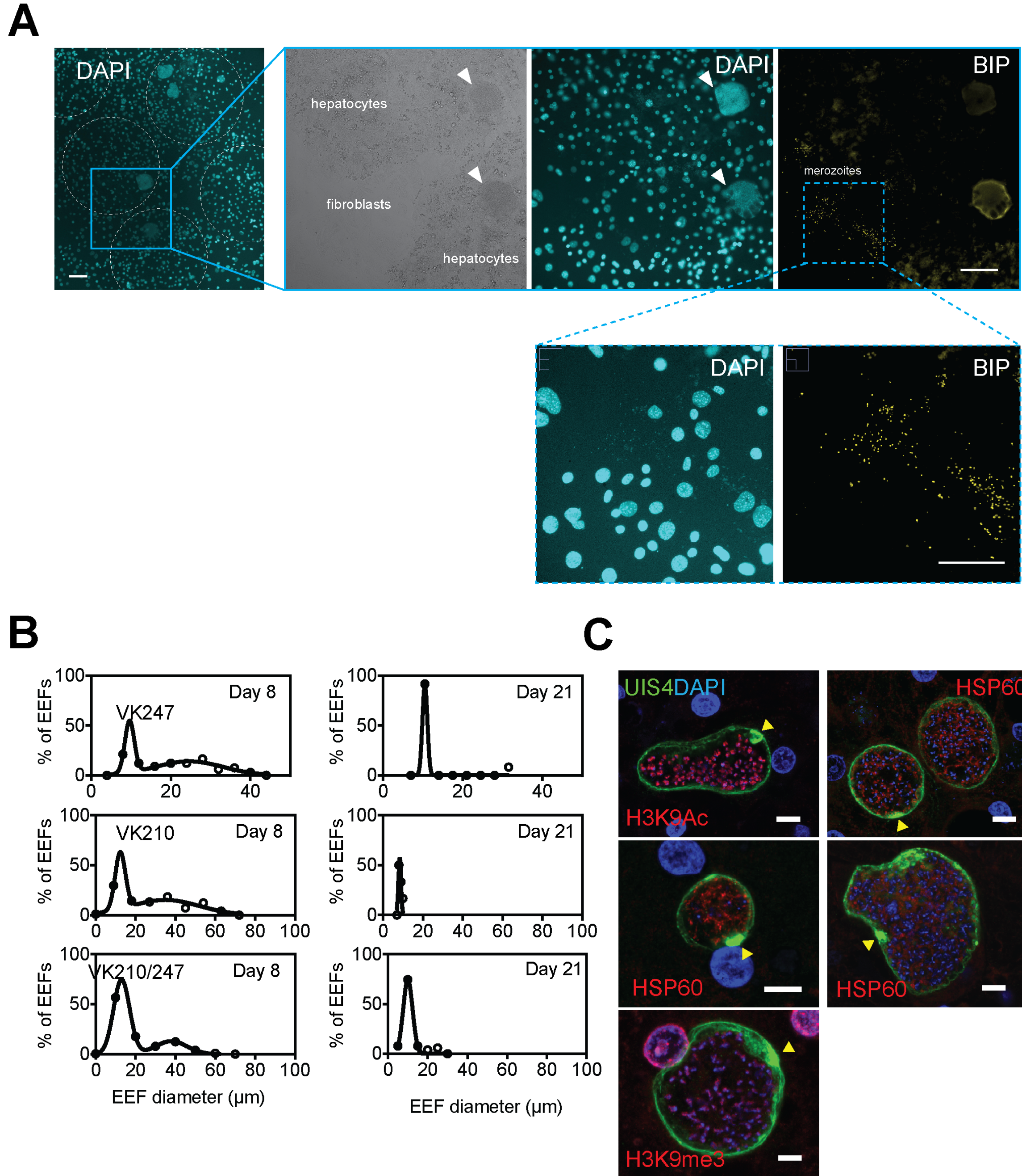


**Figure S1. Hypnozoites persist and schizonts develop in the MPCCs. (Related to Figure 1).**
(A) Free merozoites in MPCCs. *P. vivax* (VK210)–infected cultures were fixed on day 10 and stained with BIP and DAPI. Top left panel depicts DAPI staining of cultures. Hepatocyte islands are marked with white dashed circles. Close up of islands reveal two schizonts (white arrows) and free merozoites (on day 11, four out of seven wells and two out of six wells, in two independent experiments) that are positive for both *P. vivax* BIP and DAPI (bottom panels). Scale bars, 100 µm.
(B) Histograms of EEF diameters (on days 8 and 21) of 3 independent experiments with 3 separate clinical isolates denoted VK247, VK210 or, in one case, a mixed infection.
(C) UIS4 staining of large forms revealed prominence. A coalescence of UIS4 staining (yellow triangles) was observed in large forms as well as small forms (**Fig. 1c**). This staining pattern of UIS4 is observed in ~40% of all schizonts on day 8 (2 independent experiments, triplicate wells per experiment). Scale bars, 10 µm.

