

**Amplify *pfama1* gene with heminested primers containing multiplex identifying (MID) sequences**

- The heminested (two-step) PCR approach increases ability to amplify *pfama1* from low parasitemia samples.
- Nested PCR is performed in duplicate to improve haplotype frequency estimates and identify potential false haplotypes due to PCR errors.
- MID sequences are “barcodes” located at the 5'-end of the forward PCR primer that provide a unique ID when PCR amplicons are pooled (in this case 24 separate MID).

**Visualize PCR amplicons via agarose gel electrophoresis**

- PCR amplicons are visualized using gel electrophoresis and positives are selected based on the correct size band.
- PCR amplicons are purified to remove primers, PCR enzymes, salts, and dNTPs.
- Nucleic acid concentrations for positive PCR amplicons are determined to ensure that the same concentration of nucleic acid from each amplicon is added during index preparation.

**Index MID amplicon sets**

- Amplicon pools are constructed so that each unique MID is represented only once in each of the pools.
- A unique index (another barcode) is then ligated to each pool, making each initial PCR amplicon uniquely identifiable based on the combination of MID and index barcode sequence.

**Prepare deep sequencing libraries**

- A sequencing library is then prepared for each indexed amplicon pool according to the manufacturer's protocol adding appropriate sequencing adapters (in this case IonTorrent).

**Run amplicon deep sequencing on the Ion Torrent PGM Platform**

- Library nucleic acid concentration is determined for each library so that equal concentrations of each library are added to the Ion 318 chip.

**Perform data analysis using the SeekDeep Bioinformatics Pipeline**

- Extractor step: demultiplexes samples based on MID sequence/barcodes, performs quality filtering steps.
- Qluster step: determines haplotypes and frequency data from sequencing reads for each sample.
- ProcessClusters step: removes chimeric sequences, compares replicates, determines haplotypes/haplotype frequencies for each sample, and produces final population haplotypes.