Your Co-IP Experiment

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Analyzing Co-IP experiments is one of the more common analyses we perform here at PSR. Because of this we have put together a quick overview discussing the concerns, procedures, and data analysis methods used in this experiment. Among the topics discussed:

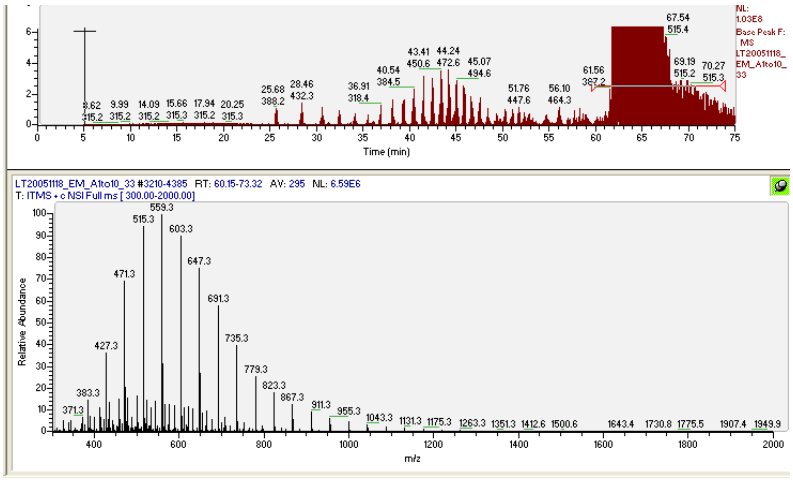
- Preparing the samples for submission.
- Our extraction and bench-work procedures.
- Mass Spectrometer analysis and data collection.
- Data analysis and interpretation.
- Approximate costs

Preparing Your Samples

Because of the procedures we use here there are a couple of things you'll want to be aware of when preparing your samples for submission.

1) Volume is an issue.

To remove the detergent and other things that may be harmful to the mass spectrometer we run the sample into a SDS-page gel. Many detergents produce strong ion series in the instrument which dwarf and suppress the signal from peptides in the sample. An



example of this can be seen on the right.

Well volume is limited to 30ul on the gel and SDS concentrations much over 10% may prevent the proteins from moving into the gel. A good goal is to have the final elution volume be under 300ul of 1% SDS. Our extraction procedure accomplishes this nicely, and is suitable in most cases. It is described on later on in this document.

2) How much protein do you need?

It can be difficult to know ahead of time just how much protein you will get from an immunoprecipitation. However it is crucial we have enough to do a good experiment. Normally you'll want to have a few micrograms of total protein after elution. As a rule of thumb, if you can see some banding on a coomassie-stained gel, you are probably in a good range for a successful Co-IP. On our end we will perform a check before we run the samples, and inform you if there looks to be too little protein to proceed.

3) What is your bait protein's sequence and the species used for the IP?

We will likely need you to provide the amino acid sequence of your bait protein. This is even more critical if the sequence differs from the native form, or is from a different species than the proteins that it is interacting with. Having the bait protein sequence helps act as an internal control and ensure the accuracy of the database search results. It is best if the sequence is sent in a text file, and stripped of spaces and other characters such as numbers indicating sequence position. However we can always do this manually if necessary.

Mutant Sequence for Co-IP

GGNFSTADQLEMMTELLGGDMVNQSFICDPDDETFIKNIIIQDCM
WSGFSAAAKLVSEKLASYQAARKDSTSLSPARGHSVCSTSSLYLQD
LTAAASECIDPSVVFPYPLNDSSSPKSCTSSDSTAFSPSSDSLLSSES
SPRASPEPLVLHEETPPTTSSDSEEEQEDEEEIDVVSVEKRQTPAK
RSESGSSPSRGHSKPPHSPLVLKRCHVSTHQHNYAAPPSTRKDYP
AAKRAKLDSGRVLKQISNNRKCSSPRSSDTEENDKRRTHNVLERQ
RRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYILSIQADEH
KLTSEKDLLRKRREQLKHKLEQLRNSGA

4) Ask us ahead of time.

Do you have questions about extraction, salt concentrations in your sample, or other things? Please send us an e-mail or call. It's much easier to work out the problems before a valuable sample has been eluted in a hard-to-work-with buffer.

Benchwork Methods

What follows is our procedure for extracting from the IP beads. This can be done either in your lab, or by a PSR employee. Volumes can be varied based on the amount of beads present. However the final SDS concentration needs to be kept below 10% for the subsequent gel run-in. Our standard

gel lane holds up to 30ul of liquid; meaning a maximum of 300ul of 1% SDS can be used for extraction.

IP Elution

Add 150ul of 1% SDS to the beads.

