Purification of Human Multiprotein Complexes using OneSTrEP Technology (PROT41)

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Last reviewed: 26 Sept 2008 by Adam Cook. Laboratory of Geneviève Almouzni, UMR218, Institut Curie, Paris

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Introduction

Here we describe a strategy for isolation of multiprotein complexes from human HeLa S3 cells in a scale and purity optimized for characterization by mass spectrometry. For this purpose, we use stably expressed One-*STrEP*-tag ® fusion proteins. This approach was successfully used in characterization of histone chaperone Asf1 complexes (Groth et al., 2007), and we have recently optimized it further. Using this protocol we routinely obtain complexes in amounts sufficient for visualising single protein bands by Coomassie Blue staining (Figure 1).

The *Strep*-tag®II (SAWRHPQFGG) and its ?double? sister, the One-STrEP-tag (tandem arrangement of *Strep*-tag®II, here called OneStrep), are reasonably small protein tags that usually do not influence protein folding and function. However, always check by appropriate control experiments that the tagged protein is functional *in vivo*. Vectors for both N- and C-terminal fusion are available from IBA Tagnology (Germany), who has developed these tags and binding matrices. We recommend using the OneStrep-tag for purification of protein complexes from mammalian cells. The *Strep*-tag® has a strong affinity towards engineered streptavidin (*Strep*-Tactin®) which allows efficient one-step purification with high stringency

washing to obtain highly pure protein complexes. Elution of the complexes by competition with D-biotin is efficient and can be done in a variety of buffer conditions allowing biochemical active complexes to be preserved. For more theoretical background and a protocol for purification of recombinant *Strep*-tag® fusion proteins from E.coli please refer to Schmidt and Skerra (2007).

In comparison to other commonly used purification strategies (i.e. HA and FLAG double-tag purification, Tagami et al., 2004), the advantages of the OneStrep-tag are:

1. Purification in only one step;
2. Strong affinity to *Strep*-Tactin® matrix allowing stringent washing;
3. Highly efficient elution;
4. Broad range of available *Strep*-Tactin® matrices including columns for gravity flow and HPLC as well as magnetic beads and spin-columns for small-scale purification;
5. And cost-effectiveness.

Note that a two-step purification strategy may be advantageous to identify weak interactors.

Procedure

Generation of HeLa S3 expressing YPI (Your Protein of Interest) tagged with OneStrep.

We strongly recommend that you generate stable cell lines rather than working with transiently transfected cells. This will reduce the non-physiological interaction due to overexpression and allow time for YPI-OneStrep to be incorporated into even highly stable complexes. After transfection of HeLa S3 cells with plasmids carrying the OneStrep fused to N- or C-terminal of YPI, clones can be isolated in 96 wells following 2 weeks of G418 selection (**note 1**). We recommend to isolate several clones and characterize them by a few simple criteria:

1. Western blotting to check expression level (see protocol) and proper cellular distribution of OneStrep-YPI (**note 2**). Antibodies against YPI should allow to determine the extent of overexpression as the tag introduces a mobility shift;
2. FACS analysis of DNA content to check the cell cycle profile;
3. Viability and cell growth (i.e. using Trypan blue and cell counting).

Protein extracts

We use a protocol for making cytosolic and nuclear extracts from HeLa S3 cells grown on dishes that has been developed in the Almouzni laboratory (modified from Li and Kelly (1984) and Martini et al. (1998)). These extracts are competent for in vitro DNA replication (Li and Kelly, 1984) and nucleosome assembly assays (Martini et al. 1998; Groth et al., 2005). In principal, any of your favourite lysis buffer could be used, but it is important to check that none of the components interfere with binding of the OneStrep-tag to the Strep-Tactin® matrix (IBA Tagnology website: http://www.iba-go.com/prottools/prot_fr01_01.html). We recommend the pre-fractionation step as it reduces the complexity of the starting material and can provide functional insights if the cytosolic and nuclear extract yield distinct complexes.

