Background proteins in immunoprecipitations: what they are & how to reduce them

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Introduction

Immunoprecipitation (**IP**) is a mainstream tool to study protein-protein interactions

Nonspecific background proteins occurring in IP's are a major problem, resulting in :

- Increased sample complexity, noise
- False positives
- False negatives

Goals

1) Characterize nonspecific background proteins in IP's

2) explore the origins of the background

3) find ways to reduce it

Survey : Identify and align protein lists from 10 negative control IP's

10 mock IP's {
Varying : lab of origin, cell line, lysis method, IP protocols
Constant : human cells, Ab-based, use ProteinA-Sepharose
Constant : analytical pipeline by GeLC-MS , data analysis

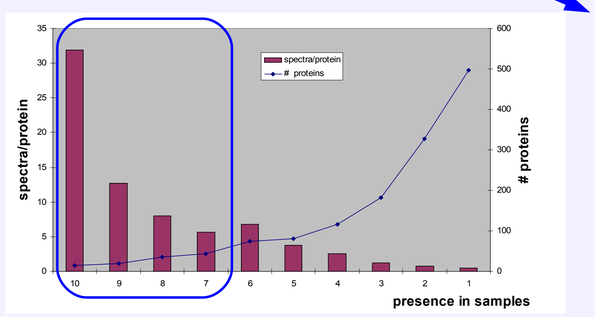
Results :

1393 proteins identified combining all samples (1 pep. >95% , prot.>95% probability)

Great variability : only 15 proteins present in all samples

However : most ubiquitous proteins are most abundant ones (based on spectral counts)

Define arbitrarily : "**frequent contaminants**" detected in 7 or more samples : **115 prots**



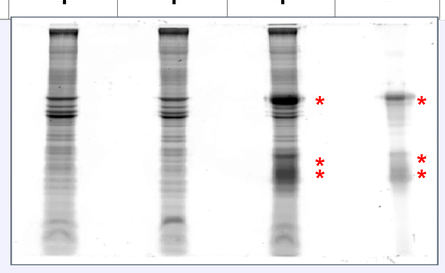
Ribosomal proteins	30
Heterogeneous nuclear ribonucleoproteins	11
Keratins	7
Heat shock proteins	6
Elongation factors	6
Helicases	5
Nucleolar proteins	3
Replication factors	3
Other chaperones	3
Tubulins	2
Histones and associated	2
Exportins	2
Poly(RC)-binding proteins	2
ATP synthases	2
Actin and actin-bdg.	2
Chaperones	2
Others	29

Due to the large variability in the pool of recovered proteins, the data cannot be used to determine conclusively a list of contaminant proteins.

Frequent contaminants are constituted in majority by proteins from a few functional families.

Which component generates the background ?

Sepharose CL-4B	+	-	-	-
ProteinA-Sepharose-CL4B	-	+	+	-
Human IgGs	-	-	+	+
Incubation w. cell lysate	+	+	+	-



Background appears to be due mainly to protein binding to the Sepharose CL-4B resin

* IgG light, heavy chain

Conclusions :

1 - Survey of 10 mock IP's shows great variability and low predictive value but gives indications on the nature of the most recurrent background proteins

2 - A large portion of the background is due to protein binding to Sepharose beads

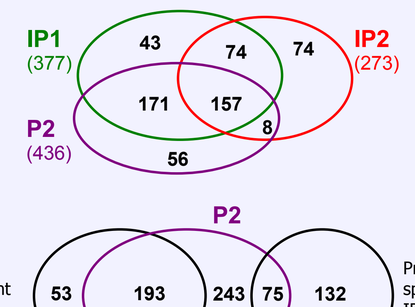
3 - Pelletable aggregates form during 4°C incubation of lysates. Pellet is larger if the lysate is treated with benzonase (RNase+DNase activity)

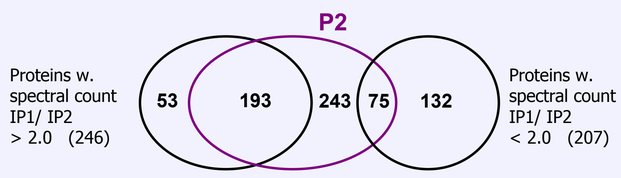
4 - Proteins found in the aggregate pellet contain most of the "frequent contaminants" found in IP's.