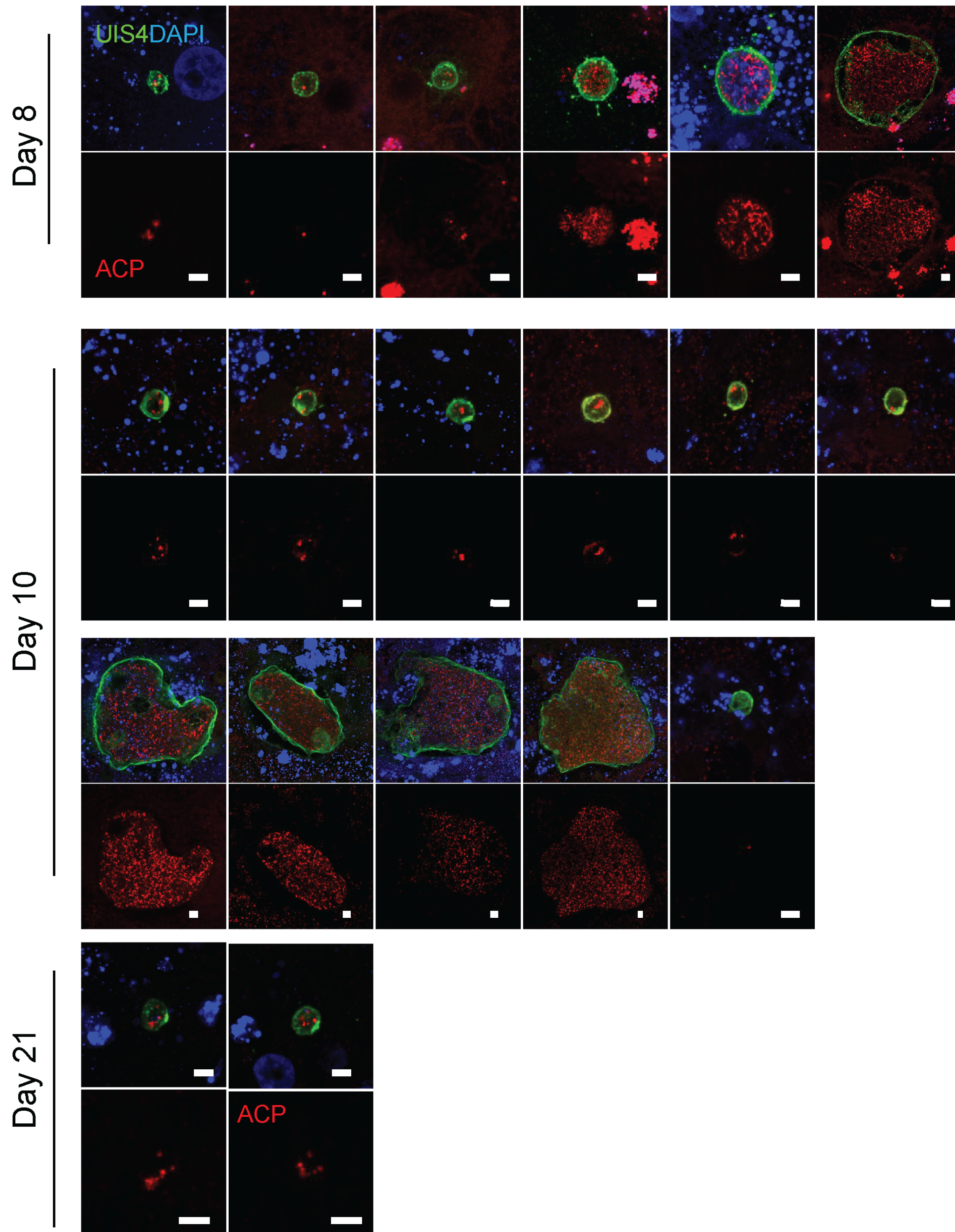


**Figure S2. The apicoplast of the parasites develop in the MPCCs. (Related to Figure 1).**
The apicoplast expands as the parasite develops. A collection of parasites from cultures fixed on days 8, 10, and 21 are shown. The apicoplast was visualized by immunofluorescence with an antibody against ACP (red), and the combined channels (top image in each pair) mark the locations of DAPI nuclear (blue) and UIS4 membrane (green) stains. Scale bars, 5 µm.

