Assaying for enzymatic activity is a persistent bottleneck in biocatalyst and drug development. Existing high-throughput assays for enzyme activity tend to be applicable only to a narrow range of biochemical transformations, whereas universal enzyme characterization methods usually require chromatography to determine substrate turnover, greatly diminishing throughput. We present an enzyme activity assay that allows the high-throughput mass-spectrometric detection of enzyme activity in complex matrices without the need for a chromatographic step. This technology, which we call probing enzymes with click-assisted NIMS (PECAN), can detect the activity of medically and biocatalytically significant cytochrome P450s in cell lysate, microsomes, and bacteria. Using this approach, a cytochrome P450BM3 mutant library was successfully screened for the ability to catalyze the oxidation of the sesquiterpene valencene.