

**Figure 3 – supplement 1. Workflow of focused classification with residual signal subtraction for the AcrABZ-TolC/MBX3132 pump reconstructed with C3 symmetry imposition.** (A)A soft mask is generated in the target region of the pump complex for focused classification. (B) Generation of three maps with the part of the complex to be ignored in focused classification. (C) Generation of modified particle images for focused classification by subtraction of the 2D projections of the maps in (B) from raw images. The modified images are CTF corrected. (D) The focused 3D classification procedure yielded two principal classes, only one class showed ligand density in the hydrophobic trap of AcrB. (E) Particles were classified by their ligand states (TTT, LTT, LLT) according to the classification result in d. The LLL state (not shown) was only observed in a few particles. (F) FSC profile for the particle reconstructions with C3 symmetry using particle images classified as TTT state. The dotted grey line shows the profile in which the phases were randomized at 5 Å resolution. (G) Colour-coded 3D map of AcrABZ-TolC/MBX3132 based on resolution variations obtained with ResMap (Methods).