

PRG2010 Summary. ID# 20091

We operate an academic proteomics core focusing on both fee-for-service and collaborative research. Samples were analyzed by a facility member with less than 6 months of hands on mass spectrometry experience, although several additional members of the facility, each with many years of experience, assisted with experimental design and data analysis. Our identification of the contents of tube 1, an explanation for the differences in ubiquitin ligase activity between tubes 1-3, and our investigation of the nature of the doublet observed in tube 1 are described below:

Identify the contents of Tube 1:

In order to understand why the ubiquitin ligase reaction failed in tube 2 but was restored in tube 3, we decided to use a shotgun approach which we thought would—given the relatively low complexity of the samples based on supplied gel images—identify all of the major/minor components of each tube. The contents of each tube were first resuspended in 20 µl of ammonium bicarbonate, pH 8 containing 0.1 % Rapigest. A protein assay revealed that each tube had ~1.5 µg or less protein, and since we were not attempting to quantify protein levels across the samples, we simply subjected 5 µl of each sample to reduction/alkylation and overnight digestion with 1:50 trypsin. After workup, we analyzed each sample by 1D-UPLC-MS/MS with a Q-TOF instrument operating in DDA mode using a 120 min gradient from 5-40% acetonitrile (Note: based on the supplied gel images, we would have used a 30 min gradient for an actual customer sample). Raw data was processed using Mascot Distiller and data was searched against the SwissProt database using fixed [carbidomethyl (C)] and variable [deamidated (NQ); GG (K); oxidation (M)] modifications as well as ¹⁵N metabolic labeling for identifying the isotopically labeled proteins in tubes 2-3. Search results were analyzed and visualized using Scaffold.

The contents of tube 1 contained peptides derived from human keratin (at low levels), porcine trypsin (from auto-proteolysis) as well as bovine serum albumin, which presumably was present as a stabilizer of one of the proteins. In addition, we identified:

Uniprot	Name	N Isotope	Confidence	Unique Peptides	% Coverage	Notes
P35222	Human catenin beta-1	14	High	17	25	1
Q9HB71	Human calyculin-binding protein	14	High	6	24	
P63208	Human S-phase kinase-associated protein 1	14	High	7	26	
Q8IUQ4	Human Siah1	14	High	8	18	
P62988	Human ubiquitin	14	High	2	33	
P0A6Y8	E. coli DnaK	14	High	3	6.7	2
P30801	Rabbit S100-A6	14	Med	1	7.8	3

Notes:

1. The protein sequence given for the recombinant beta-catenin began with **GGIL**HAVVNLINYQDDAELATR, where GGIL was derived from an epitope tag and His134 was the first residue of beta-catenin which was expressed in the recombinant protein. However, we identified the tryptic peptides ¹²⁴LAEPSQMLK¹³³¹³⁴HAVVNLINYQDDAELATR¹⁵² and, representing residues 124-133 of beta-catenin. We concluded that either the sample contained some native catenin, or the supplied sequence was incorrect.

2. The chaperone DnaK commonly co-purifies with recombinant proteins expressed in E. coli.

3. Only one peptide to S100-A6 (calcyclin) identified. However, since calcyclin-binding protein was also present in this tube, and because S100-A6 was also identified in tubes 2-3, we have a higher than normal confidence in this ID.

Identify what is different in Tube 2 that might explain why the reaction failed and is restored in Tube 3 that might explain why the reaction is functional again.

None of the contents of tube 1 were ¹⁵N-labeled. However, both beta-catenin, DnaK and an additional protein were ¹⁵N-labeled in tubes 2-3. Tube 2 contained ¹⁵N-labeled E. coli yodA (6 unique peptides; 20% coverage) rather than the ubiquitin E3 ligase Siah1. This explained why the ligase reaction failed in tube 2. Dr. Quickhands appears to have mistakenly added YodA instead of Siah1. On the other hand, tube 3 correctly contained ¹⁵N-labeled Siah1, consistent with the ubiquitin ligase activity being restored in this last tube.

What is the nature of the unusual doublet that is present only in Tube 1, but does not seem to be related to function?

To interrogate the nature of the doublet in tube 1, we subjected the remaining protein to 1D-SDS-PAGE, and after staining with Colloidal Coomassie, performed an in-gel tryptic digestion of the resolved doublet bands. Extracted peptides were analyzed as described above, except that a 30 min acetonitrile gradient was used for LC separation. Beta-catenin was identified as the principal component of each of these bands. However, we could not conclusively determine any differences between the doublet bands. The upper band had the only hit to the peptide ¹³⁴HAVVNLINYQDDAELATR¹⁵² and many more hits to the peptide ¹²⁴LAEPSQMLK¹³³ as compared to the lower band. Although the source of these peptides is as yet unresolved (see Note 1, above), we can only speculate that, relative to the upper band, the lower band represents an N-terminal truncated form of the beta-catenin protein.