculate the percent of protein coding versus non-protein coding expression, gene  
203 expression was averaged across replicates after separation by time point, MOI and depletion  
204 method (**Figure 4C**). Across both time points we see an average of 2.8x more non-protein  
205 coding expression when combining rRNA and polyA depletion, with the highest proportion  
206 occurring at an MOI of 0.1 (3.4x at 1 hour and 4.9x at 24 hours) (**Figure 4C**). Pie charts  
207 highlight that the majority of expression comes from protein-coding genes. However, as  
208 identified in the PCA plots earlier, non-protein coding expression contributed to the separation  
209 of depletion methods. By characterising the most common biotypes, we see mitochondrial  
210 rRNA (MT rRNA), small nucleolar RNAs (snoRNA), miscRNA and long intergenic non-  
211 coding (lincRNA); but without any visible trends separating time points, depletion methods or  
212 MOI (**Figure 4D**).

213 To identify potentially influential non-protein coding genes, we used the top 200 expressed  
214 genes from both depletion methods and extracted a subset of genes that occur frequently

215 (across 3 or more conditions) (**Figure 4E**). Of the 12 genes identified, 5 were snoRNAs which  
216 are involved with RNA modifications, and are among the most highly abundant non-coding  
217 RNAs (ncRNAs) in the nucleus [43]. The MT-RNR1 (12S RNA) and MT-RNR2 (16S RNA)  
218 genes encode the two rRNA subunits of mitochondrial ribosomes, and are generally always  
219 highly expressed within eukaryotic cells [44]. LincRNAs include CCAT1, which is linked to  
220 cell growth and regulation of EGFR [45], while MALAT1 and NEAT1 co-localise to hundreds  
221 of genomic loci, predominantly over active genes [46].

### 222 **Increasing infection highlights minimal changes to highly expressed host and chlamydial** 223 **genes**

224 To determine whether the host or chlamydial transcriptional-profile changes in relation to the  
225 ratio of EBs per cell, highly expressed genes were compared against an MOI of 1. Chlamydial  
226 transcripts were examined from the combined depletion replicates, as more transcripts were  
227 captured (**Figure 1C**), thus giving a more representative profile. Host reads were taken from  
228 just the rRNA depleted replicates, as these were shown to contain more of an accurate  
229 representation of protein coding and non-protein coding genes (**Figure 4**).

230 Each of the four panels (**Figure 5**) contains two graphs. The first graph contains the top 50  
231 expressed genes taken from an MOI of 1, while the second graph shows the ranked-positions  
232 of these top expressed genes. At 1 hour, there is slightly less chlamydial expression at an MOI  
233 of 0.1, and slightly more expression when additional EBs are introduced at an MOI of 10  
234 (**Figure 5A**). The ranking chart to the right shows that 9/10 of the top expressed genes remain  
235 the same across the three MOIs. The top 25 genes from the host's response at 1 hour share  
236 highly similar expression profiles (**Figure 5B**); with only two mitochondrial-based genes (MT-  
237 RNR1 and MT-RNR2) at an MOI of 10 standing out with lower expression. The ranking chart  
238 shows 7/10 top expressed genes remaining constant across the three MOIs, similar to the  
239 chlamydial profile. At 24 hours, similar expression profiles of the top 25 expressed chlamydial

240 genes are seen, irrespective of MOI (**Figure 5C**). Rankings are also similar, with only slight  
241 variations in the top ten genes, and 16/20 of the top expressed genes remain identical. The host  
242 expression profile at 24 hours is consistent at an MOI of 1 and 0.1, whereas the expression  
243 pattern is more widely distributed at an MOI of 10; again with MT-RNR1 and MT-RNR2  
244 exhibiting lower expression (**Figure 5D**). Although the top ranked genes exhibit more  
245 variability within their rankings compared to 1 hour, 90% of the top expressed host genes  
246 appear at both time points. Functional characterisation of the genes shows their involvement  
247 with general cell-based growth events, such as ribosomal-based processes, metabolism, and  
248 cytoskeletal components (**Supplementary File 1**). Many top ranked host genes are also non-  
249 protein coding as identified by an asterisk (\*). However, with limited annotation available,  
250 their characterisation in to infection-association functions are limited. Of the annotated non-  
251 protein coding genes, they appear to be involved with general cell regulatory processes. Only  
252 seven chlamydial genes overlap both time points, which was anticipated, as two different  
253 biological events are occurring at these times, including infection mechanisms at 1 hour, and  
254 growth-related processes at 24 hours. Characterisation of these overlapping genes identifies  
255 membrane proteins and transcription/translation machinery, which are needed throughout the  
256 developmental cycle (**Supplementary File 1**).

### 257 **Comparative analysis between MOIs show increased expression of inflammatory and** 258 **immune-based host genes**

259 To observe and compare how the host expression responded to increased infection, we  
260 examined differentially expressed (DE) genes comparing MOIs. At 1 hour, the majority of  
261 genes (87% from 0.1 to 1, and 67% from 1 to 10) exhibited an increase in regulation as the  
262 MOI increased (**Figure 6A**). By enriching DE genes which are up-regulated and overlap both  
263 comparisons, pathways that exhibit an increase in expression as the MOIs increase were  
264 identified (**Figure 6B**). The same method was applied to down-regulated genes. However, no

265 continuously down-regulated pathways were identified. The top four up-regulated pathways  
266 highlight similar host immune regulated functions that include (*TNF signalling*), (*NF- $\kappa$ B*  
267 *signalling*), (*NOD-like receptor signalling*) and (*Cytokine-cytokine receptor interaction*); with  
268 the proinflammatory cytokine TNF exhibiting almost double the combined score of the next  
269 highest. We had anticipated seeing a strong immune-based response, as these pathways are  
270 associated with primary defence mechanisms; so, they should increase as the bacterial threat  
271 increases.

272 To further examine influential genes underlying these pathways, ‘trended-genes’ were  
273 extracted. The criteria consisted of an expression profile that at least doubled (fold-change >2)  
274 for each comparison, in addition to showing a continued increase from an MOI of 0.1 to 10. In  
275 total, 46 genes were identified that trended-upwards (**Figure 6C**), no genes trended  
276 downwards. These trended-genes further highlight that the underlying host-mechanisms to  
277 increased infection at initial stages are predominately immune system associated 24/46 (52%),  
278 encompassing cytokine signalling, chemokines and interleukins.