1. Grow cells on 150mm dishes until 80-90% confluence. We routinely use 6-8 150mm plates/experiment (note 3 and note 4).
2. All following steps are done at 4°C in a cold room. Remove the medium and rinse cells 2x with ice-cold PBS. Drain well after the second wash.
3. Add 10 ml Buffer E (-inhib.)/150mm plate and incubate for 8-10 min to swell the cells.
4. Remove, drain and add 10ml of Buffer E (+inhib.)/150mm plate. Incubate for 8-10 min.
5. Remove and drain well by leaving the dishes in vertical position for about 2 min.
6. Scrape the cells and transfer into douncer (1 ml, Wheaton).
7. Homogenize using a loose pestle with 25 strokes.
8. Transfer to 2 ml Eppendorf tubes and pellet the nuclei by centrifugation at 1500 g for 5 min.
9. Transfer the supernatant to a new tube and clear it by spinning 15 min in a tabletop centrifuge at 4°C max. speed (13.000 rpm). This is your cytosolic extract containing cytoplasmatic and soluble nuclear proteins. Snap freeze and store at -80°C.
10. To the nuclei pellet from step 8 add one to two volumes of Buffer N and resuspend carefully with a P1000 pipette. Extract nuclear and chromatin bound proteins by end-over-end rotation 90 min at 4°C.
11. Spin 15 min in a tabletop centrifuge at 4°C max. speed. Transfer the supernatant to a new tube and snap freeze. This is your nuclear extract containing nuclear and chromatin bound proteins. (note 5)
12. Wash the pellet (matrix and salt-extracted chromatin) once with Buffer N, spin again, and snap freeze.

OneStrep-tag purification

We routinely use gravity flow Strep-Tactin Superflow® columns with a column volume (CV) of 200 µl (IBA Tagnology, Cat. No. 2-1207-550) (see note 6). Purification is performed at 4°C in a cold room.

1. Equilibrate the column by applying 2x CV of Washing buffer.
2. Add 5-10 mg of protein extracts to the column (not more than 1 ml). note 7
3. Wash columns 10 times with 1 ml Washing buffer.
4. Elute 6 times with 0,5 CV (100 µl)/elution step. note 8 and note 9
5. Snap freeze eluted fractions E1-E6. For the first test purification, collect also the flow-through and washes for the analyses described below.

Analysis of purified complexes

We quality check purified complexes by Western blotting (Figure 2) and silver staining (Figure 3). For Western blot detection we usually use 5-10 µl of E1-E6, and corresponding aliquots of input extracts, flow-through, and washes to judge the efficiency of binding and elution from the column. For silver staining we run 10-15 µl of E1-E6 to determine the purity. The purest fractions with highest yield can then be pooled and processed for mass spectrometry.

Western blot detection

1. Block membrane 1 hr at RT in 5% BSA, PBS-0.5%Tween. note 10

2. Wash briefly 2x with PBS-0.1%Tween
3. Incubate membrane with Strep-tactin®-HRP (IBA Tagnology , Cat. No. 2-1502-001) 1:5000 diluted in blocking buffer at RT for 1 hr.
4. Wash 3x with PBS-0.1%Tween
5. Develop by chemiluminescence as usual (we use SuperSignal® West Pico Chemiluminescent Substrate, Pierce, Cat. No. 34080)

For detection of interacting proteins by mass spectrometry we precipitate proteins before separation of complexes on NuPAGE 4-12% Bis-Tris Gradient gels (Invitrogen, Cat. No. NP0321) and Coomassie staining (Figure 1). We have a good experience with methanol/chloroform precipitation protocol from Wessel and Flugge (1984).

Protein precipitation

1. To 200 µl of protein solution add 800 µl methanol.
2. Vortex and spin briefly.
3. Add 200 µl chloroform.
4. Vortex and spin briefly.
5. Add 600 µl H_2O.
6. Vortex thoroughly for 30 seconds.
7. Centrifuge at 9000 g for 1 min.
8. The white precipitate will form the interphase. Remove the top phase.
9. Add 600 µl methanol
10. Vortex briefly.
11. Centrifuge at max. speed for 2 min.
12. Precipitate will end up at the bottom of tube. Remove the supernatant.
13. Speed vac until dry.
14. Resuspend in 1x SDS gel-loading buffer.

