Elucidating the Transcriptome of Turkey Hemorrhagic Enteritis Virus

**Running Title:** Novel Insights into Turkey Hemorrhagic Enteritis Virus Transcriptome

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## ABSTRACT

**Background:** Hemorrhagic enteritis (HE) is a disease affecting 6-12-week-old turkeys characterized by *immunosuppression (IS)* and bloody diarrhea. This disease is caused by *Turkey Hemorrhagic Enteritis Virus (THEV)* of which avirulent strains (THEV-A) that do not cause HE but retain the immunosuppressive ability have been isolated. The THEV-A Virginia Avirulent Strain (VAS) is still used as a live vaccine despite its immunosuppressive properties. *Our objective is to understand the genetic basis by which VAS induces IS*. The *transcriptome of THEV* was studied to set the stage for further experimentation with specific viral genes that may mediate IS.  
**Methods:** After infecting a turkey B-cell line (MDTC-RP19) with the VAS vaccine strain, samples in triplicates were collected at 4-, 12-, 24-, and 72-hours post-infection. Total RNA was subsequently extracted, and poly-A-tailed mRNA sequencing done. After trimming the raw sequencing reads with the FastQC, reads were mapped to the THEV genome using Hisat2 and transcripts assembled with StringTie. An in-house script was used to consolidate transcripts from all time-points, generating the final transcriptome. PCR, gel electrophoresis, and Sanger sequencing were used to validate all identified splice junctions.

## INTRODUCTION

Adenoviruses (AdVs) are non-enveloped icosahedral-shaped DNA viruses, causing infection in virtually all vertebrates. Their double-stranded linear DNA genomes range between 26 and 45kb in size, producing a broad repertoire of transcripts via a highly complex alternative splicing pattern (1, 2). The AdV genome is one of the most optimally economized; both the forward and reverse DNA strands harbor protein-coding genes, making it highly gene-dense. There are 16 genes termed “genus-common” that are homologous in all AdVs; these are thought to be inherited from a common ancestor. All other genes are termed “genus-specific”. “Genus-specific” genes tend to be located at the termini of the genome while “genus-common” genes are usually central (1). This pattern is observed in *Adenoviridae*, *Poxviridae*, and *Herpesviridae* (1, 3, 4). The family *Adenoviridae* consists of five genera: *Mastadenovirus* (MAdV), *Aviadenovirus*, *Atadenovirus*, *Ichtadenovirus*, and *Siadenovirus* (SiAdV) (5, 6). Currently, there are three recognized members of the genus SiAdV: frog adenovirus 1, raptor adenovirus 1, and turkey adenovirus 3 also called turkey hemorrhagic enteritis virus (THEV) (5, 7–10). Members of SiAdV have the smallest genome size (~26 kb) and gene content (~23 genes) of all known AdVs, and many “genus-specific” putative genes of unknown functions have been annotated (see **Figure 1**)(1, 2, 7).

Virulent THEV strains (THEV-V) and avirulent strains (THEV-A) of THEV are serologically indistinguishable, infecting turkeys, chickens, and pheasants and the THEV-V cause different clinical diseases in these birds (2, 11). In turkeys, the THEV-V cause hemorrhagic enteritis (HE), a debilitating acute disease affecting predominantly 6-12-week-old turkeys characterized by immunosuppression (IS), weight loss, intestinal lesions leading to bloody diarrhea, splenomegaly, and up to 80% mortality (11–13). HE is the most economically significant disease caused by any strain of THEV (11). While the current vaccine strain (a THEV-A isolated from a pheasant, Virginia Avirulent Strain [VAS]) have proven effective at preventing HE in young turkey poults, it still retains the immunosuppressive ability. Thus, vaccinated birds are rendered more susceptible to opportunistic infections and death than unvaccinated cohorts leading to substantial economic losses (11, 14–16). The induced IS also interferes with vaccination schemes for other infections of turkeys (11, 14). To eliminate this immunossupressive side-effect of the vaccine, a thorough investigation of the culprit viral factors (genes) mediating this phenomenon is essential. However, the transcriptome (splicing and gene expression patterns) of THEV has not been characterized, making the investigation of specific viral genes for possible roles in causing IS impractical. A well-characterized transcriptome of THEV is required to enable the next leap forward in THEV research - experimentation with specific viral genes that may mediate IS.

Myriads of studies have elucidated the AdV transcriptome in fine detail (17, 18). However, a large preponderance of studies focus on MAdVs - specifically human AdVs - thus, most of the current knowledge regarding AdV gene expression and replication is based on MAdV studies, which is generalized for all other AdVs (6, 19). MAdV genes are transcribed in a temporal manner; therefore, genes are categorized into five early transcription units (E1A, E1B, E2, E3, and E4), two intermediate (IM) units (pIX and IVa2), and one major late unit (MLTU or major late promoter [MLP]), which generates five families of late mRNAs (L1-L5). An additional gene (UXP or U exon) is located on the reverse strand. The early genes encode non-structural proteins such as enzymes or host cell modulating proteins, primarily involved in DNA replication or providing the necessary intracellular niche for optimal replication while late genes encode structural proteins. The immediate early gene E1A is expressed first, followed by the the delayed early genes, E1B, E2, E3 and E4. Then the intermediate early genes, IVa2 and pIX are expressed followed by the late genes (6, 17, 18). MAdV makes an extensive use of alternative RNA splicing to produce a very complex array of mRNAs. All but the pIX mRNA undergo at least one splicing event. The MLTU produces over 20 distinct splice variants all of which contain three non-coding exons at the 5’-end (collectively known as the tripartite leader, TPL) (17, 18). There is also an alternate 5’ three non-coding exons present in varying amounts on a subset of MLTU mRNAs (known as the x-, y- and z-leaders). Lastly, there is the i-leader exon, which is infrequently included between the second and third TPL exons, and codes for the i-leader protein (20). Thus, the MLTU produces a complex repertoire of mRNA with diverse 5’ untranslated regions (UTRs) spliced onto different 3’ coding exons which are grouped into five different 3’-end classes (L1-L5). Each transcription unit (TU) contains its own promoter driving the expression of all the array of mRNA transcripts produced via alternative splicing of the genes encoded in the unit(6, 17, 18). During translation of AdV mRNA, recent studies strongly suggest the potential usage of secondary start codons; adding to what was already a highly complex system for gene expression (17).

High throughput sequencing methods have facilitated the discovery of many novel transcribed regions and splicing isoforms. It is also a very powerful tool to study alternative splicing under different conditions at an unparalleled depth (18, 21). In this paper, a paired-end deep sequencing experiment was performed to characterize for the first time, the transcriptome of THEV (VAS vaccine strain) during different phases of the infection, yielding the first THEV splicing map. Our paired-end sequencing allowed for reading **149** bp long high quality (mean Phred Score of 36) sequences from each end of cDNA fragments, which were mapped to the genome of THEV. The generated data from our paired-end sequencing experiment should thus be reliable.

## RESULTS

**Overview of sequencing data and analysis pipeline outputs**  
A previous study by Zeinab *et al* showed that almost all THEV transcripts were detectable beginning at 4 hours (22). Therefore, infected MDTC-RP19 cells were harvested at 4-, 12-, 24-, and 72-hours post-infection(h.p.i) to ensure an amply wide time window to sample all transcripts. Our paired-end RNA sequencing (RNA-seq) experiment yielded an average of **107.1** million total reads of **149**bp in length per time-point, which were simultaneously mapped to both the virus (THEV) and host (*M.gallopavo*) genomes using the Hisat2 (23) alignment program. A total of **18.1** million reads from all time-points mapped to the virus genome; this provided good coverage/depth, leaving no regions unmapped. The mapped reads to the virus genome increased substantially from **432** reads at 4 h.p.i to **16.9** million reads at 72 h.p.i (**Table 1**, **Figure 2a**). From the mapped reads, we identified a total of **2,457** unique THEV splice junctions from all time-points, with splice junctions from the later time-points being supported by significantly more sequence reads than earlier time-points. For example all the **13** unique junctions at 4 h.p.i had less than 10 reads supporting each one, averaging a mere **2.8** reads/junction. Conversely, the **2,374** unique junctions at 72 h.p.i averaged **898.4** reads/junction, some junctions having coverage as high as **322,677** reads. The substantial increases in splice junctions and mapping reads to the THEV genome over time denotes an active infection, and correlates with our quantitative PCR (qPCR) assay quantifying the total number of viral genome copies over time (**Figure 2b**). Using StringTie (23), an assembler of RNA-seq alignments into potential transcripts, the mapped reads for each time-point were assembled into transcripts using the genomic location of the predicted THEV ORFs as a guide. In the consolidated transcriptome, a composite of all unredundant transcripts from all time points, we counted a total of **28** transcripts all of which are novel, ~~and using 3’ Rapid Amplification of cDNA Ends (3’RACE) and other methods, we further identified #### unique splice variants~~. Although some exons in some transcripts match the predicted ORFs exactly, most of our identified exons are longer, spanning multiple predicted ORFs (**Figure 3**). The complete list of unique splice junctions mapped to THEV’s genome has been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) under **accession no. XXXXXX**.

