Title
Dissecting Dynamic and Heterogeneous Proteasome Complexes Using In Vivo Cross-Linking-Assisted Affinity Purification and Mass Spectrometry

Authors
Wang, Xiaorong
Huang, Lan

Publication Date
2018

DOI
10.1007/978-1-4939-8706-1_25

Peer reviewed

HHS Public Access

Author manuscript

Methods Mol Biol. Author manuscript; available in PMC 2020 January 06.

Published in final edited form as:

Methods Mol Biol. 2018 ; 1844: 401–410. doi:10.1007/978-1-4939-8706-1_25.

Dissecting Dynamic and Heterogeneous Proteasome Complexes Using *In Vivo* Cross-linking Assisted Affinity Purification and Mass Spectrometry

Xiaorong Wang, **Lan Huang**

Department of Physiology & Biophysics, University of California, Irvine, CA 92697

Abstract

Protein-protein interactions are essential for protein complex formation and function. Affinity purification coupled with mass spectrometry (AP-MS) is the method of choice for studying protein–protein interactions at the systems level under different physiological conditions. Although effective in capturing stable protein interactions, transient, weak and or dynamic interactors are often lost due to extended procedures during conventional AP-MS experiments. To circumvent this problem, we have recently developed XAP (*in vivo* cross-linking (X) assisted Affinity Purification) MS strategy to better preserve dynamic protein complexes under native lysis conditions. In addition, we have developed XBAP (*in vivo* cross-linking (X) assisted Bimolecular tandem Affinity Purification) MS method by incorporating XAP with bimolecular affinity purification to define dynamic and heterogeneous protein subcomplexes. Here we describe general experimental protocols of XAP- and XBAP-MS to study dynamic protein complexes and their subcomplexes, respectively. Specifically, we present their applications in capturing and identifying proteasome dynamic interactors and ubiquitin receptor (UbR)-proteasome subcomplexes.

Keywords

Protein-protein interaction; AP-MS; XAP; XBAP; dynamic interactions; proteasome complexes; ubiquitin receptor-proteasome subcomplexes

1 Introduction

Protein–protein interactions (PPIs) that establish the “protein sociology” in different cellular contexts play key regulatory roles in nearly all biological processes including development, cell homeostasis, growth, and proliferation. Aberrant Protein–protein interactions lead to various human diseases including cancer [1,2]. Modulation of protein-protein interaction represents an emerging therapeutic paradigm, and protein interaction interfaces describe a new class of attractive targets for drug development. Therefore, understanding protein interacting network is critical not only for a comprehensive understanding of protein function and regulation, but also provides potential targets for better therapeutics. Several methodologies including yeast two-hybrid system, protein microarray, fluorescence imaging,

Correspondence should be addressed to Dr. Lan Huang (lanhuang@uci.edu).

and affinity purification-mass spectrometry have been developed for studying protein-protein interactions. Owing to new developments in sample preparation strategies, mass spectrometry technologies, and bioinformatics tools, affinity purification coupled with mass spectrometry (AP-MS) has become the method of choice for studying protein–protein interactions at the systems level with great sensitivity, accuracy, versatility, and speed [3–8]. While AP-MS has proven effective and powerful in capturing stable interactions, weakly bound interactors are often lost due to extended biochemical manipulation under native purification conditions. Given the complex nature of PPIs, alternative strategies would be needed to enable the capture of all types of interactions. To this end, we have previously developed the QTAX (Quantitative analysis of Tandem Affinity purified *in vivo* cross-linked (X) protein complexes) method to capture a wide range of interactions in a single experiment [9]. One of the key components in the QTAX strategy is *in vivo* formaldehyde (FA) cross-linking of living cells to freeze protein-protein interactions prior to cell lysis, thus allowing better preservation of the dynamic proteome of protein complexes during subsequent affinity purification [9]. The second key component in the QTAX method is the integration of the HB (His-Bio) tag for tandem affinity purification of protein complexes, which is versatile and can be used for protein purification under both native and fully denatured conditions [10,9,11–13]. In combination with quantitative mass spectrometry, the QTAX strategy has been successfully employed to capture and identify stable and transient/weak interactors of protein complexes including proteasomes [9,14]. While the composition of protein complexes resulting from denaturing purification can be readily determined, subunit stoichiometry can be misrepresented due to variance in protein cross-linking efficiency, leading to varying protein absolute abundances. Therefore, to isolate functional protein assemblies for proteomic analysis, native purification would be preferred to maintain the integrity of protein complexes. Previously, it has been shown that mild cross-linking can be coupled with native purification to preserve protein interactions [15–17]. In order to preserve the dynamic proteome of protein complexes and maintain complex intactness during affinity purification under native conditions, we have developed a new AP-MS strategy, namely XAP (*in vivo* cross-linking (X) assisted Affinity Purification) MS by incorporating mild *in vivo* formaldehyde (<0.1%) cross-linking of intact cells with HB-tag based single-step affinity purification under native conditions [18,19] (Fig. 1A). The XAP method has been successfully applied to reliably capture dynamic/weak interactors of human proteasome complexes including a known proteasome interactor Ecm29, which enabled us to investigate and determine the evolutionarily conserved functional role of Ecm29 in regulating 26S proteasome disassembly mediated by oxidative stress [19]. In comparison to the QTAX strategy [9], XAP utilizes significantly less amount of formaldehyde (0.05% vs. 1%) for *in vivo* cross-linking [18,19]. Our results have further demonstrated that such mild *in vivo* cross-linking allows effective stabilization of protein structures and interacting networks without altering the integrity and functionality of protein complexes [18]. In addition, limited formaldehyde cross-linking does not interfere with native cell lysis, subsequent purification and mass spectrometric analysis.