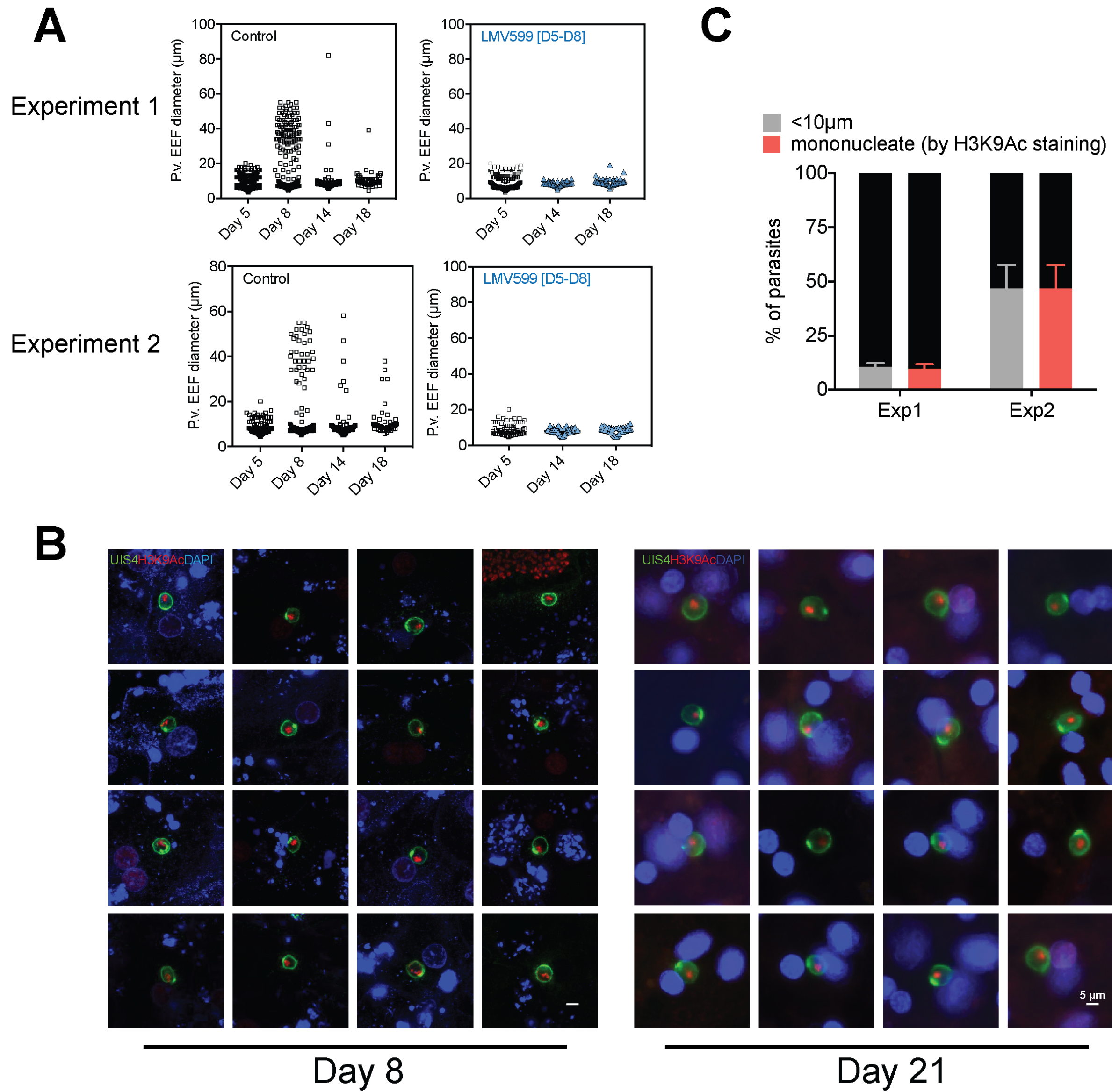


**Figure S3. *In vitro* hypnozoites are uninucleate and have the potential to reactivate. (Related to Figure 2).**
(A) MPCCs were infected with *P. vivax* (VK210) in 2 separate experiments. In both cases, cultures were either kept in media (left panels, black) or treated from day 5 to day 8 (radical cure mode) with 1 µM of a PI(4)K inhibitor (right panels, blue). Cultures that were treated with the PI(4)K inhibitor were fixed on days 14 and 18.
(B) Two different categorization methods were used to identify hypnozoites. In two independent experiments, use of either a size threshold or phenotypic histone staining patterns to measure hypnozoite frequency reveals that all hypnozoites identified as having a single nucleus (using H3K9Ac staining) were also smaller than 10 µm, indicating that size categorization may be used as a proxy for hypnozoite identification. Two representative experiments with different hypnozoite frequencies are shown (mean ± s.e.m. from triplicate wells).
(C) Hypnozoites in untreated day 8 and day 21 cultures stained for nuclei (DAPI, blue; H3K9Ac, red), and membrane (UIS4, green). Scale bars, 5µm.