**Changes in THEV splicing profile over time**  
AdV gene expression occurs under exquisite temporal control, supervised by designated promoters for each transcription unit (TU) or region. Each promoter typically produce one or few pre-mRNAs that undergo alternative splicing to yield the manifold repertoire of complex transcripts characteristic of AdVs (17, 18). To evaluate the activity of each promoter over time, Firstly, Ballgown, a program for statistical analysis of assembled transcriptomes (24) was used to estimate and normalize expression levels of all transcripts for each time point in Fragments Per Kilobase of transcript per Million mapped reads (FPKM) units. Very few unique splice junctions, reads, and transcripts were counted at 4 h.p.i; hence, this time point was excluded in this analysis.

Individually, TRXPT\_21 (DBP) - from the E2 region - was the most significantly expressed at 12 h.p.i, comprising about **33.58**% of the total transcripts. Transcripts in the E3 and E4 regions also contributed significant proportions, and noticeably, some MLP region transcripts. The later time points were dominated by the MLP region; transcripts TRXPT\_10 and TRXPT\_14 were most abundantly expressed at 24 and 72 h.p.i, respectively, as expected (**Figure 4a**). When we performed analysis of the FPKM values of transcripts per region we found a similar pattern: the E2 region was the most abundantly expressed at 12 h.p.i, after which the MLP region assumes predominance (**Figure 4b**). Secondly, we estimated relative abundances of all splice junctions for each time point using the raw reads. We counted as significantly expressed only junctions with coverage of at least 1% of the total splice junction reads counted at the given time point. At 12 h.p.i, **18** junctions meet the 1% threshold, and were comprised of predominantly early region (E1, E2, E3, and E4) junctions, albeit the MLP was the single most preponderant region overall, constituting **38.8**% of all the junctions reads. (**Table 2a**). The levels of the top most abundant junctions at 12 h.p.i were maintained also 24 h.p.i as the most significantly expressed. However, here, the MLP-derived junctions were unsurprisingly even more preponderant overall, accounting for **NA**% of all the junction reads counted (**Table 2b**). At 72 h.p.i, the trend of increased activity of the MLP continued as expected; at this time, the MLP-derived junctions were not only the most abundant overall - accounting for **67.5**% of all junctions reads - but also contained the most significantly expressed individual junctions (**Table 2c**. Also see **Supplementary Tables 1a-c**; **Figure 4c**). When we limited this analysis to only junctions in the final transcriptome, the relative abundances of the junctions for each region over time was generally similar to the pattern seen with all the junctions included (**Figure 4d**).  
Furthermore, we analyzed splice donor and acceptor site nucleotide usage over time to investigate any peculiarities that THEV may show, generally or over the course of the infection. We found that most splice donor-acceptor sequences were unsurprisingly the canonical GU-AG nucleotides.

**Early Region 1 (E1) transcripts**. This region in MAdVs is the first transcribed after successful entry of the viral DNA into the host cell nucleus, albeit at low levels (18). The host transcription machinery solely mediates the transcription of this region. After their translation, the E1 proteins in concert with a myriad of host transcription factors activate the other viral promoters (6). Only two ORFs (ORF1 [sialidase] and Hyd) are predicted in this region; however, we discovered **four** novel transcripts in this region, which collectively contain **3** unique splice junctions (**Figure 5**). Most of the encoded proteins of the novel transcripts are distinct from the predicted ORFs, although they all have the potential to encode the Hyd protein as the 3’-most coding sequence (CDS) if secondary start codon usage is considered. The 5’-most CDS of TRXPT\_1 is multi-exonic, encoding a 17.9 kilodalton (kDa) protein of 160 residues [amino acids (aa)]. The CDS begins in the first exon, starting at position 211, spans the second exon, and terminates in the third exon at position 2312. From the 5’-most start codon (SSC), TRXPT\_2 encodes the largest protein in this region - a 64.3 kDa, 580 aa protein with the same SSC as TRXPT\_1 (position 211). This CDS spans almost the entire predicted ORF1 and Hyd, coming short in two regards: it is spliced from 1655 to 1964 (ORF1’s C-terminus, including the stop codon), and it’s stop codon (STC; position 2312) is 13 bp short of the Hyd STC. However, it has an SSC 102 bp upstream and in-frame with ORF1’s predicted SSC. TRXPT\_2 CDS therefore, shares substantial protein sequence similarity with ORF1 but not with Hyd, as the SSC of Hyd is not in-frame. TRXPT\_3 is almost identical to TRXPT\_1, except for the lack of TRXPT\_1’s second exon. From our RNA-seq data, all E1 transcripts share the same transcription termination site (TTS; at position 2325bp); however, TRXPT\_3 and TRXPT\_4 seem to have transcription start sites (TSS) downstream of the TSS of TRXPT\_1 and TRXPT\_2 (at position 54bp). Given that studies in MAdVs show that E1 mRNAs share not only a common TTS but also the TSS, and only differ from each other regarding the introns (18), it is likely that TRXPT\_3 and TRXPT\_4 are incomplete, and their actual TSS just like the TTS are identical for all E1 transcripts. Regardless of the TSS considered for TRXPT\_3, the coding potential (CP) remains unaffected. Its 5’-most CDS, beginning at 1965 and sharing the same STC as TRXPT\_1 and TRXPT\_2, produces a 13.1 kDa, 115 residue protein. This CDS (ORF4) was predicted in an earlier study (25) but was excluded in later studies (1, 12); however, our data suggests it is a bona fide ORF. The CP of TRXPT\_4 is affected by the TSS considered; if we consider its unmodified TSS, then its CP is the same as TRXPT\_3 (ORF4 as the first CDS and Hyd as second CDS if the first SSC is skipped). However, if we assume that TRXPT\_4 shares the same TSS as TRXPT\_1, then the 5’-most CDS is a distinct multi-exonic 15.9 kDa, 143 aa protein with the same SSC as TRXPT\_1 and TRXPT\_2 but with a unique STC. All splice junctions of the transcripts in this region (except the junction for TRXPT\_4) have been validated by cloning and Sanger sequencing of cDNA (**Figure 5b**; **supplementary PCR methods**). Finally, during our validation of TRXPT\_2, ORF1 was present on the agarose gel and Sanger sequencing results as a bona fide transcript (**supplementary PCR methods**). This is corroborated by 3’-RACE experiment, which shows transcripts spanning the entire ORF1 and Hyd ORFs without any splicing with a poly-A tail immediately after the TTS of transcripts in this region. The 5’-most CDS of this transcript would encode ORF1. However, the SSC of the predicted ORF1 is in-frame but downstream of TRXPT\_2 SSC, suggesting that the predicted ORF1 CDS is a truncated ORF; it shares the same TSS, SSC, and TTS as TRXPT\_2, but has a unique STC.

**Early Region 2 (E2) and Intermediate Region (IM) transcripts**. The E2 TU expressed on the anti-sense strand, is subdivided into E2A and E2B and encodes three classical AdV proteins essential for genome replication: pTP and Ad-pol (E2B proteins), and DBP (E2A protein) (17, 18). Unlike MAdV where two promoters (E2-early and E2-late) were discovered (17), we discovered only a single promoter from which both E2A and E2B transcription is initiated. However, similar to MAdVs, E2A and E2B transcripts have distinct TTSs, and the E2B transcripts share the TTS of the IVa2 transcript of the IM region (17, 18) (**Figure 6**).

The E2A ORF, DBP is one of three THEV ORFs predicted to be spliced from two exons. The corresponding transcript (TRXPT\_21) found in our data matches this predicted splicing pattern exactly but with a non-coding additional exon at the 5’-end (E2-5’UTR) at position 18,684-18,751 bp. Thus a three-exon transcript with an identical encoded protein (DBP; 380 residues, 43.3 kDa). This transcript (TRXPT\_21) was also corroborated in a 3’-RACE experiment. Additionally, from the 3’-RACE, a splice variant of TRXPT\_21 which retains the second intron leading to a 2-exon transcript was found. This transcript (TRXPT\_21B), albeit longer due to retaining the second intron and possessing a short 3’ UTR, encodes a truncated isoform of DBP because the first SSC utilized by TRXPT\_21, is followed shortly by STCs in the retained intron, and does not yield any viable product. Utilizing the SSC 173 bp downstream of TRXPT\_21’s SSC yields a 346 residue, 39.3 kDa product, which is in-frame of DBP but entirely contained in the second exon. TRXPT\_21 and TRXPT\_21B share a common TSS but TRXPT\_21B as seen in our 3’-RACE data, extends 39 bp into an adenine-thymine (A-T) rich sequence before the poly-A tail sequence occur, suggesting this position (16,934bp) as the bona fide E2A TTS (**Figure 6**).