It is well known that proteasomes exists in cells as dynamic and heterogeneous populations. To dissect dynamic ubiquitin receptor-proteasome subcomplexes, we have developed the XBAP (*in vivo* cross-linking (X) assisted Bimolecular tandem Affinity Purification) method

to enable the effective purification of these subcomplexes and to define their subunit compositions [18] (Fig. 1B). In comparison to XAP, XBAP requires tagging of two constituents of a given complex, thus allowing the isolation of protein subcomplexes containing the two selected baits. To this end, the XBAP strategy employs two unique tags, i.e. HF (His-FLAG) and TB (Tev-biotin) tags for two distinct baits, permitting bimolecular purification not only under native conditions, but also under fully denaturing conditions [18]. Importantly, the employment of two tagged baits enables the dissection of heterogeneous protein complexes into subpopulations. With the XBAP strategy, we have effectively identified seven UbR-proteasome subcomplexes and quantitatively compared their subunit compositions and relative abundance based on label-free quantitative mass spectrometry [18]. Collectively, the development of the XAP- and XBAP-MS strategies enhance our ability to define dynamic and heterogeneous protein complexes in more detail, thus facilitating the determination of their structure, regulation and function. Here we describe how the two new AP-MS strategies are applied to study proteasome complexes.

2 Materials

2.1 Cell Culture

1. A HEK293 cell line stably expressing C-terminal HTBH-tagged Rpn11: 293 $^{\text{Rpn11-HTBH}}$ (*see* Note 1).

2. A HEK293 cell lines stably expressing the His-Flag-UbR and Rpn11-TB: 293 $^{\text{HF-UbR\_Rpn11-TB}}$ (*see* Note 2).

3. Culture medium: DMEM medium supplemented with 10 % fetal bovine serum, and 1 % penicillin/streptomycin.

2.2 HB-tag Based Affinity Purification for XAP and XBAP Experiments

1. 1X protease inhibitor cocktail: 1 μg/ml phenylmethylsulfonyl fluoride, leupeptin, aprotinin, pepstatin. Make a 100X stock solution for phenylmethylsulfonyl fluoride in 100% isopropanol, store at 4 °C, and 1,000X stock solutions for leupeptin, aprotinin, and pepstatin, store at −20 °C.

2. 1X phosphatase inhibitor cocktail: 5 mM NaF, 0.1 mM Na_3VO_4, 2.5 mM $Na_4P_2O_7$, 1 mM EDTA. Make a 10X stock solution, store at −20 °C.

3. 1X trypsin–EDTA solution for cell culture.

4. 1X PBS.

5. 2.5 M glycine (pH 7).

6. Lysis buffer A:100 mM NaCl, 50 mM sodium phosphate, 10 % glycerol, 5 mM ATP, 1 mM DTT, 5 mM $MgCl_2$, 1x protease inhibiter cocktail, 1x phosphatase inhibitor cocktail, 0.5 % IGEPAL CA-630, pH 7.5. Make the buffer right before the experiment.

7. 20 gauge needles.