Materials & Reagents

Buffer E	• 20 mM HEPES-KOH pH 7.8 (1 M stock) • 5 mM Potassium Acetate (1 M stock) • 0.5 mM $MgCl_2$ (1 M stock) • 0.5 mM DTT (1 M stock) *Inhibitors:* • 10 µg/ml Leupeptin (stock 10 mg/ml in DMSO) • 10 µg/ml Pepstatin (stock 10 mg/ml in DMSO) • 0.1 mM PMSF (stock 100 mM in isopropanol) • 0.2 mM Sodium Vanadate (stock 0.1 M in H_2O) • 5 mM Sodium Flouride (stock 0.5 M in H_2O) • 10 mM β-GlyceroPhosphate (stock 1 M in H_2O)
Buffer N	• Buffer E • 540mM NaCl (5 M stock) • 10% Glycerol

Washing buffer	• 20 mM Tris pH 7.8 (1 M stock) • 500 mM NaCl • 0.2 mM EDTA • 0.2 % NP-40 (or Igepal CA-630, Sigma, I3021) • 5 % Glycerol • 1 mM DTT • 10 µg /ml Leupeptin • 10 µg /ml Pepstatin • 0.1 mM PMSF • 0.2 mM Sodium Vanadate • 5 mM Sodium Flouride • 10 mM β-GlyceroPhosphate
Elution Buffer	• Washing buffer • 2 mM D-biotin (Sigma, B4501) • (Use 10 mM Biotin for batch purification)
Strep-Tactin Superflow® columns	IBA Tagnology (Germany), Cat. No. 2-1207-550

Author Notes

1. We use Geneticin (Gibco, cat. no 10131-027, 50 mg/ml) at final concentration 500 µg/ml for HeLa S3.
2. We do not have a good experience with immunofluorescence detection of OneStrep-tag in HeLa S3 cells, but antibodies are available (IBA Tagnology).
3. When preparing extracts from more plates we advise to handle 6-8 plates at a time, since manipulating more plates can be difficult.
4. Protocol can easily be modified for cell cultures grown in suspension. Pellet cells at 300 g, wash cells 2x with ice-cold PBS, and swell twice in one volume of Buffer E (+inhibitors). Then follow step 7 onwards.
5. Under given conditions a significant fraction of chromatin-bound proteins will be extracted, leaving nucleosomal histones and some strongly bound chromatin proteins in the pellet. If your YPI is expected to bind strongly to chromatin you may need to increase the salt concentration in Buffer N accordingly.
6. Alternative matrices for purification are available. Gravity flow columns require relatively high elution volumes and protein precipitation may then be necessary for downstream applications. We have observed that this may result in incomplete protein recovery (using tips and tubes with low-protein binding surface may overcome this problem). We are currently optimizing a protocol for using Strep-Tactin Superflow® resin (IBA Tagnology, Cat. No.2-1206-002) to reduce the elution volume and avoid protein precipitation, which also reduces the number of handling steps. For batch purification of tagged proteins from smaller amount of cells (1x150mm plate or less) MagStrep beads (IBA Tagnology, Cat. No. 2-1601-002) may be used.
7. We have observed that when using higher amount of extracts the yield of purified complexes decreased, probably due to presence of biotinylated proteins in the extracts. Biotinylated proteins can be removed from extracts prior to purification by incubating with avidin (IBA Tagnology, Cat. No. 2-0204-015, 100 µg/10 mg extracts) for 30 min on ice, followed by 10 min max. speed spin. The cleared extracts are loaded onto columns and in this case the amount of extract can be increased.

8. We routinely use high salt Elution Buffer, however depending on the downstream application other buffer conditions may be preferable. In that case we recommend doing the last wash as well as the elution in these conditions (i.e. low salt buffer with 150 mM NaCl and no glycerol).
9. Biotin takes time to dissolve (about 30 min at RT).
10. It is important to use BSA in blocking buffer, since milk contains a high amount of biotinylated proteins leading to inactivation of Strep-Tactin®-HRP antibody.