The E2B region transcripts share the TSS of E2A - begins with E2-5’UTR - but extend thousands of base pairs downstream to reach the TTS at 2334bp in the IM region, which is immediately followed by an A-T rich sequence (position 2323-2339bp) where polyadenylation probably occurs. Interestingly, the TTS of the E1 region on the sense strand is in the immediate vicinity of this A-T rich sequence, and the A-T rich sequence is almost palindromic; hence it likely serves as the polyadenylation signal for both E1 and E2B/IM transcripts. The E2B transcripts, TRXPT\_6 and TRXPT\_7 are almost identical except for an extra splice junction at the 3’-end of TRXPT\_6, making TRXPT\_6 a five-exon transcript and TRXPT\_7, four (**Figure 6**). TRXPT\_7 has the CP for IVa2 and both classical proteins (pTP and Ad-pol) encoded in this region, of which the pTP ORF is predicted to be spliced from two exons just like in all other AdVs. The predicted splice junction of pTP is corroborated by our data; however, the full transcript is markedly longer than the predicted ORF: there are two novel 5’ exons, the third exon (containing the SSC of pTP) is significantly longer than predicted, and the last exon containing the bulk of the CDS is more than triple the predicted size of pTP. The first two exons are 5’-UTRs because the SSC here is immediately followed by STCs; hence, the 5’-most SSC (position 10,995bp) of the third exon which matches the predicted SSC is utilized. The encoded product is identical to the predicted pTP ORF (597 residues; 70.5 kDa). If secondary SSC (secSSC) usage is considered, with SSC at 6768bp and STC at 3430bp, the encoded product is identical to the predicted Ad-pol ORF (1112 residues; 129.2 kDa). ~~finish trxpt\_7 and then discuss trxpt\_6~~.

The IM region is a single-transcript TU, encoding a single classical protein, IVa2. The promoter expressing this single transcript (TRXPT\_5) is embedded in E2B region and shares a TTS with E2B transcripts (17, 18). TRXPT\_5 is a two-exon transcript located on the reverse strand spliced at 3447-3615. The first exon is an UTR, except the last 2 nucleotides, which connect with the first nucleotide of second exon to form the 5’-most SSC. This first SSC is 4 codons upstream and in-frame of the predicted IVa2 SSC. Regardless of the SSC considered, the encoded protein (IVa2) is largely unaffected. Except for the four extra residues at the N-terminus (considering the 5’-most SSC), the entire protein sequence is identical.

The splice junction of ~~TRXPT\_5 add trxpts from E2~~ were confirmed by cloning and Sanger sequencing of cDNA (**supplementary PCR methods**).

**Early Region 3 (E3) transcripts**.  
The E3 region is wholly contained in the MLTU and encodes proteins involved in modulating and evading the host immune defenses. In MAdVs, this region contains seven ORFs expressed from several transcripts which share the same TSS (from the E3 promoter) but have different TTSs (6, 17, 18). However, some E3 transcripts use the MLP as their TSS. Due to sharing the same TSS, in MAdVs, secSSC usage is heavily relied on for gene expression in this region except for 12.5K and transcripts using the MLP as TSS, as utilizing only the first SSC cannot produce all the other transcripts in this TU (17).

In THEV, only one ORF (E3) was predicted in this region. However, we identified six transcripts here (**TRXPT\_22, TRXPT\_23, TRXPT\_24, TRXPT\_25, TRXPT\_26, TRXPT\_27**), all of which are novel (**Figure 7**). We identified two distinct TSSs - one similar to the classic MAdV E3 TSS (position 18,230bp) and the other about 500 bp downstream at 18,727bp. The E3 transcripts collectively have the CP for several THEV ORFs: 100K, 22K, 33K, pVIII, E3, Fiber (IV), and ORF7 belonging to the MLTU; however, some CDSs are nonidentical due to unpredicted splicing. For instance, 33K is one of the few THEV ORFs predicted to be spliced from two exons; however, we discovered it to be a four-exon ORF expressed from TRXPT\_24. The first two exons were not predicted but the last two, match the predicted exons precisely and the CDS is in-frame. However, the first 20bp of the predicted 33K (including the SSC) is spliced as as part of the second intron of TRXPT\_24. Thus, the bona fide 33K is a 19.8 kDa, 171 residue protein spanning four exons. TRXPT\_24 can also encode the ORFs, pVIII and E3 if we consider downstream SSC usage. TRXPT\_22 and TRXPT\_23 share the TSS of TRXPT\_24 (E3 promoter; 18,230bp) but have distinct TTSs. Considering the first SSCs, they both encode novel 73 residue proteins that is over 80% similar but differ at the C-termini. Considering downstream SSC usage, both transcripts can encode pVIII and E3 in that order, but TRXPT\_23 being longer, has the CP for the Fiber ORF also. TRXPT\_25 is the largest transcript in the TU. It also utilizes the classic E3 TSS but has distinct TTS. It is a two exon transcript, encoding a novel protein (t100K; 543 residues), which is a shorter isoform of the predicted 100K ORF. Considering secSSC usage on this transcript yields the predicted ORF, 22K. It also has the CP for pVIII and E3 in that order. Furthermore, during the validation of TRXPT\_25’s splice junction using primers that span the junction (18350-18717bp), we noticed a DNA band that corresponds to the full unspliced sequence (**supplementary PCR methods Table 1**). As TRXPT\_25 only falls short of encoding the complete 100K protein due to its splice junction, this band (which we cloned and validated by Sanger sequencing) suggests that the predicted 100K is indeed expressed. This transcript (TRXPT\_25B) although not seen in our RNA-seq data, likely shares the same TSS and TTS as TRXPT\_25. Lastly, TRXPT\_26 and TRXPT\_27 share the same TSS, unique from the other transcripts in this region but have distinct TTSs. TRXPT\_26 is a three-exon transcript but the first two are UTRs - only the terminal exon encodes proteins. It encodes pVIII as the 5’-most ORF and has the CP for E3 and Fiber in that order. TRXPT\_27 on the other hand, is only a two-exon transcript but similarly, only the terminal exon contains the CDSs. It encodes Fiber as the 5’-most ORF and ORF7 downstream with secSSC usage. TRXPT\_13, which seems to be an E3 transcript that uses the MLP as TSS is discussed under the MLTU transcripts.

**Early Region 4 (E4) transcripts**. This transcription unit (TU) is the found at the tail-end (3’-end) of the genome, on the reverse strand. Based on nucleotide position, ORF7 and ORF8 were predicted in this region (1); however, as ORF7 is neither on the same strand as ORF8 nor transcribed from a promoter in the E4 region, only ORF8 can legitimately be classified as a transcript in this TU. This is corroborated by our RNA-seq data, as only one transcript was identified in this region on the reverse strand (**Figure 8**). The transcript (TRXPT\_28) spans 25192-26247 and is spliced at 25701-26055; hence, a two-exon transcript. The second exon fully matches the predicted ORF8 with 12 extra base pairs at the 3’-end; however, the encoded protein is an exact match. There is a SSC in the first exon at position 26246 (second nucleotide of the transcript). The encoded protein from this SSC is in-frame with the SSC of ORF8 in the second exon; hence, the C-terminus of this longer protein (26.4 kDa, 229 aa) would be the identical to the predicted ORF8 protein. ~~The splice junction of TRXPT\_28 was validated by cloning and Sanger sequencing of cDNA (~~**~~supplementary PCR methods~~**~~).~~

**Major Late Transcription Unit (MLTU) or MLP Region transcripts**.

## DISCUSSION/CONCLUSIONS

In the original study where the ORFs of THEV were predicted, ORF4 was predicted in the E1 region spanning the Hyd gene. However, later studies predicted and preferred Hyd instead of ORF4; hence, the current prediction map. However, this study shows that while both Hyd and ORF4 may be both expressed, ORF4 is most likely the bona fide gene. For fig2a: There is a dramatic increase of mean coverage/depth from **2.42** at 4 h.p.i to **95,042** at 72 h.p.i, strongly demonstrating an active infection. Unexpectedly, the pileup of reads seems consistently skewed over similar regions of the genome. We could speculate that the temporal gene expression regulation of THEV is different from MAdVs or this could simply mean that the infection was not well synchronized. However, the relative proportions over these similar regions shows some variation over time. For fig2b: titer reaching a plateau at 120 h.p.i, probably due to high cell death TRXPT\_2 and ORF1 are isoforms Presumably, if the junction reads were normalized, MLTU would not be predominant at 12hpi. The TTSs were all in the context of A-T rich sequences; which presumably serve as polyA signals.

## MATERIALS AND METHODS

### Cell culture and THEV Infection

The Turkey B-cell line (MDTC-RP19, ATCC CRL-8135) was grown as suspension cultures in 1:1 complete Leibovitz’s L-15/McCoy’s 5A medium with 10% fetal bovine serum (FBS), 20% chicken serum (ChS), 5% tryptose phosphate broth (TPB), and 1% antibiotics solution (100 U/mL Penicillin and 100ug/mL Streptomycin), at 41oC in a humidified atmosphere with 5% CO2. Infected cells were maintained in 1:1 serum-reduced Leibovitz’s L15/McCoy’s 5A media (SRLM) with 2.5% FBS, 5% ChS, 1.2% TPB, and 1% antibiotics solution (100 U/mL Penicillin and 100ug/mL Streptomycin). A commercially available HE vaccine was purchased from Hygieia Biological Labs as a source of THEV-A (VAS strain). The stock virus was titrated using an in-house qPCR assay with titer expressed as genome copy number(GCN)/mL, similar to Mahshoub *et al* (26) with modifications. Cells were infected at a multiplicity of infection (MOI) of 100 GCN/cell and samples in triplicates were harvested at 4-, 12-, 24-, and 72-h.p.i for RNA-seq. The infection was repeated but samples in triplicates were harvested at 12-, 24-, 36-, 48-, and 72-h.p.i for PCR validation of novel splice sites. Still one more independent infection was done at time points ranging from 12 to 168-h.p.i for qPCR quantification of virus titers.