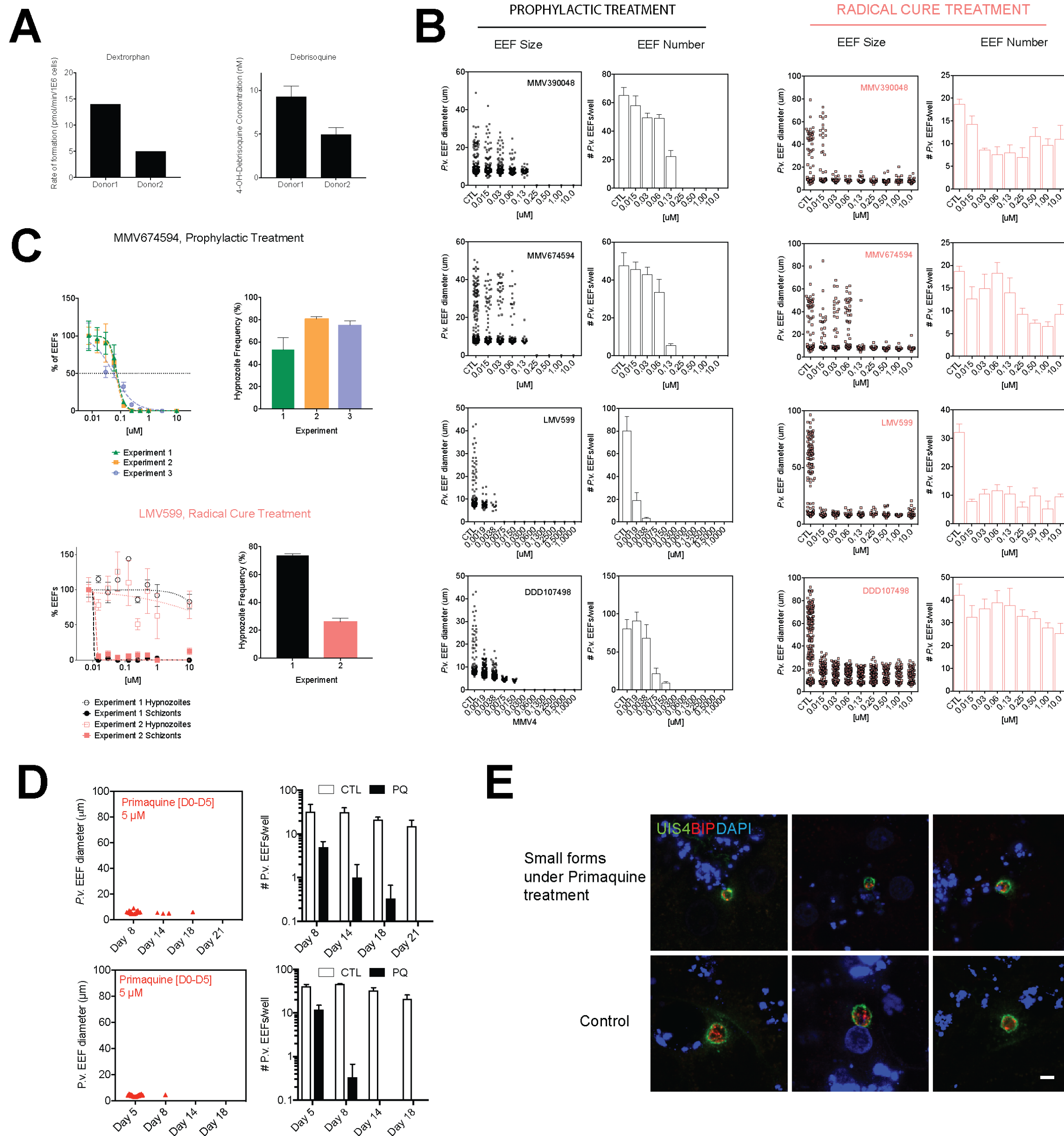


**Figure S4. MPCCs as an antimalarial testing platform reveal unique phenotypes. (Related to Figure 3).**
(A) Rate of formation of Dextrorphan by the two donors tested, as supplied by the vendor, was plotted (left panel). 100uM of Debrisoquine, in media, was added to wells and collected 1 hour later. The amount of 4-OH Debrisoquine was measured in the collected supernatant (right panel).
(B) Six antimalarials (4 shown here, rest in Figure 3C-D) were tested in prophylactic (left, black) and radical cure (right, pink) regimens (range: 1.9 nM to 10 µm). Cultures were fixed on day 8 and the diameter (squares) and number (black bars) of remaining EEFs were assayed (mean ± s.e.m. from triplicate wells).
(C) MMV674594 was tested, in prophylactic mode, in 3 independent experiments with 3 clinical isolates. IC50s were plotted. Hypnozoite frequencies, in control wells, of the 3 clinical isolates used are indicated in the bar graphs on the right.
(D) In 2 independent experiments, cultures were treated with primaquine in prophylactic mode. Size (red triangles), numbers (bars) and IC50s (circles) of parasites remaining in culture were plotted. Numbers of parasites under primaquine treatment was compared to numbers of parasites in control wells (mean ± s.e.m. from triplicate wells). All remaining forms were small in size and cleared at time points later than day 8.
(E) Residual small forms remain in culture after treatment with primaquine (5 µM). A population of small forms remained in culture on day 8 (3 days after the removal of primaquine). Scale bar, 5 µm.