279 The number of DE genes at 24 hours show an even distribution of fold-changes compared to 1  
280 hour, with 49% up-regulated comparing MOIs 0.1 and 1, and 50% comparing 1 and 10 (**Figure**  
281 **6D**). Enriched pathways that are continuously up-regulated include (*TNF signalling*) and (*NF-*  
282  *$\kappa$ B signalling*), which are the same top two pathways found at 1 hour, and strongly linked to  
283 inflammation [47]. We also see two enriched pathways that become down-regulated as the  
284 MOI increases: (*Carbon metabolism*) and the (*Citrate cycle (TCA cycle)*) (**Figure 6E**). This  
285 decrease in key metabolism is likely due to cells prioritising defence over growth as the  
286 infection escalates.

287 Examining trended-genes at 24 hours uncovers 1 gene exhibiting decreased expression  
288 (TXNIP), and 14 genes with increased expression (**Figure 6E**). TXNIP (Thioredoxin  
289 Interacting Protein) is a thiol-oxidoreductase involved in redox regulation which protects cells

290 against oxidative stress [48]. Chlamydial-specific studies have identified an increase in reactive  
291 oxygen species (ROS) at early time points, but expression is rapidly reduced shortly afterwards  
292 [49]. A further study has suggested that the redox state within a cell could be a regulator in  
293 *Chlamydia*-induced apoptosis [50]. However, it is difficult to know if this decreased regulation  
294 is directly linked to chlamydial infection and what advantages an oxidative cellular  
295 environment would provide at this developmental stage. Genes with increased expression fall  
296 into three main categories: cytokines and inflammation (6 genes), viral-based immune response  
297 (5 genes), and ubiquitin-related immune responses (3 genes). As anticipated and seen at 1 hour,  
298 expression of key immune related genes increases with an increased burden. Only 4 genes  
299 overlapped both time points that also increased expression across MOIs (CXCL1, CXCL2,  
300 CXCL8 and IL6), indicating their importance as immune mediators against infection.

### 301 **Comparative analysis of chlamydial expression between MOIs**

302 DE genes were also identified to explore chlamydial-based changes attributed to different  
303 MOIs. The number of DE genes at 1 hour reflected the underlying minimal expression profiles  
304 already identified (**Figure 2**), with 47 DE genes comparing MOIs 0.1 and 1, and 23 genes  
305 comparing 1 and 10 (**Figure 7A**). At 24 hours, the increase in underlying expression resulted in  
306 an increase in DE genes, with 81 comparing MOIs 0.1 and 1, while over half (56%) of the  
307 chlamydial genome (566/1008 genes) showed a significant change in regulation comparing  
308 MOIs 1 and 10 (**Figure 7B**).

309 No chlamydial genes continuously increased across MOIs at either time point. Only two genes  
310 showed a continued decreased in expression: SCLA1|TEF25 (Succinyl-CoA Synthetase) at 1  
311 hour, and CT726 (tRNA) at 24 hours. The decrease of transfer RNAs (tRNA) at 24 hours is  
312 slightly surprising, considering they are an important component of translation, and would  
313 likely be in abundance during this growth phase of the developmental cycle. Also surprising is  
314 a decrease in Succinyl-CoA synthetase, which is involved with the citric acid cycle and cellular

315 metabolism [51]. We can theorise from these genes, as more EBs are introduced, the likelihood  
316 of multiple infections within a cell is greatly increased and perhaps some inclusions are  
317 benefitting from effector proteins already circulating within the cell from existing inclusions.

318 Due to low numbers of DE genes at 3 of the 4 comparisons, enrichment was only possible  
319 comparing MOIs 1 and 10 at 24 hours (**Figure 7C**). Down-regulated functions comprise genes  
320 that show decreased expression at a higher MOI. Results also show unexpected functions such  
321 as ‘*ATP-binding*’ and ‘*Lipid biosynthesis*’, which would generally be associated with  
322 chlamydial growth. This may highlight the possibility that inclusions may benefit from effector  
323 proteins already in existence, likely reducing the need to express these genes and associated  
324 processes. Up-regulated genes cover a wider range functions, with half associated with  
325 different binding mechanisms facilitating transcription and growth (*RNA-binding*, *rRNA-*  
326 *binding*, *Metal-binding* and *Nucleotide-binding*); which is expected at this stage of the  
327 developmental cycle, especially with a ten-fold increase in EBs.

328

## 329 **Discussion**

### 330 **How much influence is associated with increasing the MOI**

331 There is a finite balance when infecting monolayers to accurately measure both host-cell and  
332 chlamydial transcriptional responses. This experiment used the universally standard MOI of 1,  
333 in addition to a ten-fold increase (MOI 10) and decrease (0.1), to directly observe what changes  
334 occur. One reason to increase the MOI is to examine early time points of infection when  
335 chlamydial transcripts are in low quantities as seen in (**Figure 2A**). In this experiment, when  
336 increasing the MOI to 10 at both time points, an increased capture rate of chlamydial  
337 transcripts was observed, confirming the suitability for early times (**Figure 2A-B**). However a  
338 challenge when working with higher MOIs is that some cells may have formed multiple

339 inclusions which may skew host-cell responses beyond what may be seen in a real-world  
340 infection setting [52]. When looking at an MOI of 10 at 24 hours, we see over 60% of total  
341 captured transcripts from *Chlamydia*. Although this may not be representative of an *in vivo*  
342 infection, it is highly useful when focusing on chlamydial-based mechanisms. However, this  
343 does raise a question regarding a theoretical maximum proportion of chlamydial reads that can  
344 exist within a host cell during the developmental cycle, particularly at the later stages of  
345 infection. This was highlighted from **(Figure 1B)** at 24 hours, where we see a single replicate  
346 showing a staggering 74% of all transcripts associated with chlamydial expression. What was  
347 difficult to determine was if the increase in EBs had a corresponding influence in reducing the  
348 length of the developmental cycle due to possible synergistic interactions and shared resources.  
349 A future dual-RNA-seq study following the time course of [26], but replacing biovars for  
350 MOIs would be intriguing.