### RNA extraction and Sequencing

Total RNA was extracted from infected cells using Thermofishers’ RNAqueous™-4PCR Total RNA Isolation Kit (#AM1914) per manufacturer’s instructions. An agarose gel electrophoresis was performed to check RNA integrity. The RNA quantity and purity was initially assessed using nanodrop, and RNA was used only if the A260/A280 ratio was 2.0 ± 0.05 and the A260/A230 ratio was >2 and <2.2. Extracted total RNA samples were sent to LC Sciences, Houston TX for poly-A-tailed mRNA sequencing where RNA integrity was checked with Agilent Technologies 2100 Bioanalyzer High Sensitivity DNA Chip and poly(A) RNA-seq library was prepared following Illumina's TruSeq-stranded-mRNA sample preparation protocol. Paired-end sequencing was performed on Illumina's NovaSeq 6000 sequencing system.

### Validation of Novel Splice Junctions

All splice junctions identified in this work are novel except one predicted splice site each for pTP and DBP, which were corroborated in our work. However, these predicted splice junctions had not been experimentally validated hitherto, and we identified additional novel exons, giving the complete picture of these transcripts. The novel splice junctions in this work discovered in the assembled transcripts using the StringTie transcript assembler which we validated by PCR and Sanger Sequencing are shown in supplementary PCR methods Table 1. Briefly, we designed primers that crossed a range of novel exon–exon boundaries for each specific transcript in a transcription unit (TU) paired with their respective universal primers for the TU (~~supplementary PCR methods). Each forward primer contained a KpnI restriction site and reverse primers, an XbaI site. After first-strand cDNA synthesis with SuperScript™ III First-Strand Synthesis System, these primers were used in a targeted PCR amplification, the products analyzed with agarose gel electrophoresis to confirm expected band sizes, cloned by traditional restriction enzyme method, and Sanger sequenced to validate these splice junctions at the sequence level.

### 3’ Rapid Amplification of cDNA Ends (3’-RACE)

We performed a rapid amplification of sequences from the 3’ ends of mRNAs (3’-RACE) experiment using a portion of the extracted total RNA of infected MDTC-RP19 cells used for the RNA-seq experiment as explained above. We followed the protocol described by Green *et al* (27) with modifications. Briefly, 1ug of total RNA was reverse transcribed to cDNA using SuperScript™ IV First-Strand Synthesis System following the manufacturing instructions using an adapter–primer with a 3’-end poly(T) and a 5’-end BamHI restriction site. A gene-specific sense primer with a 5’-end KpnI restriction site paired with an anti-sense adapter-primer with a 5’-end BamHI site were used to amplify target sections of the cDNA using Invitrogen’s Platinum™ Taq DNA polymerase High Fidelity, following manufacturer’s instructions. The PCR amplicons were restriction digested, cloned, and Sanger sequenced.

### Computational Analysis of RNA Sequencing Data: Mapping and Transcript characterization

Analysis of our sequence reads were analyzed following a well established protocol described by Pertea *et al* (23), using SNAKEMAKE 7.24.0 to drive the pipeline. Briefly, sequencing reads were trimmed with the FastQC - version 0.11.9 (28) program to achieve an overall Mean Sequence Quality (Phred Score) of 36. Trimmed reads were mapped to the complete sequence of avirulent turkey hemorrhagic enteritis virus strain Virginia (<https://www.ncbi.nlm.nih.gov/nuccore/AY849321.1/>) and *Meleagris gallopavo* (<https://www.ncbi.nlm.nih.gov/genome/?term=Meleagris+gallopavo>) using Hisat2 - version 2.2.1 (23) with default settings without relying on known splice sites. The generated BAM files from each infection time-point were filtered for reads mapping to the THEV genome and fed into StringTie - version 2.2.1 (23) using a gff3 file from NCBI cont m. aining the predicted ORFs of THEV as a guide. A custom script was used to consolidate all transcripts from all time-points without redundancy, generating the final transcriptome of THEV.

## SCRIPTS AND SUPPLEMENTARY MATERIALS

### DATA AVAILABILITY

### CODE AVAILABILITY

All the code/scripts written for analysis of the data are available on github (<https://github.com/Abraham-Quaye/thev_transcriptome>)

## ACKNOWLEDGMENTS

LC Sciences - RNA sequencing was done here Eton Bioscience, Inc, San Diego, CA - All Sanger sequencing validations was done here

