



**Figure S4. Reaction of Alexa-680 labeled Ubc1~Ub<sup>K48R</sup> with both untethered ubiquitin and Ubc1-Ub<sup>Cys</sup> disulfide.** Coomassie stained image (top) and fluorescent image at 700 nm (bottom) of Ubc1~Ub<sup>K48R</sup> thioester reactions. Thioester reactions were performed as described in the text for 30 min at 37 °C and reacted with Ubc1-Ub<sup>Cys</sup>, ubiquitin, or Ub<sup>K48R</sup>. Reaction components are listed above each gel with a (+) or (-). All components for thioester formation are signified in lane 1 (\*), while Ubc1~Ub<sup>K48R</sup> thioester for all other lanes contain no E1 or ATP. Unconjugated E1 and E2 are visualized with reactive Alexa dye, and DTT is used for reducing disulfide bonds (lanes 4, 7 and 9). Reaction products are shown with filled black / yellow circles. Molecular weight standards are listed to the left and protein species to the right of each gel.