### 351 **What is an optimal MOI**

352 We know from existing studies that different MOIs need to be used when examining different  
353 stages of the developmental cycle. For example, early time points generally require a higher  
354 MOI as limited transcription from *Chlamydia* occurs, resulting in low capture rates that can be  
355 difficult to interpret [19, 20]. During mid-stages, an MOI of 1 is often used to capture events  
356 based around growth and replication [53]. Towards the latter stages, almost all chlamydial  
357 genes are transcribed, making biological interpretations challenging [15]. Most studies  
358 examining a range of developmental stages use an MOI of 1 and this has generally been  
359 considered suitable. However, MOIs are generated from serial dilutions, so lower MOIs such  
360 as 1, may not actually have 1 EB per cell. Results from this experiment show a substantial  
361 increase in capture rates and transcription from an MOI of 1 to 10, suggesting that a slightly  
362 higher MOI may be optimal. There are however implications that need to be taken into  
363 consideration when using MOIs higher than 1. These include that EBs preferentially infect

364 cells together rather than spread out evenly, which can result in all variations of the intended  
365 MOI. For example, a starting MOI of 5 will likely see an MOI range between 0-5 across a  
366 population of cells. As a result, the overall captured signal may be difficult to interpret,  
367 particularly with large MOIs. Furthermore, the length of the developmental cycle is generally  
368 shortened when many EBs are internalised due to an increased burden on the host cell.

### 369 **Combining depletion methods increases capture rate of chlamydial transcripts**

370 By combining rRNA and polyA depletion methods, we clearly observe an increased capture  
371 rate of chlamydial transcripts. These additional transcripts do not appear to be from a small  
372 subset of genes dominating capture, but from a wide range expressed genes (**Figure 3**).  
373 However, host-based expression is affected, with expression of non-protein coding genes  
374 increasing (**Figure 4**); suggesting it may only be beneficial for future chlamydial-specific  
375 sequencing approaching to use both depletion methods.

### 376 **Experimental limitations**

377 Unfortunately, this experimental design did not include any mock infected replicates from  
378 either time point, limiting some analyses. Future experiments would benefit from their  
379 inclusion, helping to separate general cell proliferation events from infection-relevant results.

380 A further limitation was not having the ability to determine if the timeframes of the  
381 developmental cycle are affected relative to increasing the MOI. The possibility of this  
382 occurring is quite likely, particularly as more EBs are internalised, resulting in more inclusions  
383 putting an increased burden on the host cell. Perhaps a future experiment could include a  
384 fluorescent tag that could be quantified as cells become lysed, thereby providing a  
385 measurement relative to the MOI and length of the developmental cycle. Alternatively,  
386 chemical-assisted methods can arrest at different cell cycle stages, ensuring all cells are



387 synchronised at a specified cell cycle phase and removing this as a potential confounding  
388 factor.

### 389 **How dynamic is the chlamydial response**

390 Differentially expressed and trended genes (**Figure 6**) identified transcriptional responses the  
391 host cell uses during an infection, which appears to be from a similar subset of key genes at  
392 both times. Genes are associated with immune related pathways, specifically inflammation;  
393 with increased expression as the MOI increases. As the concentration of EBs increases  
394 provoking this increased immune response, host cells will likely become overwhelmed if the  
395 numbers of EBs become too high. We hypothesise this could be an advantage for *Chlamydia* if  
396 a large proportion of host cell expression is focused towards immune responses, and if they  
397 already have a way of countering these, then other host processes may be easier to interfere  
398 with and possibly hijack. We anticipate this would most likely occur at higher MOIs where we  
399 have observed the most difference, particularly at 24 hours or latter stages of the  
400 developmental cycle.

401

### 402 **Conclusion**

403 This work highlights how future bacterial-specific RNA-seq studies can increase sequence  
404 capture rates by combining rRNA and polyA depletion methods. This is particularly relevant  
405 for chlamydial-based expression studies when examining early time points, as low expression  
406 is generally observed. Three different MOIs highlighted that significantly more Chlamydial  
407 transcripts were captured at both time points when using an MOI of 10. Although useful for  
408 capturing Chlamydial-specific biology, the increased burden on host cells may not be  
409 representative of *in vivo* infections. Overall, these outcomes can help influence future NGS-

410 based experimental designs to achieve more specific infection-related biological outcomes,  
411 particularly from *Chlamydia*-infected cells.

412

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417 computing cluster at UTS, with files hosted using the SpaceShuttle facility at Intersect  
418 Australia

419

### 420 **Author Contributions**

421 RH analysed, interpreted, and co-wrote the manuscript. MH performed the chlamydial  
422 infections and RNA-seq laboratory methods. WH assisted with interpretation of the data and  
423 contributed to the manuscript. GM conceived the experiments, obtained the funding, oversaw  
424 the sequencing, data analysis and interpretation, and co-wrote the manuscript.

425

### 426 **Competing interests**

427 The authors declare that they have no competing interests

428

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## 562 **Figure Legends**

563

### **Figure 1: Experimental process and design**

**A)** The process of growing cell cultures and harvesting (elementary bodies) EBs to use for downstream experiments is a time-consuming process spanning multiple days. The resulting EBs were used for three different infection ratios (multiplicity of infection) of 0.1, 1 and 10. After infections, samples were left for 1 hour and 24 hours. Each replicate was then prepared with rRNA or rRNA plus PolyA depletion, generating 32 samples in total. **B)** Showing the percent of Human and chlamydial reads across the experimental design. **C)** By combining rRNA depletion and polyA depletion, we were able to increase the capture efficiency of chlamydial transcripts at both time points and across MOIs

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### **Figure 2: Human and chlamydial mapped reads**

The number of mapped sequence reads to both human and chlamydial genomes. Green bars represent an MOI of 0.1, orange 1, and blue 10. Light shaded colours represent the rRNA depletion method, while darker shades represent rRNA and polyA depletion methods combined. **A)** Low numbers of chlamydial mapped reads at lower MOI ratios, but a substantial increase at an MOI of 10. **B)** At 24 hours the number of captured transcripts dramatically increases from 1 hour, but follows a similar distribution with a spike of reads at an MOI of 10. **C)** The increase in mapped host reads at 1 hour was to try and capture as many chlamydial transcripts as possible, as they are known to be in very low quantities this early in the developmental cycle. **D)** As the MOI increases at 24 hours, the number of mapped host reads declines.