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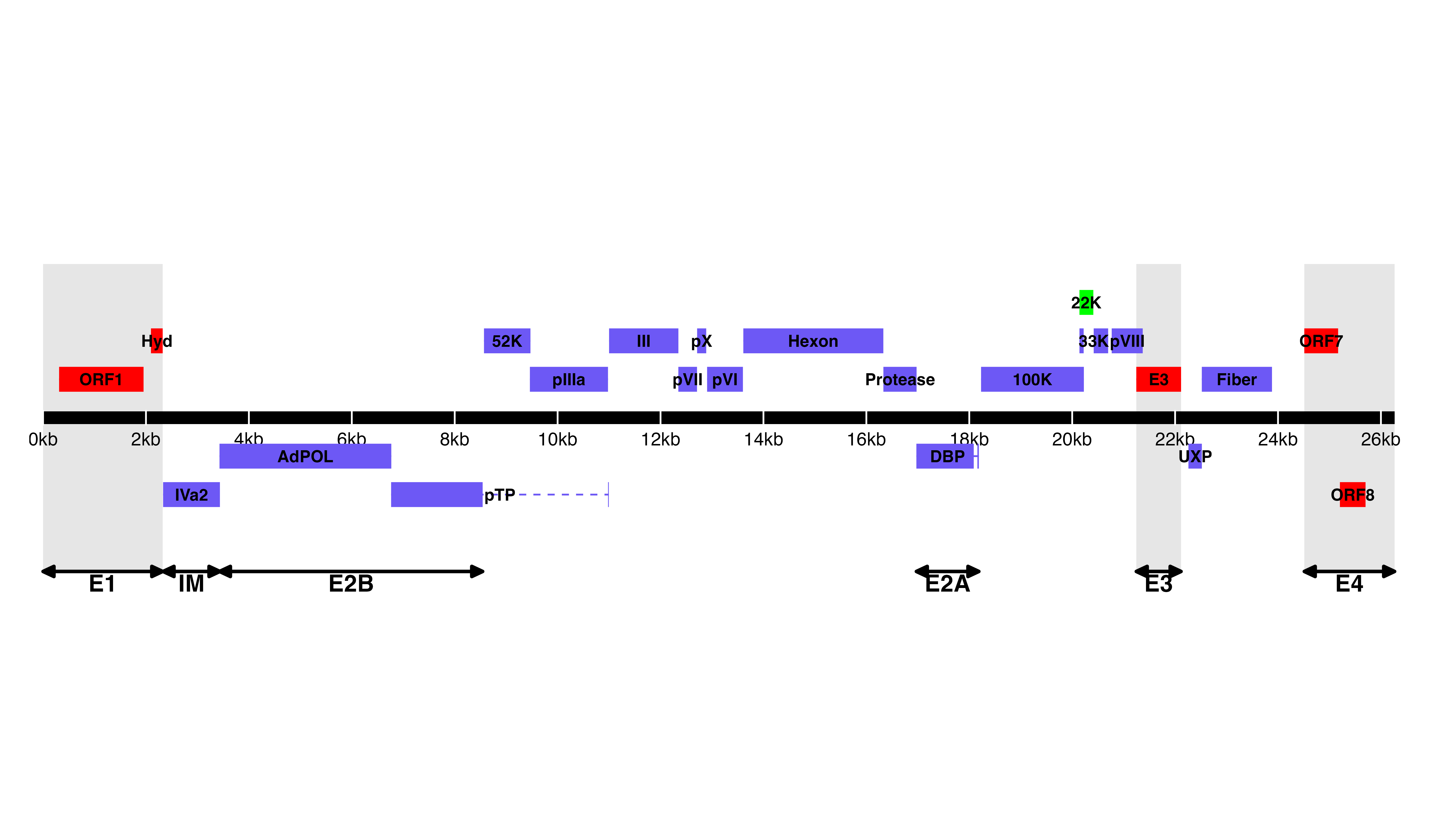
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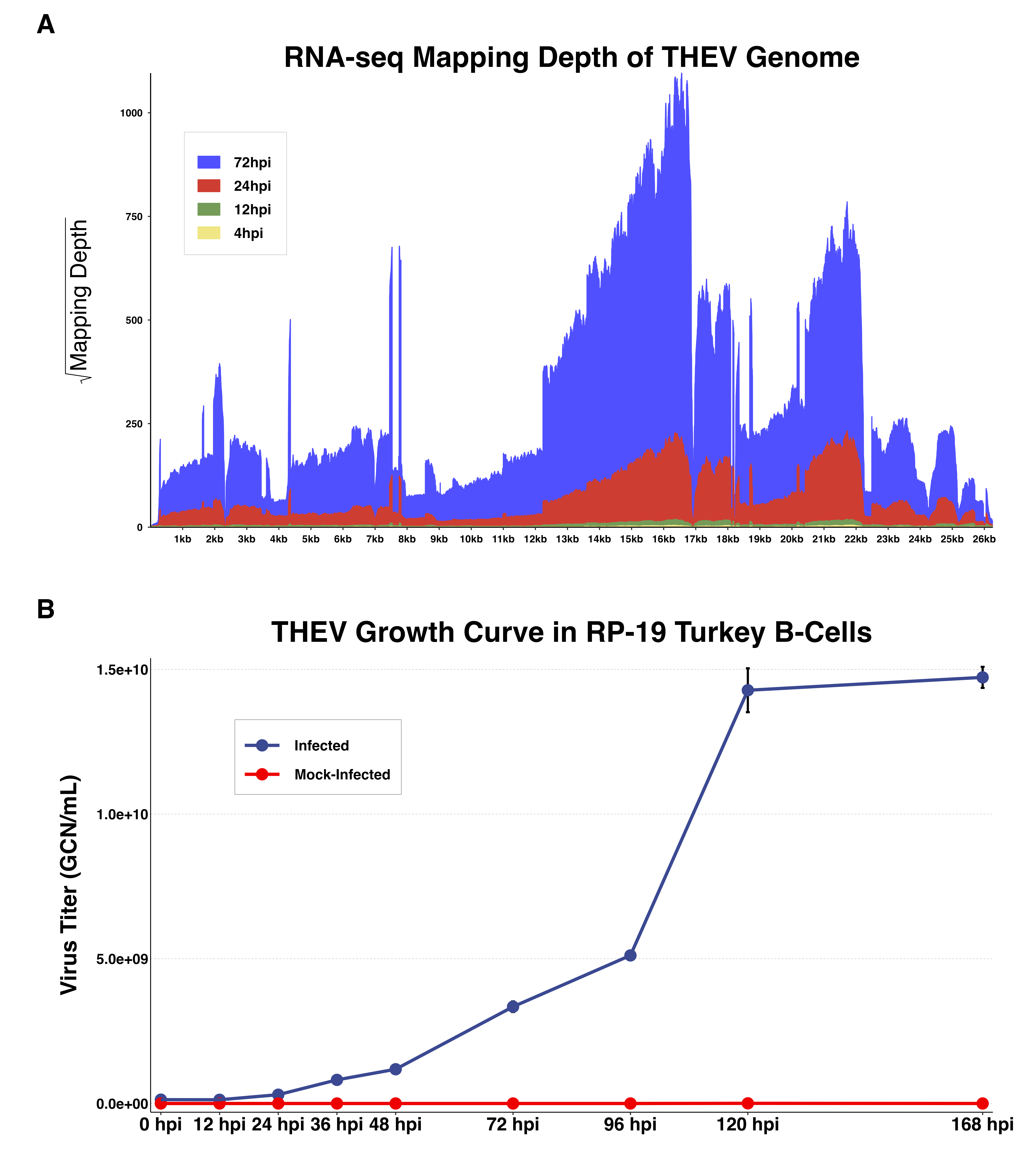
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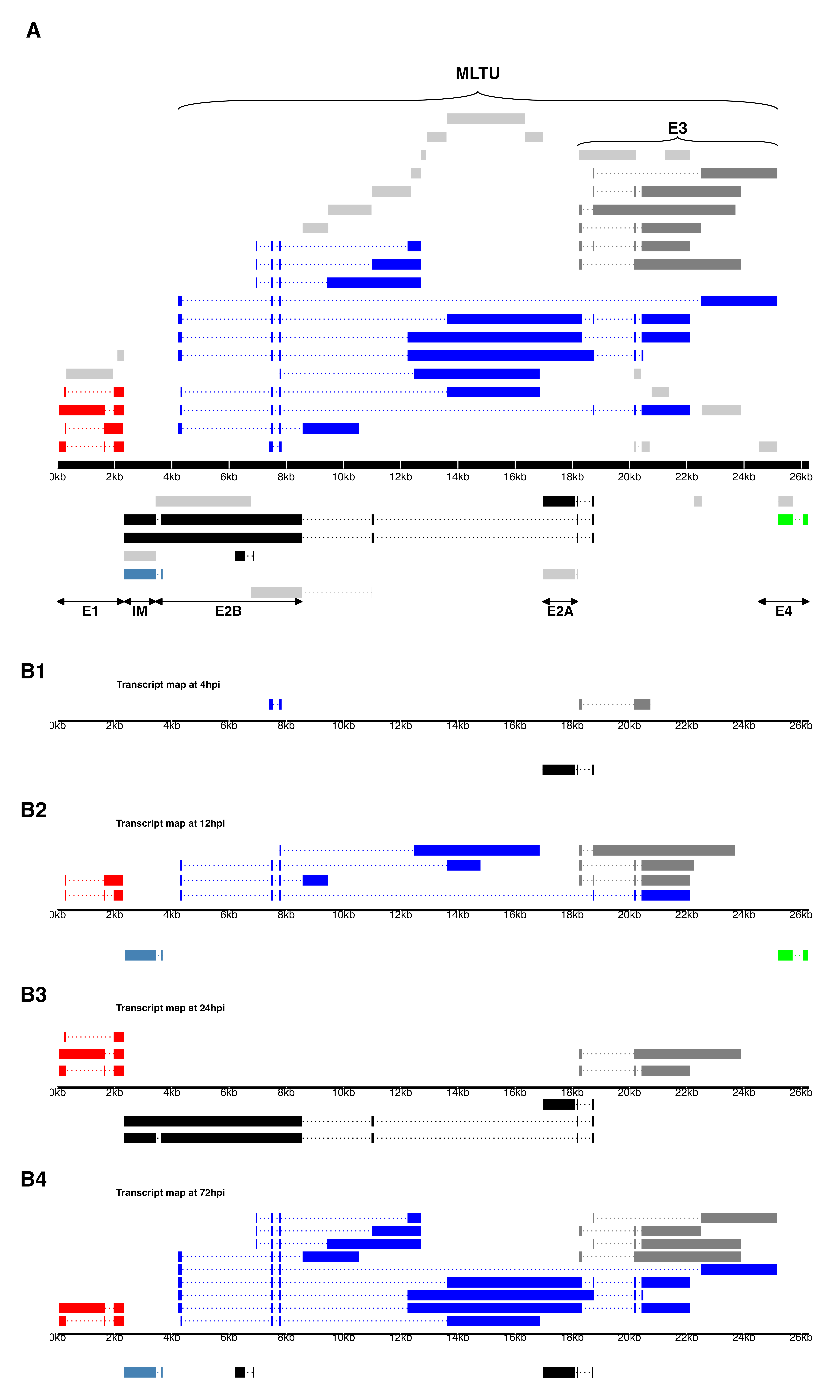
## TABLES AND FIGURES



