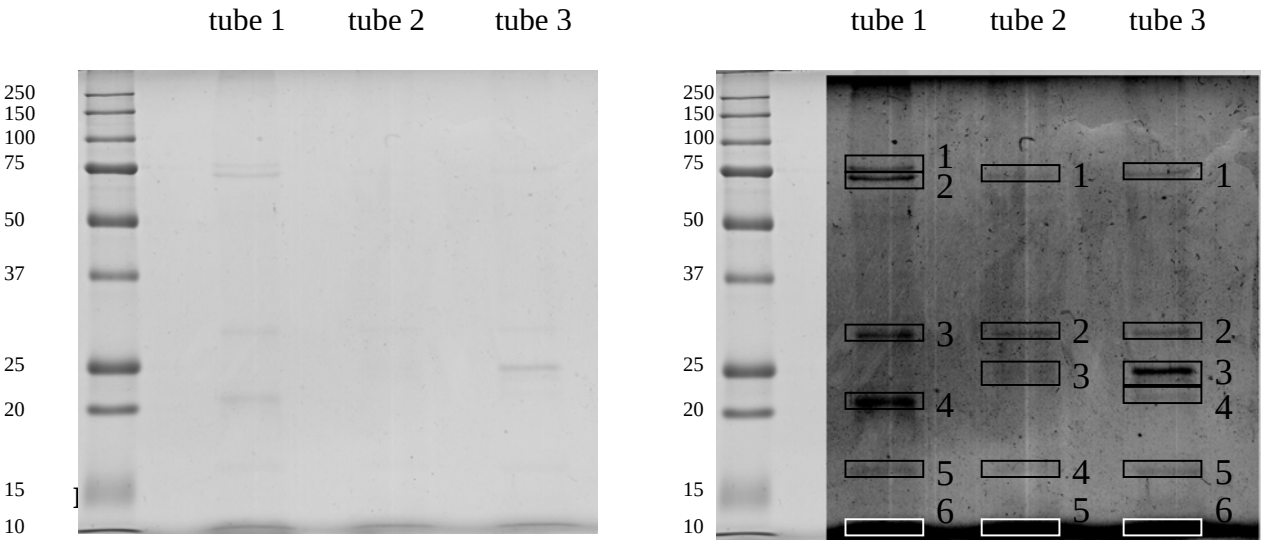
1)- 1D-SDS-PAGE

The samples were resuspended in 20 µL of TNE buffer (50 mM Tris.HCl, pH7.6, 150 mM NaCl, 2mM EDTA, pH 8.0, and 1% (v/v) NP-40). 10 µL of each were mixed 1:1 with 2xSDS Sample buffer, boiled for 5 min, and run on a 12.5% acrylamide SDS-PAGE gel. The gel was then stained with Colloidal coomassie (Images below, right image over-exposed):



The noted bands were excised from the gel and cut in two parts. A half of each band was subjected to in-gel digestion with trypsin according to the protocol described in 2). The second half of bands T2-3 and T3-3 was digested with proteinase K, as described in 3).

2) In-gel digestion with trypsin

1- Destain gel band with 50% ethanol-50mM ammonium bicarbonate (ABC), 20 min, r.t.
2- Dehydrate with 100% ethanol, 15 min, r.t.
3- Reduce with 200µL 10mM DTT-50mM ABC, 1h, 56ºC.
4- Alkykate with 200 µL 55mM IAA -50mM ABC, 30min, r.t.
5- Wash with 25mM ABC, 30 min r.t.
6- Wash with 25mM -ABC 50% acetonitrile, 2 x 30 min, r.t.
7- Dehydrate 100% acetonitrile, 10 min, r.t.
8- Rehydrate with 0.02µg trypsin (Gold, Promega) in 30 µL ABC
9- Incubate at 37ºC 3 h
10- Extract peptides adding 8 µL acetonitrile. 15 min at 37ºC
11- Extract peptides adding 130 µL 0.2% TFA, 30 min, r.t.
12- Evaporate the extract and resuspend with 25µL 0.1% formic acid (FA)

3) In-gel digestion with proteinase K

1- Destain gel band with 50% ethanol-50mMABC, 20 min, r.t.
2- Dehydrate with 100% ethanol, 15 min, r.t.
3- Reduce with 200µL 10mM DTT-50mM ABC, 1h, 56ºC.
4- Alkykate with 200 µL 55mM IAA -50mM ABC, 30min, r.t.
5- Wash with 25mM ABC, 30 min r.t.
6- Wash with 25mM -ABC 50% acetonitrile, 2 x 30 min, r.t.
7- Dehydrate 100% acetonitrile, 10 min, r.t.
8- Rehydrate with 0.02µg proteinase K in 30 µL ABC
9- Incubate at 37ºC 15min
10- Extract peptides adding 8 µL acetonitrile. 15 min 37ºC
11- Extract peptides adding 130 µL 0.2% TFA, 30 min, r.t.
12- Evaporate the extract and resuspend with 25µL 0.1% formic acid (FA)

4) LC-MS analysis

Each digest was analyzed by LC-MS. A Proxeon nano-LC system was used to separate the peptide mixtures on a 75 µm x 15 cm PepMap C18 nano column (LC Packings), coupled to the nano-ESI source of a BRUKER HCT-Ultra IonTrap Mass Spectrometer.
Mascot was used to search the MS2 spectra against the Uniprot-Swissprot database, restricting the search to either human or E. Coli proteins, or a database containing the expressed sequences of beta-catenin and Siah1 E3 ligase. Searches were performed setting the mass of nitrogen atoms to the value corresponding to 14N or 15N.