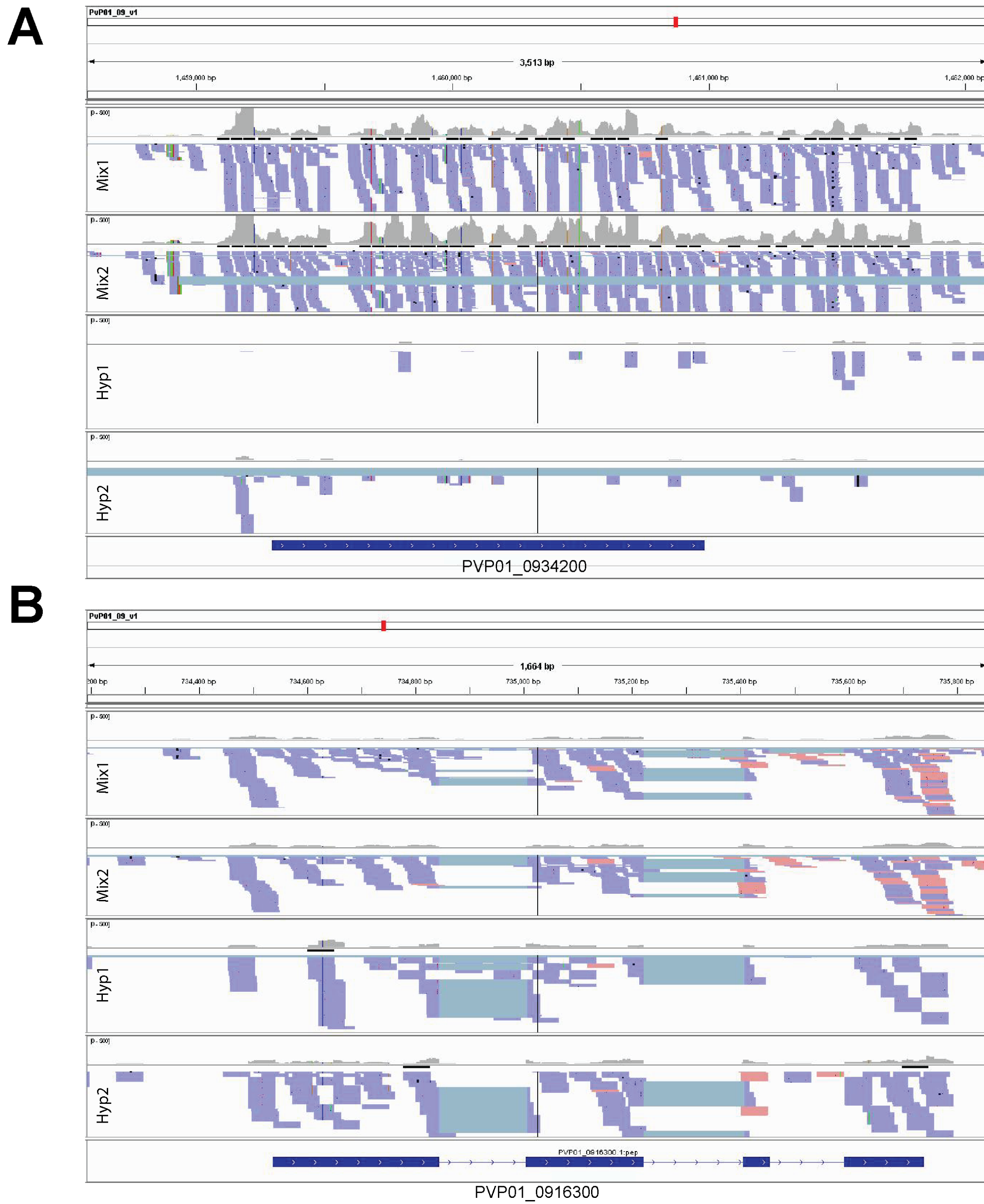


**Figure S5. RNA-seq read distribution over annotated transcripts along the *P. vivax* reference genomic sequence. (Related to Figure 4).**

A-B) Highlighting read distribution across PVP01_0934200 (AMA1) and PVP01_0916300 (AP2) which follows the annotated gene structure, with minimal intronic coverage and presence of intron-spanning reads in the hypnozoite samples. Screenshots were taken using the Integrated Genome Viewer (IGV) software.