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**Figure 3: Chlamydial-based expression differences between depletion methods**

**A)** At 1 hour, minimal separation is seen apart from at an MOI of 0.1 where replicates appear to not group or cluster together. **B)** At 24 hours, replicates group together within the same depletion method, while separation is seen between depletion methods at each MOI. Extracting the top 5% of genes driving variation from PC1 and PC2 between depletion methods. At 1 hour **C)** and 24 hours **D)**, we see subsets of genes overlapping MOIs in addition to MOI-specific subsets.

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**Figure 4: Host-based expression differences of protein coding and non-protein coding genes between depletion methods**

**A)** PCA plots show tight grouping between replicates, but separation at each MOI and depletion method. **B)** Similar grouping trends to 1 hour, but with an MOI of 10 much further separated. **C)** An overall increase in non-protein coding expression is observed when combining depletion methods. **D)** The majority of expression for all conditions is from protein coding genes. While non-protein coding genes are dominated by four biotypes, with Mt rRNAs the most highly expressed. **E)** Non-protein coding genes that are within the top 200 expressed genes at each MOI, and overlap 3 or more conditions.

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**Figure 5: Top 25 expressed host and chlamydial genes across MOIs**

Each quadrant contains two graphs: the first showing the top 50 expressed genes across each MOI (taken from an MOI of 1), while the second ranks those same genes for direct comparison. **A)** Chlamydial expression at 1 hour shows a slight upwards trend as the MOI increases, with high similarity in the ranked order. **B)** Host expression at 1 hour shows



consistent expression across MOIs apart from two mitochondrial genes (MT-RNR1 and MT-RNR2) with low expression at an MOI of 10. Rankings of the top ten genes are also highly similar. **C)** At 24 hours, chlamydial expression seems to be less influenced by MOI. **D)** Host expression at 24 hours shows a slight increase in overall expression, while the MOI of 10 has begun to have more of a varied range of expression compared to 1 hour. Ranked genes also remain highly similar.

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**Figure 6: Comparison of differentially expressed host genes across MOIs**

**A)** An increase of up-regulated genes at both comparisons is seen at 1 hour. **B)** Extracting and enriching genes that overlap both comparisons and are also up-regulated, show pathways involved with immune and inflammatory responses. **C)** Trended genes are determined from exhibiting a fold-change  $> 2$  and following the same regulation pattern at both comparisons. At 1 hour, 46 up-regulated ‘trended-genes’ further highlight the association with the immune system with over 50% of genes grouped in to cytokine signalling, chemokines and interleukins. **D)** At 24 hours the numbers of up and down-regulated genes is much more even (49% and 50%) than 1 hour. **E)** The top two up-regulated pathways are repeated at both time points, while down-regulated pathways are associated with metabolism and likely indicate cells shifting into defence mode as infection increases. **F)** Trended-genes further highlight immune responses, in addition to viral and ubiquitin-related immune responses.

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**Figure 7: Comparison of differentially expressed chlamydial genes across MOIs**

**A)** Volcano plots after differential comparisons between MOIs at 1 hour show minimal differentially expressed (DE) genes. **B)** Differential comparisons at 24 hours show a slight increase in DE genes comparing MOIs 0.1 and 1, with a further considerable increase

comparing ratios 1 and 10. C) The increase in DE genes comparing MOIs 1 and 10 at 24 hours allowed enrichment of up and down-regulated genes.

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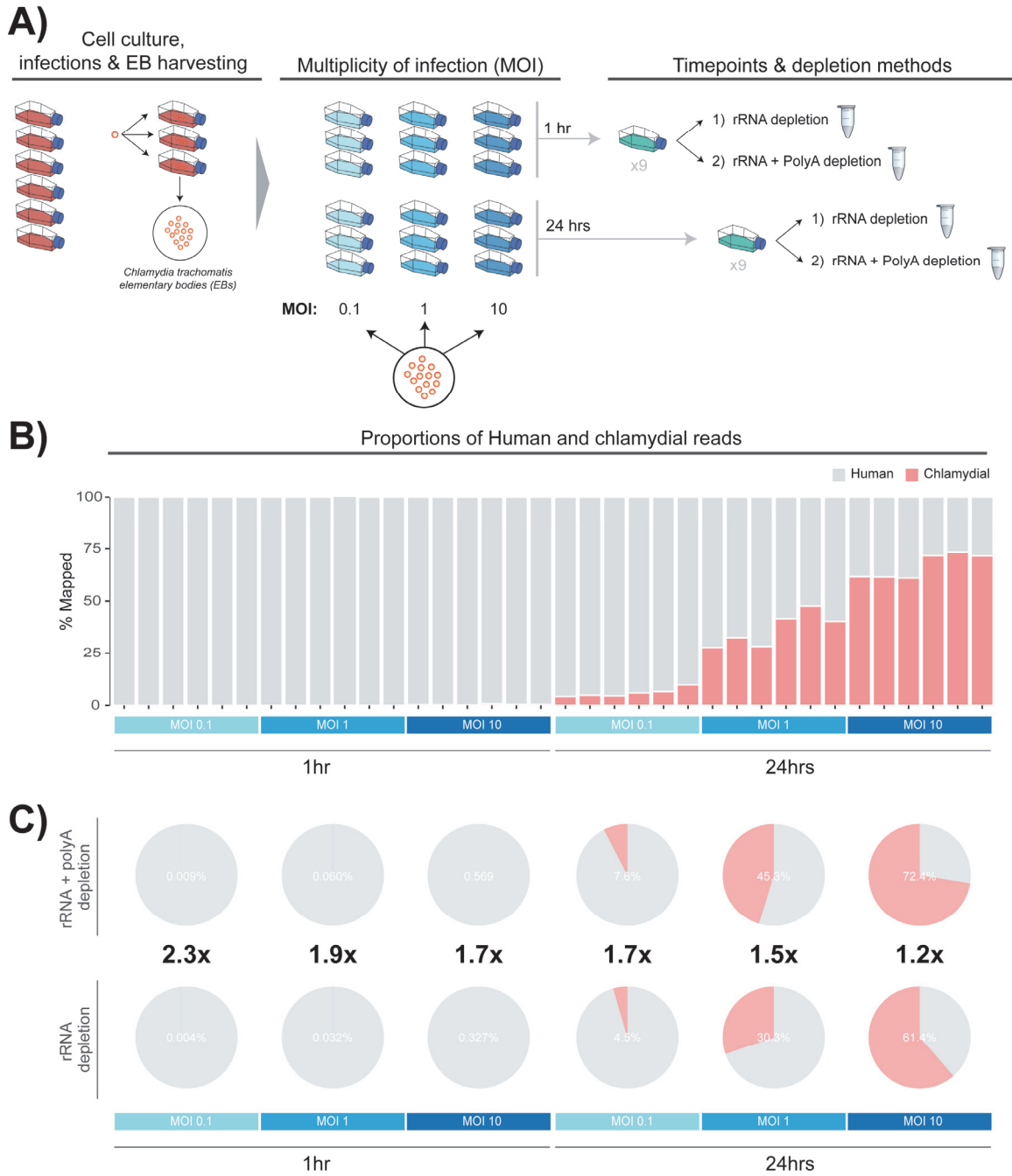
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578 **Figures**