Vortex and put on a shaker for 5 minutes.

Centrifuge at 5000rpm for 5 min to pellet beads.

Pull off the supernatant and store in another vial.

Add 150ul of diH_2O to the beads.

Vortex and put on a shaker for 5 minutes.

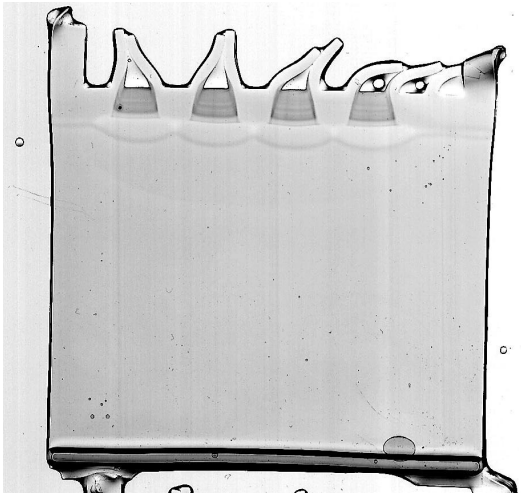
Centrifuge at 5000rpm for 5 min to pellet beads.

Pull off the supernatant and store with the previous pull-off.

Take the collection of supernatants and filter through a 0.45 micron spin filter (Millipore UFC30HV00); centrifuge at 5000rpm for 5 min.

Take the samples to dryness in a Speed-Vac or similar evaporator in preparation for gel.

Gel run-in



After the extraction the samples are then run into an SDS-page gel. The intent here isn't to generate a nice image for a publication; as you can see in the image at right the results aren't exactly picturesque. Instead we have 2 goals; the first is to filter out any detergent, salts, etc. that can interfere with the analysis. The second is to grab a quick image to verify there's a sufficient amount of protein present.

The samples are brought up in 30ul of 1x running buffer containing reducing agent, and loaded into a NuPAGE 10% Bis-Tris Gel (NP0301BOX or 148856 from research stores). Samples are never loaded in adjacent lanes to protect against any spill-over. The gel is run at 200V for 5-8 minutes.

Run times can vary as the high percentages of detergent can interfere with the protein's progression into the gel. We will usually start at 5 minutes, and add more time if the samples aren't progressing to our liking.

After the gel has finished running, it is rinsed for 5 minutes in deionized water. Next comes 30 minutes of staining in Imperial Protein Stain (Thermo Scientific, #24615), and three 10-minute long water washes to de-stain. While the times listed are less than the recommend amounts for the stain, they are more than sufficient to visualize protein that will be detectable with the mass spectrometers.

Following this the samples are imaged and cut. The region from half-way up the wells to below the dye front is excised for each sample, and chopped up into smaller fragments before being put through PSR's normal in-gel digestion protocols.

Mass Spectrometry and Data Analysis

MS/MS Analysis

Analysis on the mass spectrometer is basically the same for an IP sample as for other samples. Run times are either 90min/sample or 140min/sample and depending on the amount of material seen on the gel; unless otherwise specified. More time on the instrument is given for samples that appear more complex, simpler samples are run with less time to reduce the cost of the experiment. Control samples are run first to minimize any problems with carryover.

Data Analysis

The typical database for an IP sample will consist of the bait protein sequence, the species for the proteins you're attempting to bind, a list of about 180 common contaminate proteins (BSA, Keratins, etc.) as well as reversed sequences of all the proteins which is used as an internal control. Ideally we will get any protein sequence that has a reasonable chance of being present into the database.

Results Files

In a typical experiment results will be sent via e-mail to you, with 4 different files attached. The bulk of your results should be in the excel spreadsheet which contains the list of protein identifications. The list has been filtered somewhat to hide contaminate (i.e. keratins, BSA, etc.) and redundant entries. If you wish to view these select "all" from the dropdown menu in cell E2. The .pdf file is a walk-through of the spreadsheet, with explanations of all the rows and columns.

The 'peptide' text file contains a list of all the identified peptides in your sample. If you'd like to view this in more detail, you can open it directly in excel, and the .pdf also has some hints and tips for formatting to make it more readable. Finally the 'protein' text file is an unedited/unformatted version of the spreadsheet, and is provided as a backup in case something should happen to the original. We also will keep copies of all the data archived here for a time in case you happen to lose your data, or would like it further analyzed in the future.