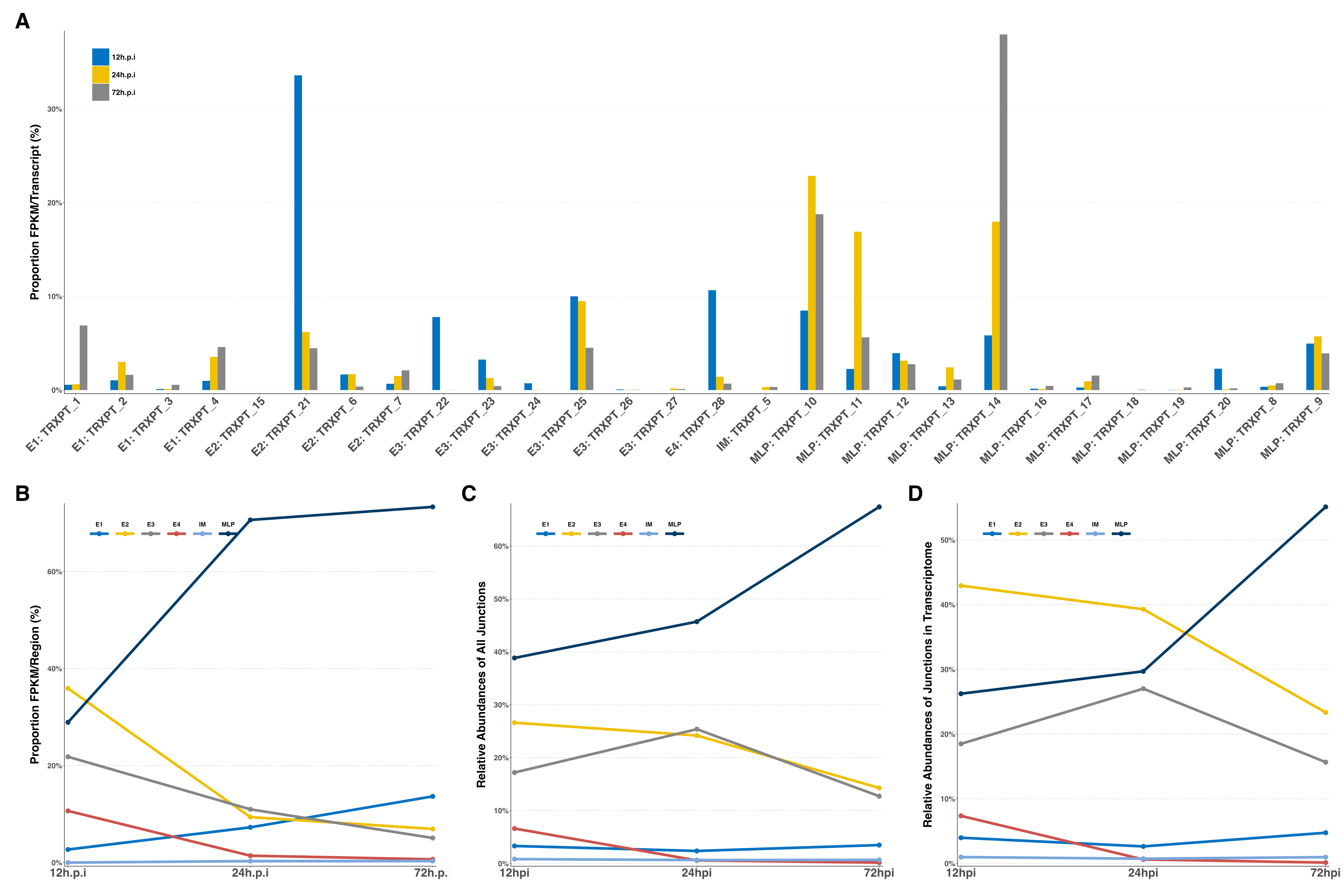
**Figure 1. *Genomic map of THEV avirulent strain annotated ORFs***. The central horizontal line represents the double-stranded DNA marked at 5kb intervals as white line breaks. Blocks represent viral genes. Blocks above the DNA line are transcribed rightward, those below are transcribed leftward. pTP, DBP and 33K predicted to be spliced are shown as having tails. Shaded regions indicate regions containing “genus-specific” genes (colored red). Genes colored in blue are “genus-common”. Gene colored in light green is conserved in all but Atadenoviruses. The UXP (light blue) is an incomplete gene present in almost all AdVs. Regions comprising the different transcription units are labelled at the bottom (E1, E2A, E2B, E3, E4, and IM); the unlabeled regions comprise the MLTU.

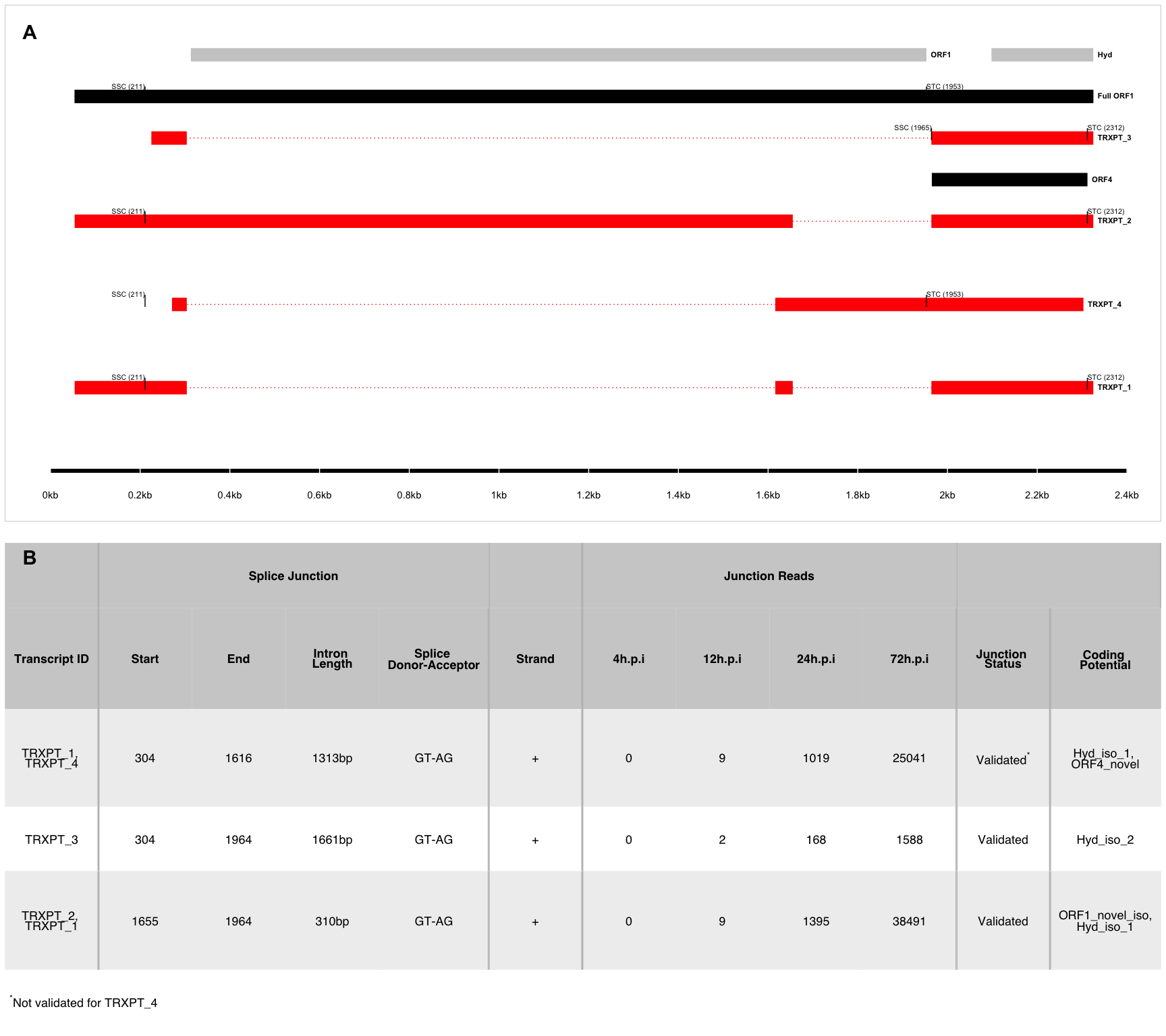


**Figure 2: Increasing levels of THEV over time. a) *Per base coverage of sequence reads mapping to THEV genome by time point***. The pileup of mRNA reads mapping to THEV genome at the base-pair level for each indicated time point. **b) *Growth curve of THEV (VAS vaccine strain) in MDTC-RP19 cell line***. Virus titers were a quantified with a qPCR assay. There is no discernible increase in virus titer up 12 h.p.i, after which there is a steady increase in virus titer is measured. The virus titer expands exponentially beginning from 48 h.p.i, increasing by orders of magnitude before reaching a plateau at 120 h.p.i. GCN: genome copy number.



**Figure 3. a) Composite transcriptome of THEV from RNA-seq**. THEV transcripts assembled from all time points by StringTie are unified forming this final transcriptome (splicing map). Transcripts belonging to the same transcription unit (TU) are located in close proximity on the genome and are color coded and labeled in this figure as such. The organization of TUs in the THEV genome is unsurprisingly similar to MAdVs; however, the MAdV genome shows significantly more transcripts. The TUs are color coded: E1 transcripts - red, E2 - black, E3 - dark grey, E4 - green, MLTU - blue. Predicted ORFs are also indicated here, colored light grey. **b) THEV transcripts identified at given time points**. Transcripts are color coded as explained in **a**.