579

580 **Figure 1**

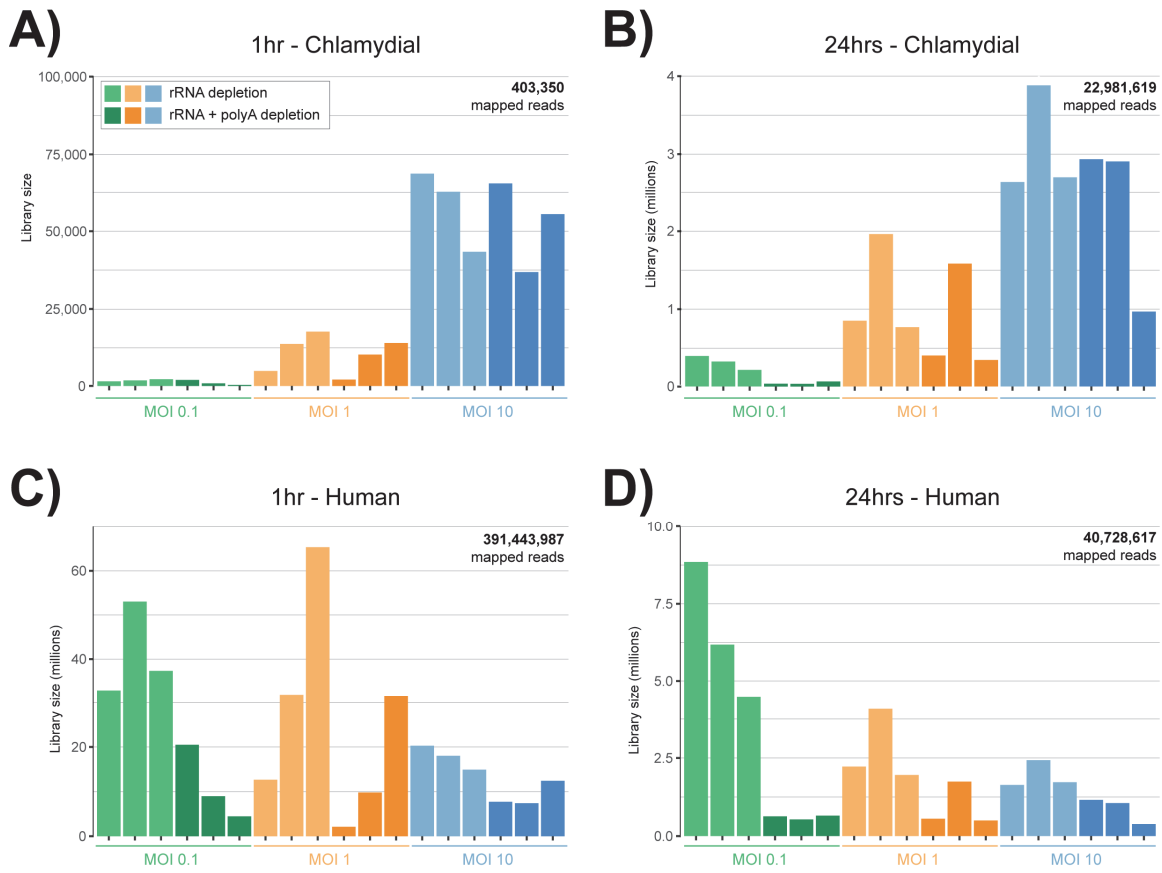
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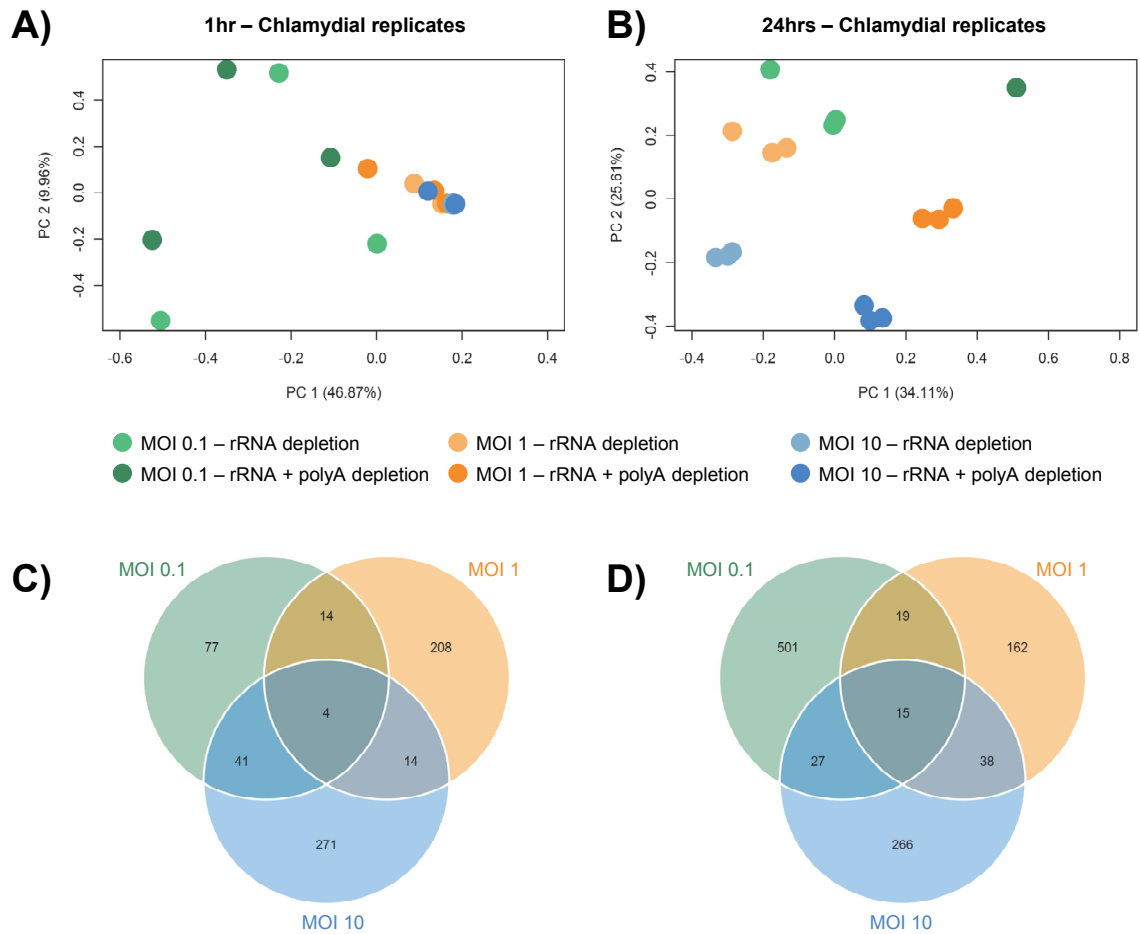
584 **Figure 2**  
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587 **Figure 3**