8. Streptavidin agarose resin (Thermo Scientific).

9. TBS buffer: 50 mM Tris-HCl (pH7.4), 100 mM NaCl, 5 mM ATP, 10% glycerol.

10. Anti-FLAG M2 affinity gel (Sigma).

11. 3X FLAG peptides (Sigma).

12. Microspin column (Bio-Rad).

13. Siliconized tubes (Axygen).

2.3 Protein Digestion and LC-MS/MS Analysis

1. 50 mM NH_4HCO_3.

2. 50 mM NH_4HCO_3 in 8 M urea.

3. Trifluoroacetic acid (TFA).

4. 1 μg/μl sequencing-grade endopeptidase LysC stock solution.

5. 0.4 μg/μl sequencing-grade trypsin stock solution: dissolve 20 μg of trypsin in 50 μl of 1 mM TFA (*see* Note 3).

6. 10 % formic acid (Thermo Scientific).

7. OMIX C18 tip (Agilent Technologies) (*see* Note 4).

8. EASY NanoLC 1000 system (ThermoFisher Scientific).

9. Mass spectrometer: LTQ-Orbitrap XL MS (ThermoFisher Scientific).

10. Nano LC solvent A: 2 % acetonitrile, 0.1 % formic acid in H_2O.

11. Nano LC solvent B: 98 % acetonitrile, 0.1 % formic acid in H_2O.

12. Beads extraction buffer: 25% acetonitrile, 0.1%formic acid.

13. Gel loading tip.

2.4 Database Searching for Protein Identification and Quantification

1. LC-MS/MS data extraction: instrument specific scripts from the manufacturer.

2. Protein identification and quantitation software: Protein Prospector (University of California, San Francisco).

3. Methods

3.1 Cell Culture and *in vivo* formaldehyde treatment

1. Culture HEK93$^{Rpn11\text{-}HTBH}$ or HEK293 $^{HF\text{-}UbR\_Rpn11\text{-}TB}$ cells to (90%) confluence in complete DMEM medium.

2. Wash the cells with 1X PBS once.

3. Treat the cells 0.05% FA for 10 min in PBS at 37 °C in the incubator (*see* Note 5).

4. Quench with a final concentration of 0.125 M glycine for 5 min.

5. Wash the cells once in 1X PBS to remove the formaldehyde and glycine.

3.2 HB-tag Based Affinity Purification for XAP

1. Detach cells with trypsin–EDTA solution and wash them once with 1X PBS buffer.

2. Collect cell pellets and lyse cells using lysis buffer A by pushing the lysate through a 20 gauge needle 15 times. Centrifuge the lysates at maximum speed of a microcentrifuge for 15 min to remove cell debris.

3. Incubate the supernatant with 25 μl bead volume of streptavidin resin per plate for the desired amount of time at 4 °C (*see* Note 6).

4. Wash the streptavidin beads with 50 bed volumes of lysis buffer A without protease and phosphatase inhibitors (*see* Note 7).

5. Wash the beads with 10 bead volumes of TBS buffer.

3.3 Bimolecular tandem affinity purification strategies for XBAP

1. Follow the same procedure for cell lysis as steps 1–2 in **3.2**.

2. Incubate the supernatant with anti-FLAG M2 affinity gel at 4 °C for 3 h, add 20 μl bead volume of FLAG affinity gel per 150 mm plate cells (*see* Note 8).

3. Wash the FLAG affinity gel with 30 bead volumes of lysis buffer A without protease and phosphatase inhibitors.

4. Wash the FLAG affinity gel with 15 bed volumes of TBS buffer.

5. Elute the bound proteins with 150 μg/ml of 3X FLAG peptide in TBS buffer by incubating at 4 °C for 30 min. Discard the beads.

6. Incubate the resulting eluent with streptavidin resin at 4 °C for 2 h. Use 10 μl bead volume of streptavidin resin per 150mm plate cells (*see* Note 9).

7. Wash the streptavidin resin with 50 bed volumes of TBS buffer.

3.4 Protein Digestion and LC-MS/MS Analysis

To avoid keratin contamination in your samples, you need to wear a hair net, sleeves, and clean gloves for the following procedure. To minimize sample loss, bound protein samples are directly subjected to subsequent LysC/trypsin digestion and mass spectrometric analysis. Each purification experiment needs to be repeated once to obtain two biological replicates to assess reproducibility.