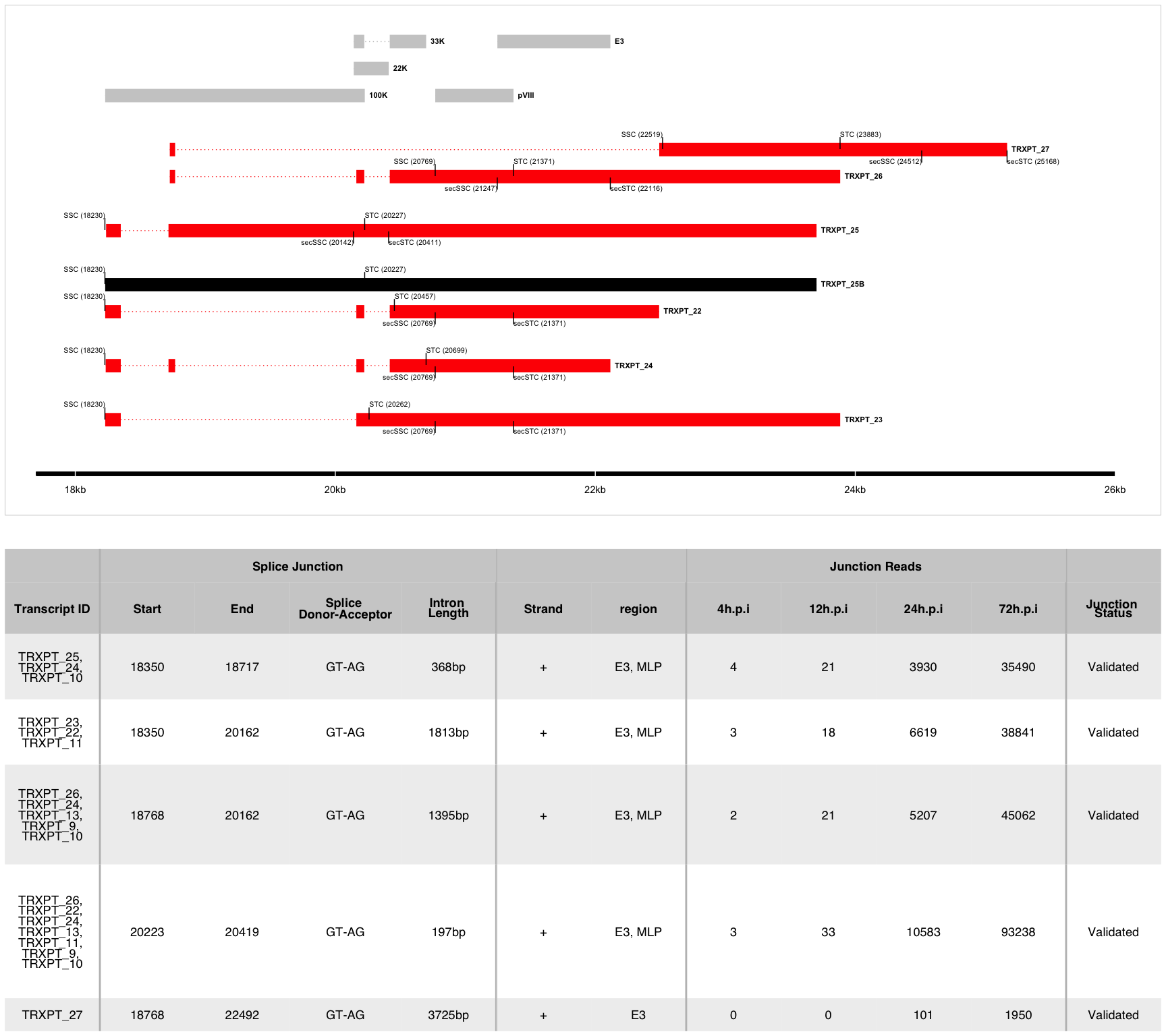
 **Figure 4: Changes in splicing and expression profile of THEV over time.** **a)** Expression levels of transcripts over time.**b)** Expression levels of transcripts by region over time.**c)** Relative abundances of all splice junctions over time. **d)** Relative abundances of junctions in transcriptome.



**Figure 5: E1 region transcripts. a)** The splice map of the E1 transcription unit. Exons are depicted as boxes connected by introns (dotted lines). Transcripts from RNA-seq data are colored red, predicted ORFs are colored grey, and the full ORF1 transcript and previously annotated ORF4 are colored black. Each transcript or ORF is labelled with its name to the right. The start codon (SSC) and stop codon (STC) of the 5’-most CDS of each transcript is indicated with the nucleotide position in brackets. The region of the virus is depicted at the bottom as a black line with labels of the nucleotide positions for reference **b)** The sequence reads covering the splice junctions are indicated with information about their validation status using cloning and Sanger sequencing.

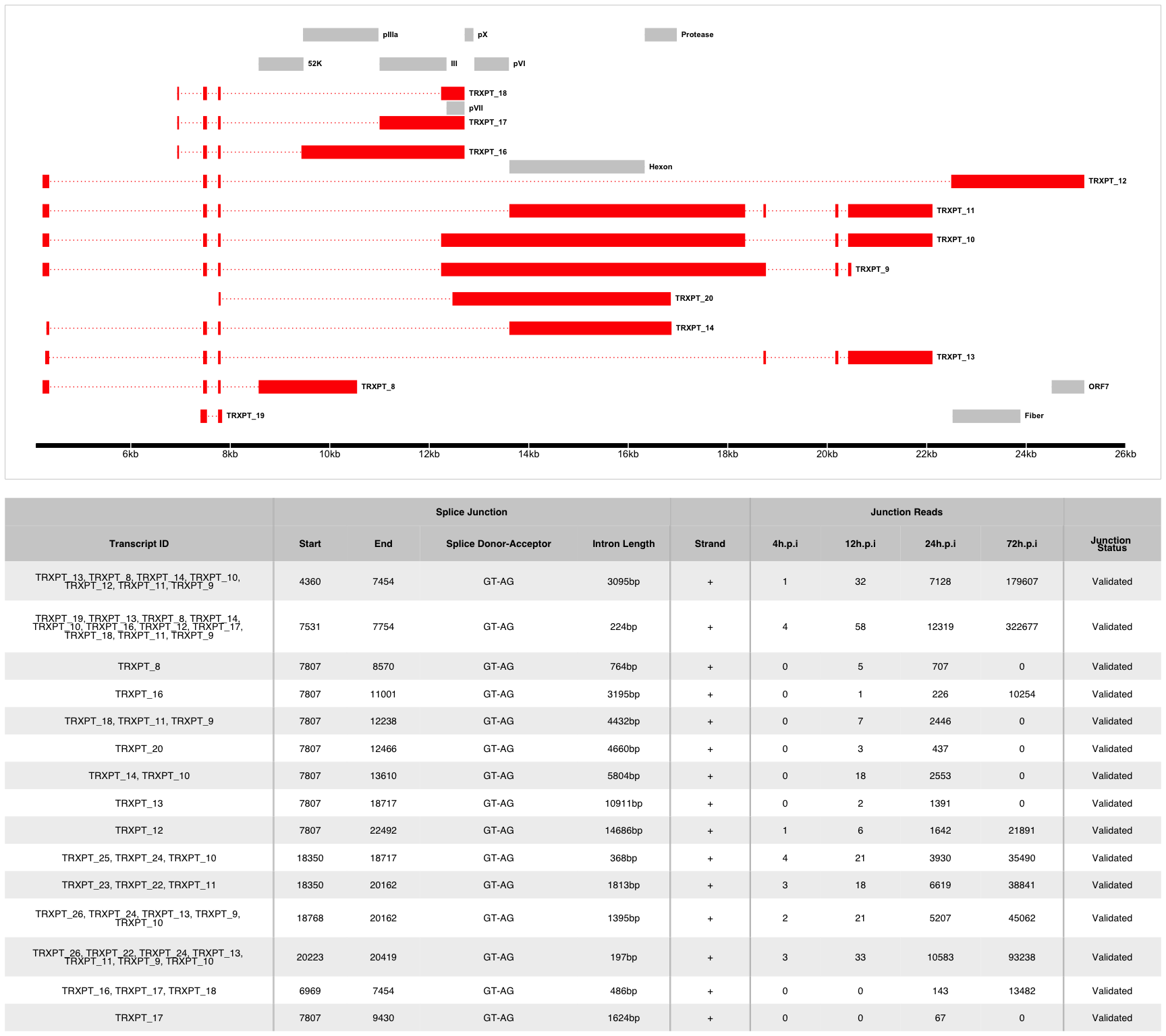


**Figure 6: E2 and IM region transcripts. a)** The splice map of the E2 and IM transcription units. Exons are depicted as boxes connected by introns (dotted lines). Red transcripts are generated from RNA-seq data and predicted ORFs are colored grey. Each transcript or ORF is labelled with its name to the right. The start codon (SSC) and stop codon (STC) of the 5’-most CDS of each transcript is indicated with the nucleotide position in brackets. The region of the virus is depicted at the bottom as a black line with labels of the nucleotide positions for reference **b)** The sequence reads covering the splice junctions are indicated with information about their validation status using cloning and Sanger sequencing.

 **Figure 7: E3 transcripts. a)** The splice map of the E3 transcription unit. Exons are depicted as boxes connected by introns (dotted lines). Red transcripts are generated from RNA-seq data and predicted ORFs are colored grey. Each transcript or ORF is labelled with its name to the right. The start codon (SSC) and stop codon (STC) of the 5’-most CDS of each transcript is indicated with the nucleotide position in brackets. The region of the virus is depicted at the bottom as a black line with labels of the nucleotide positions for reference **b)** The sequence reads covering the splice junctions are indicated with information about their validation status using cloning and Sanger sequencing.



**Figure 8: E4 region transcripts. a)** The splice map of the E4 transcription unit. Exons are depicted as boxes connected by introns (dotted lines). The transcript from RNA-seq data is colored red and the predicted ORF, grey. The transcript and ORF are labelled with their names to the right. The start codon (SSC) and stop codon (STC) of the 5’-most CDS is indicated with the nucleotide position in brackets. The region of the virus is depicted at the bottom as a black line with labels of the nucleotide positions for reference **b)** The sequence reads covering the splice junction are indicated.