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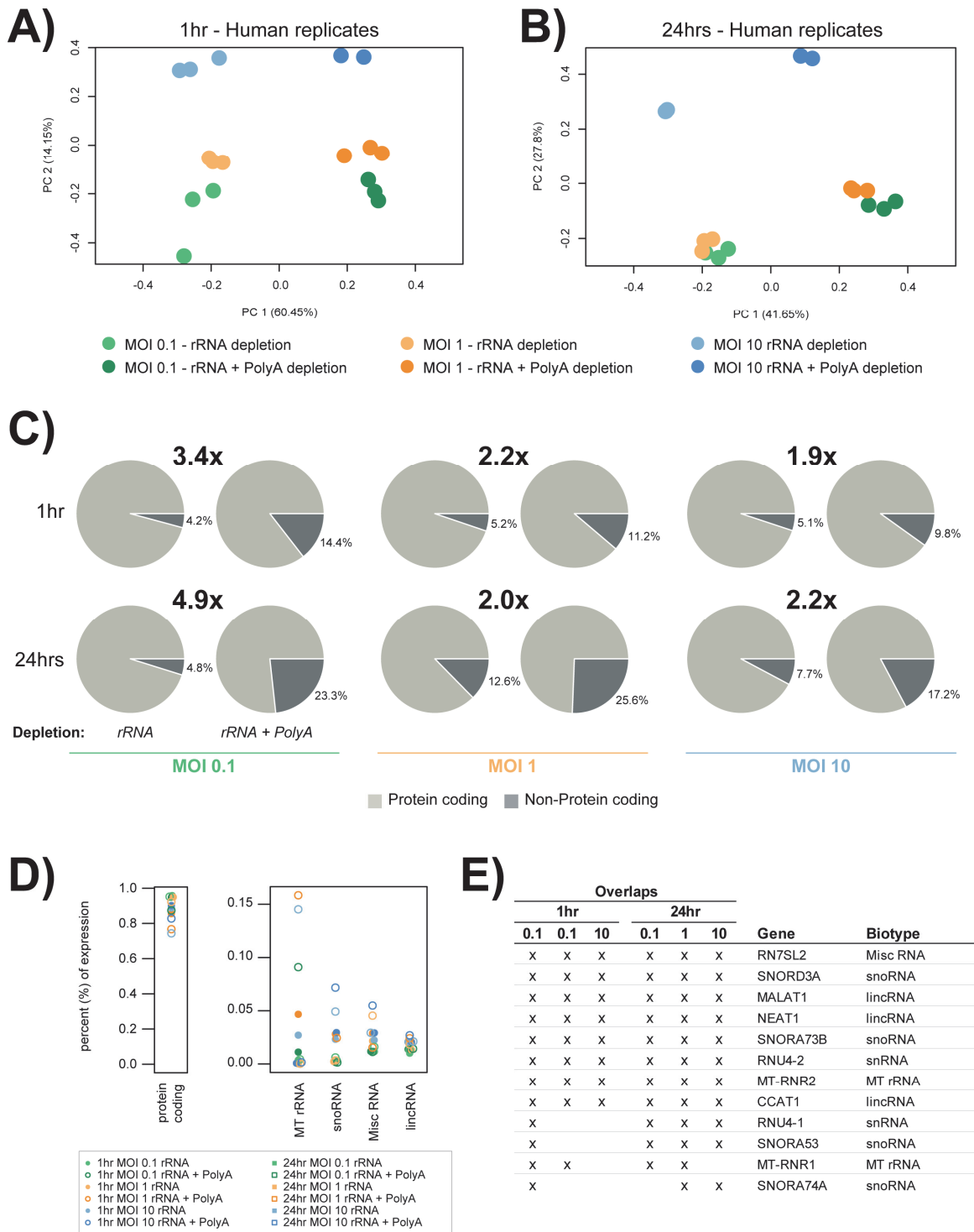