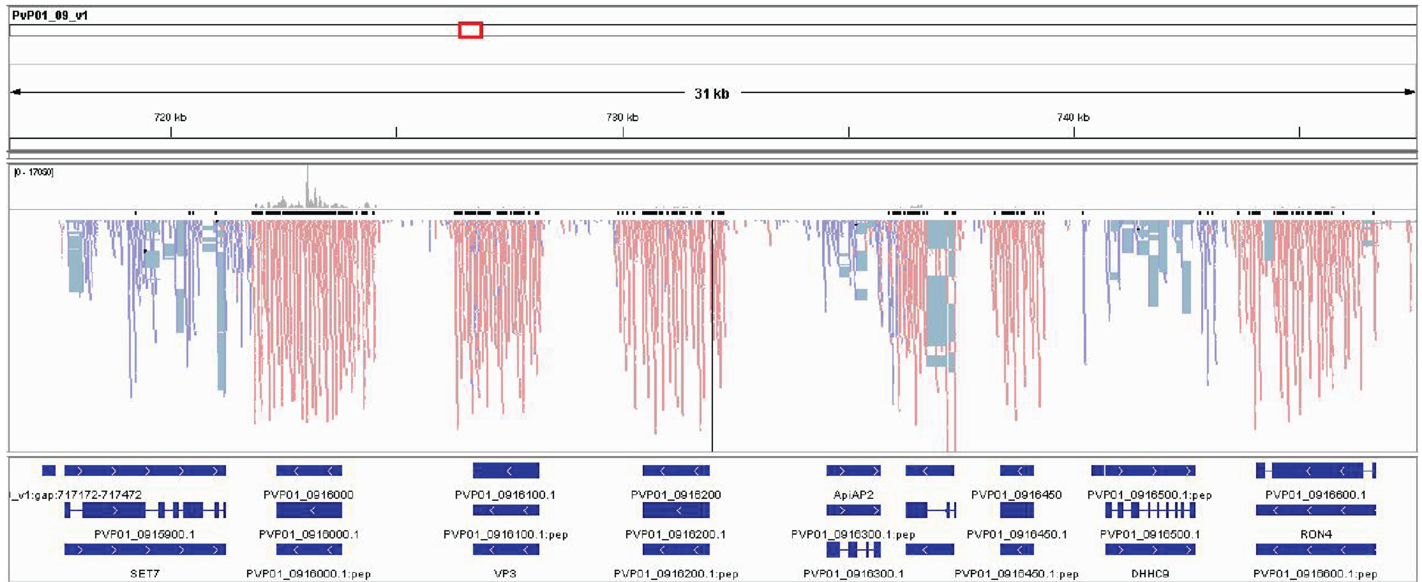


**Figure S6. Strand specificity of hybrid capture RNA-seq transcriptome (Related to Figure 4).**
Purple read pileups correspond to genes encoded on the 5' --> 3' strand; pink read pileups correspond to genes encoded on the 3' --> 5' strand, as expected based on the reverse-strand library-preparation protocol. Example provided from Mix1 samples.

| Gene | Annotation | Forward | Reverse |
|---|---|---|---|
| PVP01_0934200 | apical membrane antigen 1 | CACAGTTCTGGGGTTCGAGTGGATTT | AACCTCCATCCTTCAGCCTCTGATCT |
| PVP01_1255900 | elongation factor 2, putative | AAGCTAGGGCAAATTACCTACACA | CCACACAGTGCACCTTAATTTCAT |
| PVP01_1024200 | phosphatidylinositol 4-kinase, putative | GGACACCTCATCCACATAGACTAC | TTCTGACTTTTCTCCGTCCATGAT |
| PVP01_0216000 | AP2 domain transcription factor AP2-L, putative | AATAACGTTGGTGGGGGCAGCA | TTTGACATCCGGGTTGGCACCA |
| PVP01_1016100 | AP2 domain transcription factor AP2-Q, putative | CGGAAGAGATGCAGCGCGATGAATCA | ACATTCCCCCGCTCGATGTTCAAACC |
| PV01_0916300 | AP2 domain transcription factor, putative | AAGGGAACGAACAGGCCAGCTT | TTTGGCGCGACTTAGGTGCACA |
| PVP01_1213400 | 60S ribosomal protein L18-2, putative | ACGAATTTTGGATGCCGGGGGA | TTTCTTCCCTTGGAGCGGACGT |

**Table S2. Primers used for quantitative RT-PCR. (Related to Figure 4).**