**Figure 9: MLTU transcripts. a)**

Table 1: Overview of sequencing results

| **Metric** | **4h.p.i** | **12h.p.i** | **24h.p.i** | **72h.p.i** | **Total** |
| --- | --- | --- | --- | --- | --- |
| **Total reads** | 1.17e+08 | 7.63e+07 | 1.20e+08 | 1.15e+08 | 4.28e+08 |
| **Mapped   (Host)** | 1.04e+08  (89.06%) | 6.79e+07  (89.0393%) | 1.06e+08  (88.2719%) | 8.38e+07  (72.9802%) | 3.62e+08 |
| **Mapped   (THEV)** | 4.32e+02  ( 0.0004%) | 6.70e+03  ( 0.0088%) | 1.18e+06  ( 0.9841%) | 1.69e+07  (14.6904%) | 1.81e+07 |
| **Mean Per Base   Coverage/Depth** | 2.42 | 37.71 | 6,666.96 | 95,041.7 | 101,749 |
| **Total unique   splice junctions** | 13 | 37 | 236 | 2,374 | 2,457 |
| **Junction coverage   Total (at least 1 read)** | 37 | 605 | 115,075 | 2.13e+06 | 2.25e+06 |
| **Junction coverage   Mean reads** | 2.8 | 16.4 | 487.6 | 898.4 | 351.3 |
| **Junction coverage   (at least 10 reads)** | 0 | 13 | 132 | 1,791 | 1,936 |
| **Junction coverage   (at least 100 reads)** | 0 | 1 | 53 | 805 | 859 |
| **Junction coverage   (at least 1000 reads)** | 0 | 0 | 18 | 168 | 186 |

Table 2a: Most abundant splice junctions at 12h.p.i

| **Timepoint** | **Strand** | **Start** | **End** | **Splice\_Site** | **Splice   Acceptor-Donor** | **Region** | **Reads** | **Intron Length** | **Reads\_Percentage** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 12hpi | - | 18,087 | 18,159 | GT-AG | T-A | E2 | 103 | 72 bp | 103 (17%) |
| 12hpi | + | 18,189 | 18,684 | CT-AC | T-A | MLP | 97 | 495 bp | 97 (16%) |
| 12hpi | + | 7,531 | 7,754 | GT-AG | T-A | MLP | 58 | 223 bp | 58 (9.6%) |
| 12hpi | - | 25,701 | 26,055 | GT-AG | T-A | E4 | 37 | 354 bp | 37 (6.1%) |
| 12hpi | + | 20,223 | 20,419 | GT-AG | T-A | E3 | 33 | 196 bp | 33 (5.5%) |
| 12hpi | + | 4,360 | 7,454 | GT-AG | T-A | MLP | 32 | 3,094 bp | 32 (5.3%) |
| 12hpi | - | 18,751 | 20,668 | GT-AG | T-A | E2 | 22 | 1,917 bp | 22 (3.6%) |
| 12hpi | + | 18,350 | 18,717 | GT-AG | T-A | E3 | 21 | 367 bp | 21 (3.5%) |
| 12hpi | + | 18,768 | 20,162 | GT-AG | T-A | E3 | 21 | 1,394 bp | 21 (3.5%) |
| 12hpi | + | 7,807 | 13,610 | GT-AG | T-A | MLP | 18 | 5,803 bp | 18 (3%) |
| 12hpi | + | 18,350 | 20,162 | GT-AG | T-A | E3 | 18 | 1,812 bp | 18 (3%) |
| 12hpi | - | 18,189 | 18,684 | GT-AG | T-A | E2 | 14 | 495 bp | 14 (2.3%) |
| 12hpi | - | 18,751 | 21,682 | GT-AG | T-A | E2 | 10 | 2,931 bp | 10 (1.7%) |
| 12hpi | + | 304 | 1,616 | GT-AG | T-A | E1 | 9 | 1,312 bp | 9 (1.5%) |
| 12hpi | + | 1,655 | 1,964 | GT-AG | T-A | E1 | 9 | 309 bp | 9 (1.5%) |
| 12hpi | - | 18,087 | 18,163 | GT-AG | T-A | E2 | 8 | 76 bp | 8 (1.3%) |
| 12hpi | + | 7,807 | 12,238 | GT-AG | T-A | MLP | 7 | 4,431 bp | 7 (1.2%) |
| 12hpi | + | 7,807 | 22,492 | GT-AG | T-A | MLP | 6 | 14,685 bp | 6 (1%) |

Table 2b: Most abundant splice junctions at 24h.p.i

| **Timepoint** | **Strand** | **Start** | **End** | **Splice\_Site** | **Splice   Acceptor-Donor** | **Region** | **Reads** | **Intron Length** | **Reads\_Percentage** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 24hpi | - | 18,087 | 18,159 | GT-AG | T-A | E2 | 18,825 | 72 bp | 18,825 (16.4%) |
| 24hpi | + | 18,189 | 18,684 | CT-AC | T-A | MLP | 17,670 | 495 bp | 17,670 (15.4%) |
| 24hpi | + | 7,531 | 7,754 | GT-AG | T-A | MLP | 12,319 | 223 bp | 12,319 (10.7%) |
| 24hpi | + | 20,223 | 20,419 | GT-AG | T-A | E3 | 10,583 | 196 bp | 10,583 (9.2%) |
| 24hpi | + | 4,360 | 7,454 | GT-AG | T-A | MLP | 7,128 | 3,094 bp | 7,128 (6.2%) |
| 24hpi | + | 18,350 | 20,162 | GT-AG | T-A | E3 | 6,619 | 1,812 bp | 6,619 (5.8%) |
| 24hpi | + | 18,768 | 20,162 | GT-AG | T-A | E3 | 5,207 | 1,394 bp | 5,207 (4.5%) |
| 24hpi | + | 18,350 | 18,717 | GT-AG | T-A | E3 | 3,930 | 367 bp | 3,930 (3.4%) |
| 24hpi | - | 18,751 | 20,668 | GT-AG | T-A | E2 | 3,870 | 1,917 bp | 3,870 (3.4%) |
| 24hpi | + | 7,807 | 13,610 | GT-AG | T-A | MLP | 2,553 | 5,803 bp | 2,553 (2.2%) |
| 24hpi | + | 7,807 | 12,238 | GT-AG | T-A | MLP | 2,446 | 4,431 bp | 2,446 (2.1%) |
| 24hpi | + | 7,807 | 22,492 | GT-AG | T-A | MLP | 1,642 | 14,685 bp | 1,642 (1.4%) |
| 24hpi | + | 1,655 | 1,964 | GT-AG | T-A | E1 | 1,395 | 309 bp | 1,395 (1.2%) |
| 24hpi | + | 7,807 | 18,717 | GT-AG | T-A | MLP | 1,391 | 10,910 bp | 1,391 (1.2%) |
| 24hpi | - | 18,189 | 18,684 | GT-AG | T-A | E2 | 1,124 | 495 bp | 1,124 (1%) |
| 24hpi | - | 18,751 | 21,128 | GT-AG | T-A | E2 | 1,124 | 2,377 bp | 1,124 (1%) |
| 24hpi | + | 20,223 | 20,894 | GT-AG | T-A | E3 | 1,208 | 671 bp | 1,208 (1%) |

Table 2c: Most abundant splice junctions at 72h.p.i

| **Timepoint** | **Strand** | **Start** | **End** | **Splice\_Site** | **Splice   Acceptor-Donor** | **Region** | **Reads** | **Intron Length** | **Reads\_Percentage** |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 72hpi | + | 7,531 | 7,754 | GT-AG | T-A | MLP | 322,677 | 223 bp | 322,677 (15.1%) |
| 72hpi | + | 4,360 | 7,454 | GT-AG | T-A | MLP | 179,607 | 3,094 bp | 179,607 (8.4%) |
| 72hpi | - | 18,087 | 18,159 | GT-AG | T-A | E2 | 161,336 | 72 bp | 161,336 (7.6%) |
| 72hpi | + | 18,189 | 18,684 | CT-AC | T-A | MLP | 146,425 | 495 bp | 146,425 (6.9%) |
| 72hpi | + | 20,223 | 20,419 | GT-AG | T-A | E3 | 93,238 | 196 bp | 93,238 (4.4%) |
| 72hpi | + | 7,807 | 13,610 | GT-AG | T-A | MLP | 81,420 | 5,803 bp | 81,420 (3.8%) |
| 72hpi | + | 7,807 | 12,238 | GT-AG | T-A | MLP | 77,616 | 4,431 bp | 77,616 (3.6%) |
| 72hpi | + | 18,768 | 20,162 | GT-AG | T-A | E3 | 45,062 | 1,394 bp | 45,062 (2.1%) |
| 72hpi | + | 1,655 | 1,964 | GT-AG | T-A | E1 | 38,491 | 309 bp | 38,491 (1.8%) |
| 72hpi | + | 18,350 | 20,162 | GT-AG | T-A | E3 | 38,841 | 1,812 bp | 38,841 (1.8%) |
| 72hpi | + | 18,350 | 18,717 | GT-AG | T-A | E3 | 35,490 | 367 bp | 35,490 (1.7%) |
| 72hpi | + | 304 | 1,616 | GT-AG | T-A | E1 | 25,041 | 1,312 bp | 25,041 (1.2%) |
| 72hpi | - | 18,751 | 20,668 | GT-AG | T-A | E2 | 26,338 | 1,917 bp | 26,338 (1.2%) |
| 72hpi | + | 7,807 | 12,904 | GT-AG | T-A | MLP | 21,946 | 5,097 bp | 21,946 (1%) |
| 72hpi | + | 7,807 | 22,492 | GT-AG | T-A | MLP | 21,891 | 14,685 bp | 21,891 (1%) |