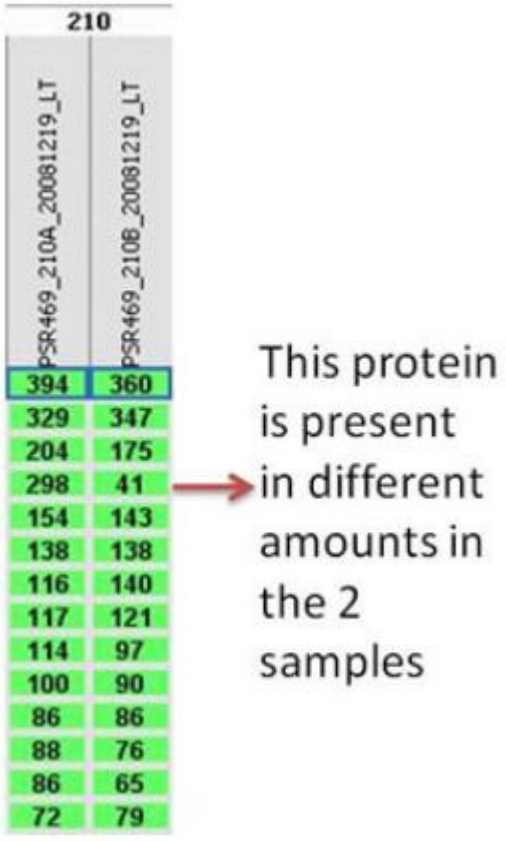
Description	Counts	Unique	UniqFra	SEA128	SEA128
[redacted] mutant sequence	25	25	1	10	15
Desmoplakin OS=Mus musculus GN	54	54	1	39	15
Nuclear factor of activated T-cells, cy	3	3	1	1	2
Carboxypeptidase D OS=Mus musc	52	52	1	27	25
Ig kappa chain V-II region 26-10 OS=	38	38	1	17	21
Ig kappa chain V-V region HP R16.7	2	2	1	2	0
Ig kappa chain V-III region PC 2880/I	6	2	0.333	4	2
Ig kappa chain V-III region ABPC 22/	4	3	0.75	1	3
Ig kappa chain V-III region CBPC 10	8	2	0.25	5	3
Ig heavy chain V-III region A4 OS=M	2	2	1	2	0
Ig kappa chain C region OS=Mus mu	70	70	1	33	37
Ig gamma-2A chain C region, A allel	28	27	0.964	13	15
Ig gamma-2B chain C region OS=Mu	9	8	0.889	3	6
Ig gamma-1 chain C region secreted	34	34	1	14	20

Quick peek at a formatted results file.

How do I know what is different?

Looking for differences in bound proteins is the most common goal of an immunoprecipitation experiment, so how do you know what proteins are present or absent between samples?

Differences in proteins found in the various IP samples is followed by the numbers of assigned MS/MS spectra (spectral counts) to individual proteins. Spectral count numbers are related to a particular protein's abundance. Ideally, the interacting proteins will only have spectra assigned in the experimental sample and not in the control sample. We also like to see at least 5 spectra assigned to a protein in the experimental sample when there are no spectra assigned to it in the control sample. This is because when proteins are in very low abundance, there is randomness in the ion selection process that produces MS/MS spectra. If the protein's abundance is extremely low and it is producing fewer than 5 assigned spectra in the



experimental sample, it could have actually been present in the control sample, but been missed due to the randomness of the ion selection process.

Sometimes it is also possible to identify potential binding partners based on differences in the relative abundance of a protein present in both the control and experimental samples. At minimum to call something different between samples we look for a total spectral count of around 10 between the two samples (so 8 counts in one sample and 2 in the other would give 10 total counts) and at least a 5-fold difference. The fold difference between the samples can be less as the total spectral count goes up (errors due to random sampling becoming relatively smaller). By the time you're around 40-50 total counts a 3-fold change should be sufficient. Of course, it is important to assure that the control IP used the same procedure as the experimental IP (same number of cells, antibody concentration, ect.).

As a disclaimer, the special count numbers you see are considered 'semi-quantitative' by the field. The quantitative data from this analysis will not be publishable on its own, and differential candidates should be further verified by repeat experiments or using other methods. Of course we are always happy to meet and discuss results, questions about the experiment, and address other concerns.

Costs

Because we charge hourly the cost of a Co-IP experiment can vary a bit from experiment to experiment. Many experiments have unique twists to them which make setting a firm price problematic. There's an adage here about no two samples being same. In the end there a number of different factors that can affect the final bill, such as:

- Do we need to create a new database from scratch?
- Are you also looking for Post-Translational Modifications such as phosphorylation?
- Did we perform the extraction step here?
- Do the samples need a shorter or longer method on the Mass Spectrometer?
- Is your lab at OHSU, or at another company or university?

Given all that, the price will usually range from about $400 to $650 for OHSU labs and about 50% higher for external for-profit companies. However searching for post-translational modifications will often drive the price above that range.

If you have questions involving pricing feel free to contact us before starting your experiment. We can always give a quote if necessary.