1. Add one bead volume of 8 M urea with 50 mM ammonium bicarbonate buffer to streptavidin resin, then add 1 μl of endopeptidase LysC stock solution to the protein complex and incubate at 37 °C for 4 h (*see* Note 10).

4. Decrease urea concentration to <1.5 M by adding an adequate volume of 50 mM NH_4HCO_3. Add trypsin to a final concentration of 5–10 ng/μl and incubate overnight at 37 °C (*see* Note 11).

5. To recover digested peptides, add 10 % formic acid to a final concentration of 1 % to the sample to stop the digestion. Mix and spin at 94 x g for 1 minute.

6. Take off the supernatant and transfer to a clean siliconized tube with a gel loading tip.

7. Add half bead volume of bead extraction buffer to the beads, shake gently for 5 minutes, spin the sample and collect the supernatant.

8. Repeat step 7 two more times.

9. Pool all the supernatant from steps 6–8 together. Dry it in a SpeedVac.

10. Add 0.1% formic acid to dissolve the pellet. Desalt the pellet with Agilent OMIX C18 tips.

11. Analyze peptide mixtures by LC-MS/MS using nanoflow reverse phase liquid chromatography (EASY NanoLC 1000 system) coupled online to a LTQ-Orbitrap XL MS. Each cycle of an MS/MS experiment includes one MS scan in FT mode (350–1400 m/z, resolution of 60,000 at m/z 400) followed by data-dependent MS2 scans in the LTQ with normalized collision energy at 35% for the top ten peaks. Elute peptides starting with nanoLC solvent A with a linear gradient 0–35 % nanoLC solvent B for 80 min at a flow of 250 nl/min. LC-MS/MS is operated in an information-dependent mode in which each full MS analysis is followed by ten MS/MS acquisitions where the three most abundant peptide molecular ions are dynamically selected for collision induced dissociation (CID) to generate tandem mass spectra (*see* Note 12).

3.5 Protein Identification Using Protein Prospector (*see* Note 13)

1. Obtain monoisotopic masses of both parent ions and corresponding fragment ions, parent ion charge states, and ion intensities from the MS/MS by using an automated version of the Mascot script from Analyst QS within Protein Prospector.

2. Use the Batch-tag program within Protein Prospector for database searching. Select trypsin as the enzyme and set the maximum number of missed tryptic cleavage sites as 2. Chemical modifications such as protein amino-terminal acetylation, methionine oxidation, amino-terminal pyroglutamine, and deamidation of asparagine residues are selected as variable modifications. These modifications, except for protein amino-terminal acetylation, need to be chosen because of their frequent occurrence during sample preparation. Set the mass accuracy for parent ions and fragment ions as ± 20 ppm and 0.6 Da, respectively. Any annotated protein databases such as SwissProt and UniProt can be used for database searching. A concatenated database composed of a normal and its reverse database can be generated in Protein Prospector for database searching. Because we purify the samples from human cell lines, Homo sapiens is selected as the restricted species.

3. General protein identification is based on at least two peptides with an expectation value cutoff of 0.01.

4 Notes

1. The HTBH-tag consists of two hexahistidine tags, a TEV cleavage site, and a signal sequence for *in vivo* biotinylation, which allows efficient purification of proteasome complexes in a single step by binding to streptavidin resins and specific elution by cleavage with TEV protease [11].

2. The His-FLAG tag consists of one hexahistidine tag and three FLAG tags. TB tag consists of a TEV cleavage site, and a signal sequence for *in vivo* biotinylation, which allows efficient purification of proteasome complexes in a single step by binding to streptavidin resins and specific elution by cleavage with TEV protease.

3. Make fresh 1 mM TFA each time from a 100 mM TFA stock solution.

4. Any C18 based desalting tips and cartridges should work.

5. If the cells are not adherent, you can do the treatment in a tube with gentle agitation.

6. For the HTBH-tag, use 25 μl of streptavidin beads per 150 mm plate of HEK293 cells for maximum specific binding efficiency with minimal background binding. Purification efficiency should be followed by western blot analysis. The optimal binding for proteasome complexes to streptavidin beads is 2 h. The binding efficiency decreases when the incubation time decreases.

7. Effective washing steps can be achieved in micro-columns from Bio-Rad, for example, to minimize the bead loss.

8. For the FLAG-tag, use 20 μl bead volume of FLAG affinity gel per 150 mm plate of HEK293 cells for maximum specific binding efficiency with minimal background binding. Purification efficiency should be evaluated by western blot analysis. The optimal binding for UbR complex to FLAG beads is 3 h. The binding efficiency decreases when the incubation time decreases.