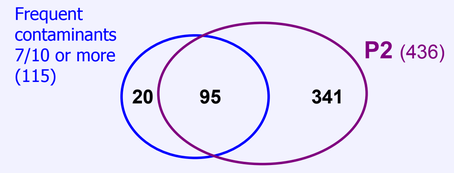
5 – A second centrifugation step before addition of beads drastically reduces level of background in IP's => **improved IP protocol !**

HYPOTHESIS : insoluble aggregates formed after lysis adhere to Sepharose beads and are responsible for a large part of the background. Formation of such aggregates is correlated with the presence of nucleic acids

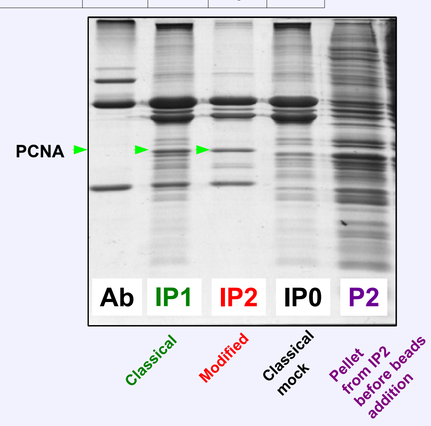
Test the modified protocol (anti-PCNA IP) and MS analysis







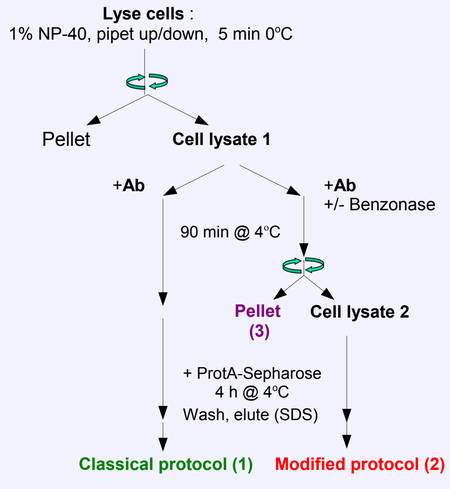
ProtA-Sepharose	-	+	+	+
α-PCNA Ab	+	+	+	-
Cell lysate	-	+	+	+
Benzonase + spin	n/a	-	+	-



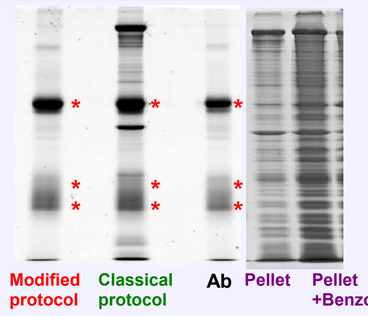
Most "frequent contaminants" are also found in pellet P2.

Aggregate removal in the **modified protocol (IP2)** leads to cleaner IP's

Which step generates the background ?



IgGs	+	+	+	+	+
ProtA-Sepharose	+	+	-	-	-
Cell lysate	+	+	-	+	+
Benzonase	+	-	+	-	+
Spin before adding beads	+	-		+	+
Pellet before IP	-	-	-	+	+



Pelletable aggregates form during 4C incubation of a 1% NP-40 cell lysate. A **modified protocol** was designed which included removal of these aggregates.

Materials & Methods :

IP's for the survey were from 6 different labs and performed in varying conditions. Common points were : 1) starting material was cultured human cells 2) IP was performed using antibodies against a protein or an epitope tag 3) Affinity resin was based on ProteinA-Sepharose.
IP's for method development: HeLa cells were lysed in 1% NP40, 1mM MgCl2, 150mM NaCl, protease inhibitors by pipetting and 5 min incubation at 4C. Human IgGs were from Sigma-Aldrich. The anti-PCNA antibody was from Santa Cruz Biotechnology. 20 ul ProteinA-Sepharose CL-4B (GE Healthcare) slurry was used for immunoprecipitations. Benzonase (Merck) was used at a concentration of 125 U/ml lysate. Other steps were as described in the boxes.

MS Analysis : all proteins were eluted from the beads by boiling in 2%SDS sample buffer and subsequently separated along 2 cm on a 10% polyacrylamide gel. Whole gel lanes were excised from the gel into 5 fractions which were digested with trypsin. Dried extracts were analysed by nano-rp-LC-MS/MS on an LTQ-Orbitrap coupled to an Agilent nano 1100 HPLC system. Exported peptide MS/MS data were pooled for each lane and used for database searches in UNIPROT using Mascot 2.2 (10 ppm mass tolerance on precursors and 0.5 Da tolerance in MS/MS). Protein lists were validated and aligned with Scaffold 2.2 with the following thresholds : at least one peptide with min. 95% probability, minimum protein probability 95%