Recent improvements in the speed and sensitivity of liquid chromatography-mass spectrometry systems have driven significant progress toward system-wide characterization of the proteome of many species. These efforts create large proteomic datasets that provide insight into biological processes and identify diagnostic proteins whose abundance changes significantly under different experimental conditions. Yet, these system-wide experiments are typically the starting point for hypothesis-driven, follow-up experiments to elucidate the extent of the phenomenon or the utility of the diagnostic marker, wherein many samples must be analyzed. Transitioning from a few discovery experiments to quantitative analyses on hundreds of samples requires significant resources both to develop sensitive and specific methods as well as analyze them in a high-throughput manner. To aid these efforts, we developed a workflow using data acquired from discovery proteomic experiments, retention time prediction, and standard-flow chromatography to rapidly develop targeted proteomic assays. We demonstrated this workflow by developing MRM assays to quantify proteins of multiple metabolic pathways from multiple microbes under different experimental conditions. With this workflow, one can also target peptides in scheduled/dynamic acquisition methods from a shotgun proteomic dataset downloaded from online repositories, validate with appropriate control samples or standard peptides, and begin analyzing hundreds of samples in only a few minutes.