Application Forum

Highly Efficient Protein and Complex Purification from Mammalian Cells Using the HaloTag® Technology

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Introduction

Technologies to purify proteins and protein complexes from mammalian cells have been challenged with the ability to efficiently and specifically capture proteins expressed at relatively low levels within complex lysates. Historically, affinity tags have been employed for both of these approaches, though due to the reversible binding to their respective resin, successful protein isolation can be inefficient due to higher off rates. Antibodies are also an option, yet can show low specificity or sensitivity. To overcome some of these challenges, the HaloTag® technology was adopted and optimized for mammalian protein purification and pull-downs. Both applications take advantage of the unique properties of the HaloTag fusion protein, which forms a highly specific and irreversible covalent bond with its resin, allowing rapid capture of low-expression proteins or protein complexes directly from mammalian cellular lysates. The protein purification system couples HaloTag protein fusion capture with subsequent proteolytic release, resulting in a highly pure protein free of tag. The protein pull-down system couples rapid binding kinetics along with low non-specific binding to improve the rate of successful complex capture and identification of binary and higher order macromolecular protein complexes. Both systems also include a fluorescent HaloTag ligand which allows detection as well as the ability to perform complementary live cell imaging studies of the same HaloTag fusion protein. In this study we demonstrate the capabilities of HaloTag compared to FLAG®, 3xFLAG® and His₆ tags.

Materials and Methods

For protein purification p65 (RelA), PKC, and PI3K, were cloned into pFC14A HaloTag CMV Flexi® Vector, and transiently expressed as C-terminal fusions in HEK293T cells (~2 x 10⁸). The cells were collected and the HaloTag fusion proteins were purified, as recommended by the manufacturer (Promega technical manual TM348). The same proteins were cloned and expressed as FLAG or His₆ tag fusion proteins in HEK293T cells and collected in a similar manner. FLAG

tagged proteins were purified using ANTI-FLAG® agarose resin (Sigma-Aldrich) and eluted by low pH or by 3xFLAG peptide, as recommended by the manufacturer. His₆ tag proteins were purified using HisLink™ resin (Promega) and eluted with 250mM imidazole.

For protein complex isolation, the same p65-HaloTag construct was used as above and transiently expressed in HeLa cells (~1 x 10⁷). The p65-HaloTag complex isolation was performed, as recommended by the manufacturer (Promega technical manual TM342).

Results and Discussion

To demonstrate the utility of HaloTag for protein purification and complex isolation, the model system of p65/NFB, a transcription factor involved in a number of cellular processes including immune and inflammatory responses, cellular growth, and apoptosis was chosen. HaloTag purification was compared to two other commonly used affinity tags, 3xFLAG, and His₆ tags for yield, purity, and recovery of the expressed protein (Fig 1A). The p65 protein was successfully purified using all tags, however yields and purity were significantly higher for p65 purified using HaloTag (Fig 1A). This is likely due to the high selectivity of the HaloLink resin, the absence of analogous endogenous mammalian proteins, and the ability

Figure 1. Purification of HaloTag, FLAG, and His₆ tagged proteins.

Figure 2. Capture and identification of NFκB protein complex.

to perform extensive washes without any loss of bound protein. The significant lower purity of the protein purified using His₆ tag was likely due to endogenous proteins with high affinity to metal ions that bind to the HisLink resin and co-purify.

To expand the comparison to other proteins, PKCγ and P13Kγ human kinases, were expressed as HaloTag-, FLAG-, 3xFLAG-, or His₆-tagged fusions and purified as described above. The purity and yields were significantly higher for protein purified using HaloTag, ≥ 95% purity and ≥ 86% yield, in comparison to the other tags (Fig 1B).

After successful protein purification, the HaloTag-p65 protein fusion was used to try to isolate known protein interactors: NFκB1 (p50, p105), NFκB2 (p52, p100), RelA (p65), RelB and c-Rel, all which can form various homodimeric and heterodimeric complexes and are further regulated by interaction with IκB proteins (inhibitors of κB). As a negative control, HaloTag protein alone was expressed. Resulting eluted complexes were run on an SDS page gel and silver stained (Fig 2A). Samples analyzed by LC/MS/MS showed capture of the expected interacting protein partners in the experimental sample containing the p65-HaloTag fusion protein and not the HaloTag control (Figure 2B). Endogenous p65 was also detected indicating the p65-HaloTag fusion dimerized with the endogenous counterpart. Use of a stable cell line expressing p65-HaloTag showed similar results (data not shown).

Both the purification and protein pull-down systems include a fluorescent HaloTag ligand, which can be used to label and detect proteins in SDS gels instead of using antibodies, and also for cellular imaging to directly observe localization. Figure

Figure 3. Live cell imaging of p65-HaloTag.

3 shows U2OS cells stably expressing the p65-HaloTag fusion protein labeled with the HaloTag TMRDirect™ fluorescent ligand. Expected cytoplasmic cellular localization, in the absence of p65 pathway stimulation, is observed. In addition, an impermeable fluorescent ligand is available enabling both temporal and spatial control over the labeling of membrane-bound fusions in the cell such that direct observation of not only localization, but also trafficking and turnover is possible.

Conclusions

These data with various proteins demonstrate that HaloTag outperforms traditional affinity tags: His₆, FLAG, and 3xFLAG tags with respect to yield, purity and overall recovery of the expressed proteins. In addition, the HaloTag pull-down experiments show a robust isolation of in vivo binary or higher order protein complexes from mammalian cells. The specific and covalent capture of the HaloTag fusion proteins onto the HaloLink resin lends itself to efficient protein and complex capture irrespective of expression levels. For the purification method, this allows extensive washes without any loss of bound protein. For the pull-down method the specific covalent nature of this system results in rapid processing to preserve protein complexes with high purity and low background. All of these features, as well as the ability to fluorescently label, differentiate HaloTag from other existing affinity tags or approaches providing considerable advantages in understanding protein functionality in mammalian cells.

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