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591 **Figure 4**

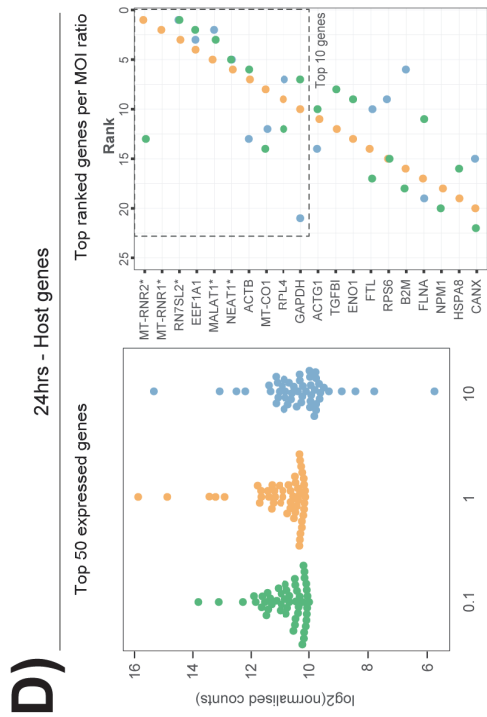
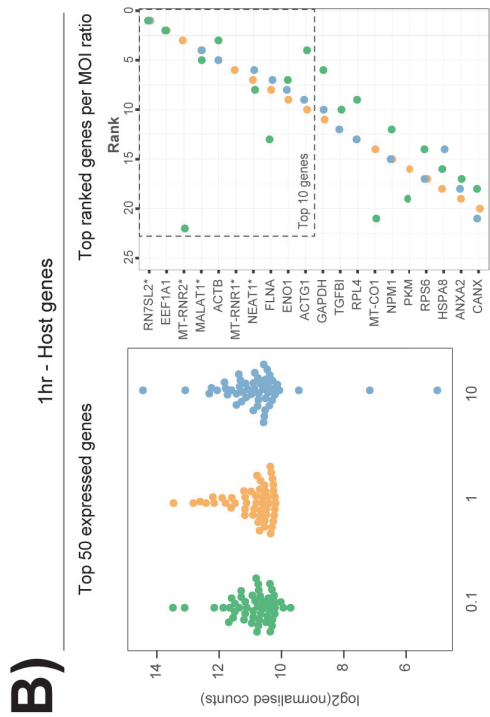
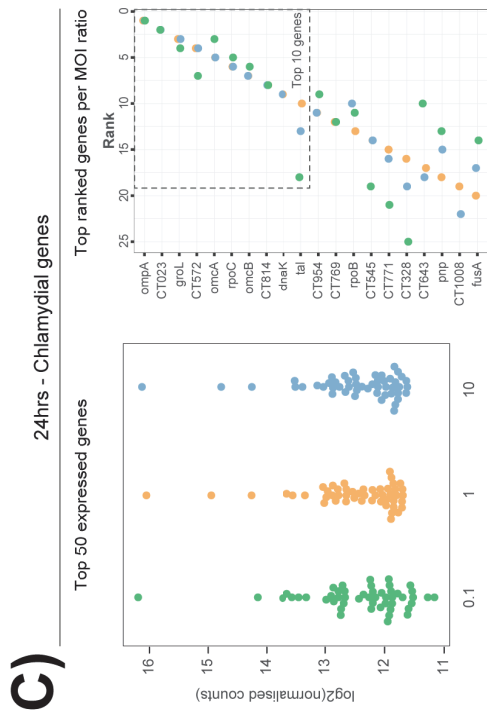
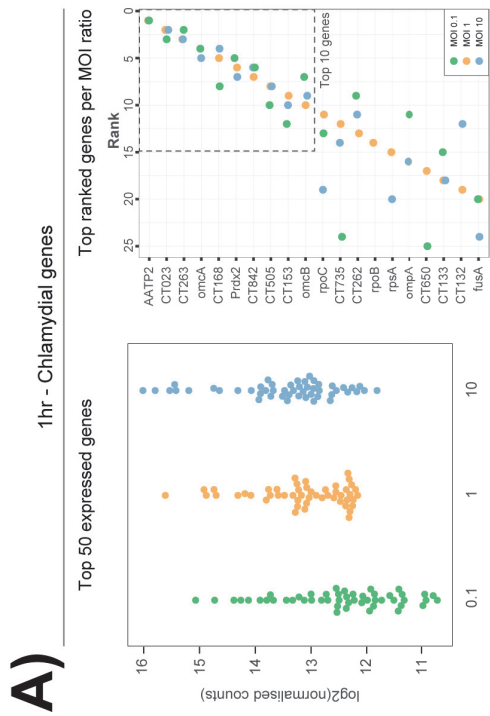
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## Figure 5