The results of protein identification in each of the bands are summarized in the following tables. All proteins identified are human, unless indicated.

Table1 : Proteins identified in Tube 1. Tryptic digestion.

Band)	Protein Name	Uniprot Accession	Label	Confidence	Unique peptides Tryp	Seq. Coverage
1	Catenin beta-1 *****	P35222	14N	High	24	34.6
2	Catenin beta-1	P35222	14N	High	31	44.6
3	Calcyclin-binding protein	Q9HB71	14N	High	11	29.4
4	E3 ubiquitin-protein ligase SIAH1	Q8IUQ4	14N	High	4	18.3
5	S-phase kinase-associated protein 1	P63208	14N	High	8	35.6
6	Ubiquitin	P62988	14N	High	5	69.7
6	Protein S100-A6	P06703	14N	Med	1	8.9

* Some peptides that are N-terminal with respect to the recombinant sequence of beta-catenin expressed were observed. The protein in this band would thus correspond to the endogenous, full-length beta-catenin.

Table2 : Proteins identified in Tube 2. Tryptic and proteinse K digestion.

Band)	Protein Name	Uniprot Accession	Label	Confidence	Unique peptides Tryp	Seq. Coverage	Unique Peptides PK (Seq. Cov.)
1	Catenin beta-1	P35222	15N	High	9	11.8	
2	Calcyclin-binding protein	Q9HB71	14N	High	6	23.7	
3	Metal-binding protein yodA (E. Coli)*	P76344	15N	High	2	11.1	4 (17.1)
4	S-phase kinase-associated protein 1	P63208	14N	High	7	34.4	
5	Ubiquitin	P62988	14N	High	5	69.7	
5	Protein S100-A6	P06703	14N	Med	1	8.9	

* 28.1% total sequence coverage by peptides observed in trypsin plus PK digestion

Table3 : Proteins identified in Tube 3. Tryptic and proteinse K digestion.

Band)	Protein Name	Uniprot Accession	Label	Confidence	Unique peptides Tryp	Seq. Coverage	Unique Peptides PK (Seq. Cov.)
1	Catenin beta-1	P35222	15N	High	9	14.1	
2	Calcyclin-binding protein	Q9HB71	14N	High	7	21.5	
3	E3 ubiquitin-protein ligase SIAH1	Q8IUQ4	15N	High	4	11.7	5 (12.7)
4	E3 ubiquitin-protein ligase SIAH1	Q8IUQ4	15N	High	2	7.5	
5	S-phase kinase-associated protein 1	P63208	14N	High	4	23.3	
5	Protein S100-A8**	P05109	14N	High	7	40.9	
5	Protein S100-A9**	P06702	14N	High	6	32.5	
6	Ubiquitin	P62988	14N	High	5	69.7	
6	Protein S100-A6	P06703	14N	Med	1	8.9	

* 24.4% total sequence coverage by peptides observed in trypsin plus PK digestion
** Some non-tryptic peptides observed. This two bands would correspond to proteolytic fragments of these proteins.

Answers to specific questions:

1) Identify the contents of Tube 1 ("I sometimes see three or four extra bands on a gel, but they are weak".

As detailed in Table 1, besides beta-catenin and Siah1 E3 ligase, the other proteins present in the mixture that could be identified are Calcyclin-binding protein (band 3) , S-phase kinase.associated protein 1 (band 5) and ubiquitin and Protein S100-A6 (band 6).

2) Identify what is different in Tube 2 that might explain why the reaction failed.

In the band that could correspond by size to the recombinant Siah1 E3 ligase (band 3), only an E. Coli protein (metal-binding protein yodA) was identified. Maybe a wrong clone was used, or the level of expression was very low. Although it can not be ruled out that some E3 ligase is also present, the concentration would be too low to observe any activity.

3) Identify what is restored in Tube 3 that might explain why the reaction is functional again.

In tube the 15N labeled 3 Siah1 E3 ubiquitin ligase is clearly identified in band 3. Band 4, of a slightly lower Mw could correspond to a degradation product of the Siah1 present in the mixture.

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

As detailed in Table 1, the upper band of the doublet (band 1) would correspond to the endogenous full-length beta catenin. This is inferred from the observation of some peptides in the tryptic digest that are N-terminal to the recombinant sequence expressed, and thus would not be present in this form.
The lower band of the doublet would correspond to the recombinant form. Only peptides within the expected sequence are observed in this case.