9. Since streptavidin resin are used for the second step binding in the XBAP experiment, the amount of beads required is less. For our bait, 10 μl bead volume per 150 mm plate cells is optimal.

10. LysC digestion can proceed from 4 h to overnight.

11. Trypsin digestion can proceed from 8 h to overnight.

12. For MS instruments with fast scanning rates such as the LTQ-Orbitrap, top ten peaks can be sequenced in each LC-MS/MS acquisition cycle.

13. You can access protein prospector from the following website: http://prospector.ucsf.edu/prospector/mshome.htm

Other database searching tools such as MaxQuant, Mascot, proteome discoverer, etc., can also be used.

Acknowledgments

This work was supported by National Institutes of Health grants RO1GM074830 to L.H, R01GM106003 to L.H. and S. R.

References

1. Ryan DP, Matthews JM (2005) Protein-protein interactions in human disease. Curr Opin Struct Biol 15 (4):441–446. doi:10.1016/j.sbi.2005.06.001 [PubMed: 15993577]
2. Kar G, Gursoy A, Keskin O (2009) Human cancer protein-protein interaction network: a structural perspective. PLoS Comput Biol 5 (12):e1000601 [PubMed: 20011507]
3. Gingras AC, Gstaiger M, Raught B, Aebersold R (2007) Analysis of protein complexes using mass spectrometry. Nature reviews Molecular cell biology 8 (8):645–654 [PubMed: 17593931]
4. Ryan CJ, Cimermancic P, Szpiech ZA, Sali A, Hernandez RD, Krogan NJ (2013) High-resolution network biology: connecting sequence with function. Nat Rev Genet 14 (12):865–879. doi:10.1038/nrg3574 [PubMed: 24197012]
5. Lambert JP, Ivosev G, Couzens AL, Larsen B, Taipale M, Lin ZY, Zhong Q, Lindquist S, Vidal M, Aebersold R, Pawson T, Bonner R, Tate S, Gingras AC (2013) Mapping differential interactomes by affinity purification coupled with data-independent mass spectrometry acquisition. Nat Methods 10 (12):1239–1245. doi:10.1038/nmeth.2702 [PubMed: 24162924]
6. Collins BC, Gillet LC, Rosenberger G, Rost HL, Vichalkovski A, Gstaiger M, Aebersold R (2013) Quantifying protein interaction dynamics by SWATH mass spectrometry: application to the 14-3-3 system. Nat Methods 10 (12):1246–1253. doi:10.1038/nmeth.2703 [PubMed: 24162925]
7. Sowa ME, Bennett EJ, Gygi SP, Harper JW (2009) Defining the human deubiquitinating enzyme interaction landscape. Cell 138 (2):389–403 [PubMed: 19615732]
8. Kaake RM, Wang X, Huang L (2010) Profiling of protein interaction networks of protein complexes Using affinity purification and quantitative mass spectrometry. Molecular & cellular proteomics : MCP
9. Guerrero C, Tagwerker C, Kaiser P, Huang L (2006) An integrated mass spectrometry-based proteomic approach: quantitative analysis of tandem affinity-purified in vivo cross-linked protein complexes (QTAX) to decipher the 26 S proteasome-interacting network. Molecular & cellular proteomics : MCP 5 (2):366–378. doi:10.1074/mcp.M500303-MCP200 [PubMed: 16284124]
10. Tagwerker C, Flick K, Cui M, Guerrero C, Dou Y, Auer B, Baldi P, Huang L, Kaiser P (2006) A tandem affinity tag for two-step purification under fully denaturing conditions: application in ubiquitin profiling and protein complex identification combined with in vivocross-linking. Molecular & cellular proteomics : MCP 5 (4):737–748 [PubMed: 16432255]