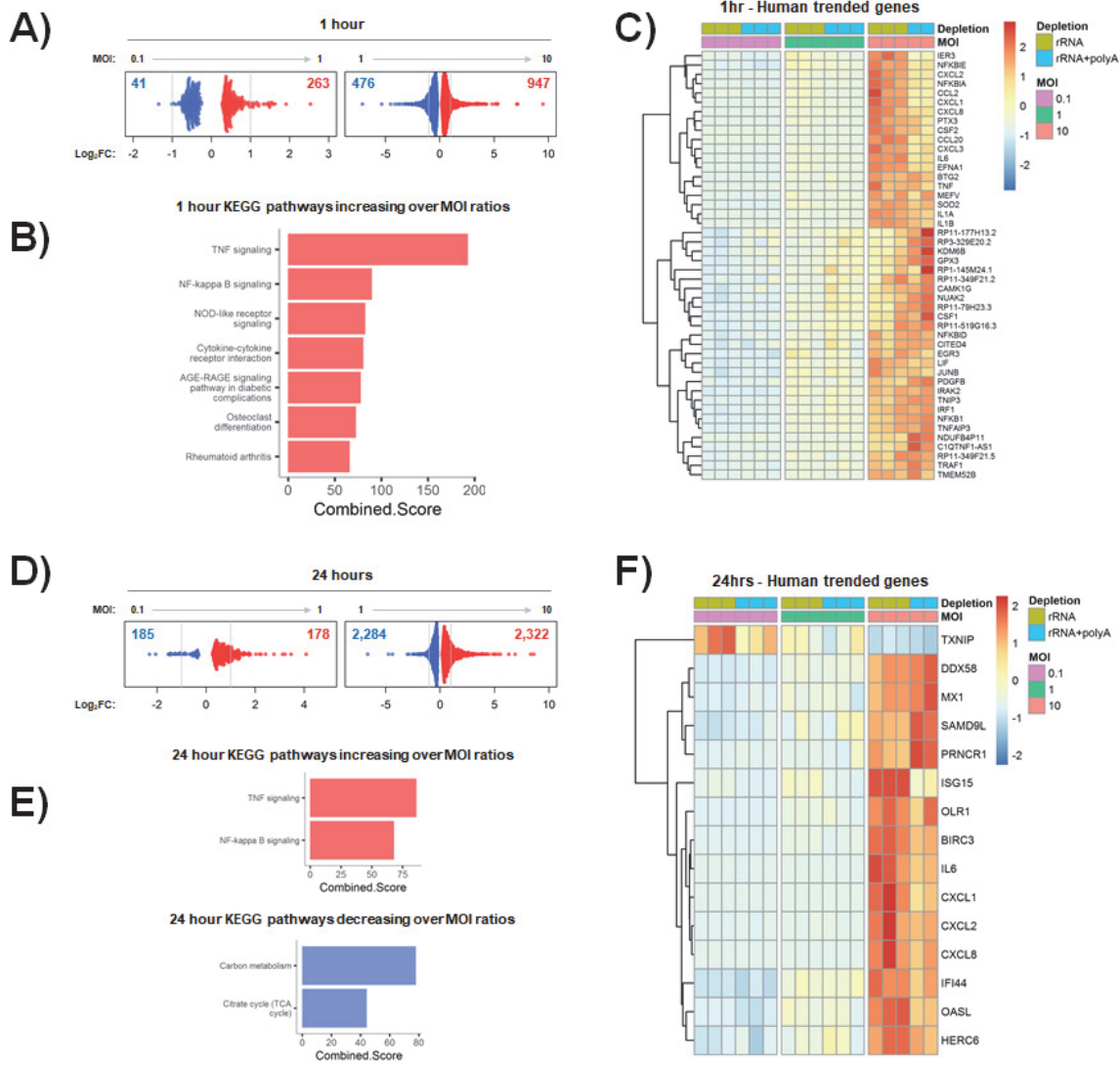
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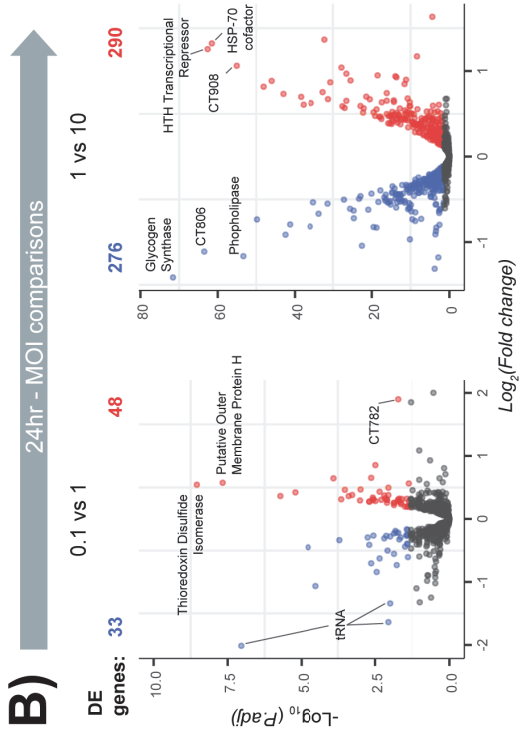
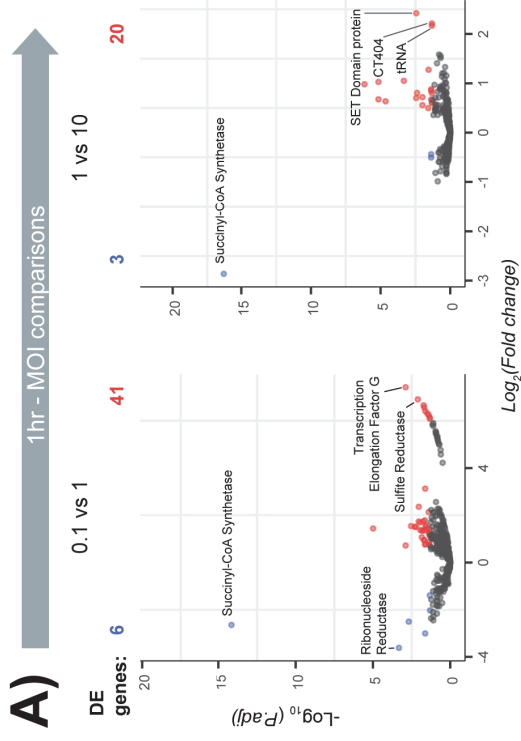
597 **Figure 6**

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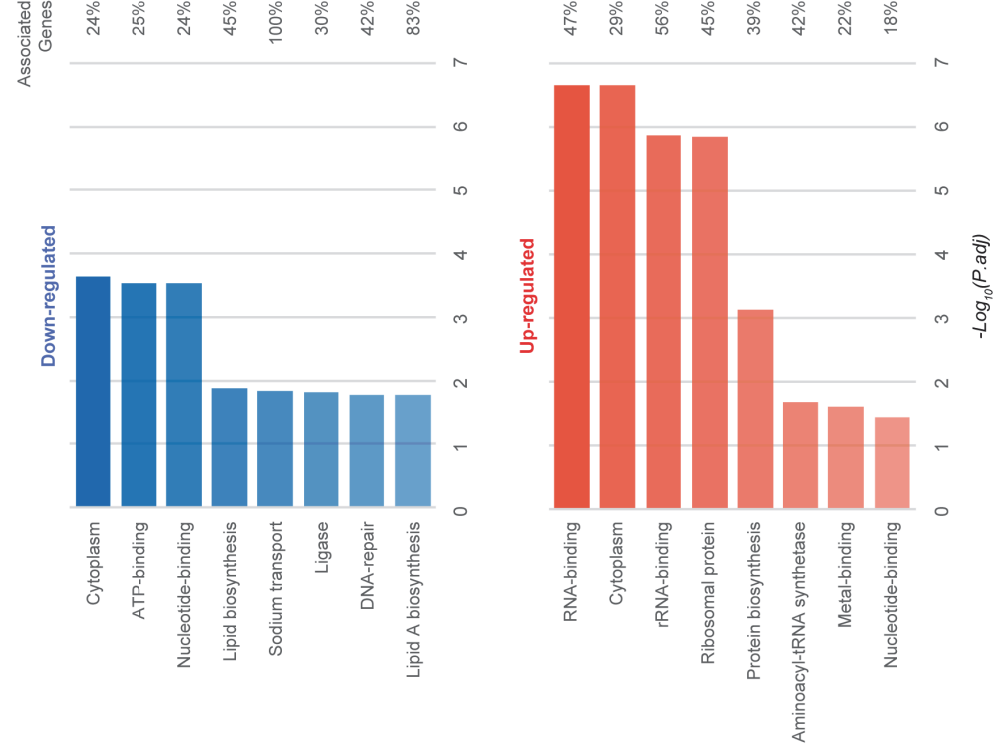




599 **Figure 7**



**C)** Enrichment at 24hrs: MOI 1 vs 10



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601 **Supplementary files**

602 **Supplementary File 1:**

603 Functional characterisation from top 25 highly expressed genes (host and chlamydial) that  
604 overlap all three MOI ratios.