Reviewer Comments

Reviewed by: Adam Cook. Laboratory of Geneviève Almouzni, UMR218, Institut Curie, Paris

1. We regularly observe that different columns, even within the same batch, have slightly but noticeably different flow rates? this does not affect the isolation of protein complexes.
2. Regarding Author note 6, I observed (in a single experiment) that the purity of the proteins eluted from MagStrep beads is lower than when using the columns. Could the authors comment on their experience of the ?cleanliness? of the purification using the beads?
   Author feedback: We get the highest purity with column purification. For smaller scale purification we prefer the Strep-tactin Superflow resin and we only use MagStrep beads for batch purification from small volumes and detection by Western.
3. For growing sufficient adherent cells for preparing cytosolic and nuclear extracts, I have also used 500 cm^2 square plates (Corning) instead of the round 15cm diameter plates, to reduce the number of plates to handle. I have found these to be quite useful.
4. Regarding preparation of cytosolic and nuclear protein fractions, if my swollen cell volume exceeds 1mL (the specified capacity of the Dounce homogeniser recommended in this protocol), I load 1 ? 2 mL in multiple batches into the same Dounce. Could the authors comment if they recommend an alternative strategy?
   Author feedback: For larger cell volumes douncers of 7, 15, or 40 ml can be used.
5. Regarding preparation of cytosolic and nuclear extracts (step 10) from a large cell number, I have experienced difficulty in resuspending the nuclei directly in the high salt Buffer N and/or obtaining a nuclear protein fraction (after the 90 min incubation and centrifugation) that is well separated from the insoluble material. I prefer to gently resuspend the nuclei in an approximately equal volume of Buffer E, and then to the resuspended nuclei, adding an equal volume of Buffer N with [2x] NaCl (1.2 M or as desired).
6. Regarding protein precipitation (step 8), even when precipitating substantial amounts of eluted proteins, I often do not observe a white precipitate at the interphase. I leave sufficient liquid at the interphase so as not to disturb the apparently invisible precipitate. The protein is successfully concentrated nonetheless.

Figures

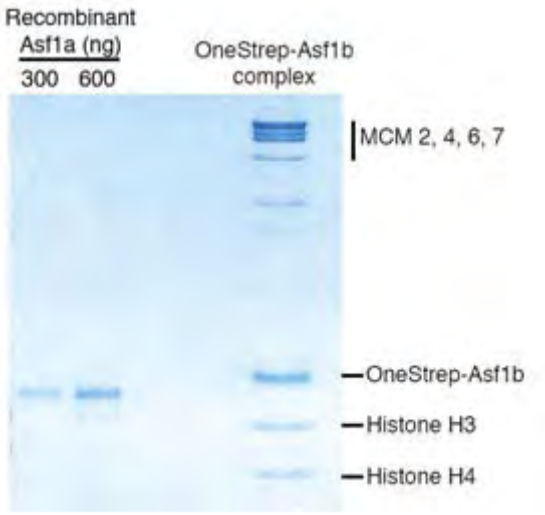

Figure 1. Purified nuclear OneStrep-Asf1b complexes from S-phase cells (24 x 150 mm plates) separated on a gradient gel and stained with Coomassie Brilliant Blue. Recombinant Asf1a is run in parallel to estimate the quantity of purified material.

Figure 2. Testing your purification. OneStrep-Asf1a complexes were purified from nuclear extracts (about 5 mg), and aliquots of elution fractions E1-E6, washes, and flow-through (Fl) were analysed by Western blotting. Identical amounts of HeLa S3 nuclear extracts were used as a negative control.

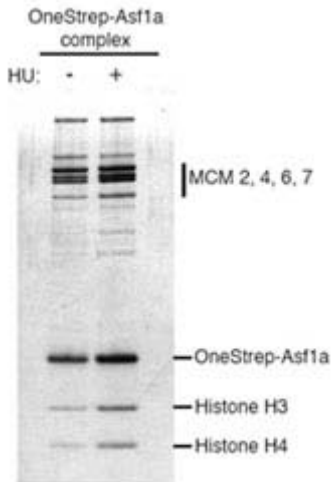

Figure 3. Silver staining of OneStrep-Asf1a complexes purified from 20 mg of nuclear S-phase extracts. 10% of elution fraction E3 was analysed.

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