11. Wang X, Chen CF, Baker PR, Chen PL, Kaiser P, Huang L (2007) Mass spectrometric characterization of the affinity-purified human 26S proteasome complex. Biochemistry 46 (11): 3553–3565 [PubMed: 17323924]
12. Fang L, Wang X, Yamoah K, Chen PL, Pan ZQ, Huang L (2008) Characterization of the human COP9 signalosome complex using affinity purification and mass spectrometry. Journal of proteome research 7 (11):4914–4925 [PubMed: 18850735]
13. Fang L, Kaake RM, Patel VR, Yang Y, Baldi P, Huang L (2012) Mapping the Protein Interaction Network of the Human COP9 Signalosome Complex Using a Label-free QTAX Strategy. Molecular & cellular proteomics : MCP 11 (5):138–147. doi:10.1074/mcp.M111.016352 [PubMed: 22474085]
14. Guerrero C, Milenkovic T, Przulj N, Kaiser P, Huang L (2008) Characterization of the proteasome interaction network using a QTAX-based tag-team strategy and protein interaction network analysis. Proceedings of the National Academy of Sciences of the United States of America 105 (36):13333–13338 [PubMed: 18757749]
15. Subbotin RI, Chait BT (2014) A pipeline for determining protein-protein interactions and proximities in the cellular milieu. Molecular & cellular proteomics : MCP 13 (11):2824–2835. doi: 10.1074/mcp.M114.041095 [PubMed: 25172955]
16. Smart SK, Mackintosh SG, Edmondson RD, Taverna SD, Tackett AJ (2009) Mapping the local protein interactome of the NuA3 histone acetyltransferase. Protein science : a publication of the Protein Society 18 (9):1987–1997. doi:10.1002/pro.212 [PubMed: 19621382]
17. Bousquet-Dubouch MP, Baudelet E, Guerin F, Matondo M, Uttenweiler-Joseph S, Burlet-Schiltz O, Monsarrat B (2009) Affinity purification strategy to capture human endogenous proteasome complexes diversity and to identify proteasome-interacting proteins. Molecular & cellular proteomics : MCP 8 (5):1150–1164 [PubMed: 19193609]
18. Yu C, Yang Y, Wang X, Guan S, Fang L, Liu F, Walters KJ, Kaiser P, Huang L (2016) Characterization of Dynamic UbR-Proteasome Subcomplexes by In vivo Cross-linking (X) Assisted Bimolecular Tandem Affinity Purification (XBAP) and Label-free Quantitation. Molecular & cellular proteomics : MCP. doi:10.1074/mcp.M116.058271
19. Wang X, Chemmama IE, Yu C, Huszagh A, Xu Y, Viner R, Block SA, Cimermancic P, Rychnovsky SD, Ye Y, Sali A, Huang L (2017) The proteasome-interacting Ecm29 protein disassembles the 26S proteasome in response to oxidative stress. The Journal of biological chemistry 292 (39):16310–16320. doi:10.1074/jbc.M117.803619 [PubMed: 28821611]

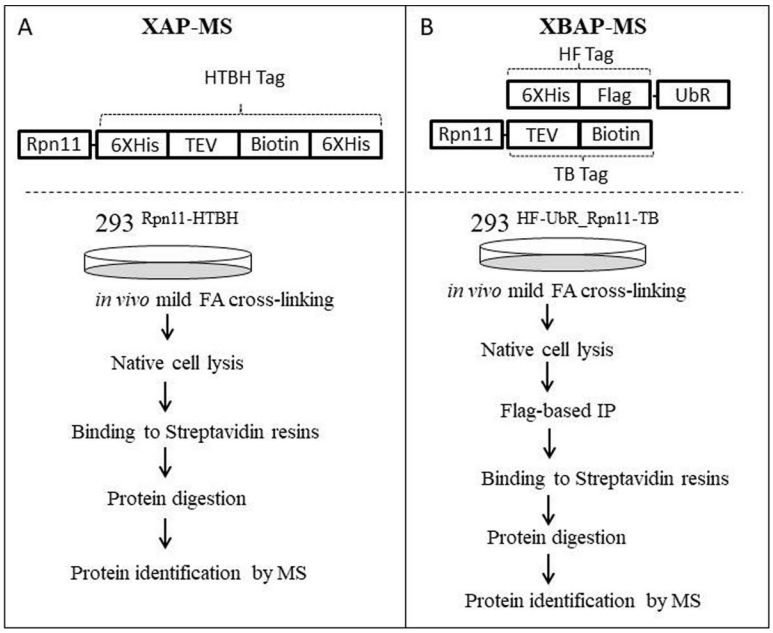

Fig. 1.

The general schemes for (A) XAP- and (B) XBAP-MS strategies. While one single bait is tagged for affinity purification in the XAP experiment, two baits are selected for tagging in the XBAP experiment.