Application Note - 004

Validation and Utility of the Ubi^{select}™ Kit (Cat# 67-0018-001)

Key Utility and Applications:

- **High affinity and selective capture of ubiquitylated proteins from Mouse Embryonic Fibroblast (MEF) cells**
- **High sensitivity detection of ubiquitylated proteins only and NOT other interacting proteins**
- **Cell-lysate based target specific ubiquitylation assays**

The above being challenging to achieve with traditional ubiquitin chain binding protein-based capture methods.

Ubi^{select} experiments facilitate:

- Selective isolation of ubiquitylated proteins from Ubi^{select}-MEF_Bio-Ub cells through the removal of ubiquitin binding proteins and ubiquitylated protein binding proteins that interact with your captured ubiquitylated protein of interest. Such selective isolation not being possible with ubiquitin chain binding protein (eg TUBE)-based capture techniques.

  NB: This protocol may be adapted to capture such interacting proteins if so desired.

- The manipulation of Ubi^{select}-MEF_Bio-Ub cells as per existing MEF cell protocols. For example if you wish to transfect with nucleic acids or in some other way modulate the cells (eg the addition of receptor activating ligands).

- Identification of mono or poly-ubiquitin attached to your protein of interest.

- Exploration of the formation of E1, E2, E3 and substrate ubiquitin-conjugates (thioester and isopeptide linked) and di-glycine signatures indicative of the ubiquitin attachment sites on conjugates isolated from Ubi^{select}-MEF_Bio-Ub cell lysates* (Franco *et al.*, 2011; Lectez *et al.*, 2014).

- Developing cell or cell-lysate based target specific ubiquitylation assays (eg FRET) utilising the biotin on the ubiquitin attached to your protein of interest.

- Isolation of candidate cysteine-ubiquitylated proteins and determination of their identity using mass spectrometry* (Lectez *et al.*, 2014).

  *NB: Both applications have been demonstrated recently using liver lysates derived from the same transgenic mouse from which the Ubi^{select}-MEF_Bio-Ub (P0) cells were derived.

BACKGROUND

The post-translational modification of proteins by ubiquitin is involved in a wide range of cellular processes (Kirkin and Dikic, 2007). In any given cell the proportion of protein modified by ubiquitin is very small therefore it has been difficult to isolate and identify this post translational modification from mammalian whole cell lysates. Ubiquitin proteomics remains challenging even though the sensitivity of Mass Spectrometry (MS) has improved dramatically through the use of innovative techniques (Sylvestersen *et al.*, 2013). Various approaches employing tagged ubiquitin or ubiquitin like molecules have been used with varying degrees of success (Peng *et al.*, 2003; Tirard *et al.*, 2012; Tsirigotis *et al.*, 2001). In recent years, the isolation of ubiquitylated proteins from neurons of *Drosophila melanogaster* using a tagged ubiquitin with a 15 amino acid long biotin-accepting peptide has been described (Franco *et al.*, 2011). This was the first demonstration that proteomics could be used to identify neuronal targets of the ubiquitin-proteasome system. This novel technique allowed for the isolation and enrichment of ubiquitin conjugates from neurons using a relatively small sample up to levels that allowed direct detection by MS and Western Blotting. In addition, di-glycine signatures indicative of the ubiquitin attachment sites could also be detected on ubiquitin conjugates. Where antibodies were available for specific substrates of ubiquitylation it was also possible to determine whether a substrate was mono- or polyubiquitylated (Franco *et al.*, 2011). Using a similar approach, hemizygous Biotinylated-Ubiquitin (Bio-Ub) and control BirA transgenic mouse models have been created (see Figure 1) which express either three moieties of Biotin-Accepting Peptide (BAP tag)-ubiquitin plus the *E. coli* enzyme BirA or the *E.coli* enzyme BirA enzyme alone (Lectez *et al.*, 2014). The Ubi^{select} kit includes both Ubi^{select}-MEF_Bio-Ub (P0) (Cat# 66-5011-001) and control Ubi^{select}-MEF_BirA (P0) (Cat# 66-5010-001) cell lines derived from 13.5 day old embryos from these transgenic mice through homogenisation and trypsinisation of the embryos minus the head and liver (Lectez *et al.*, 2014). By using cell and tissue lysates derived from Bio-Ub transgenic mice it is possible to

Transgenes in the transgenic mouse line from which Ubi^{select}-MEF_BirA (P0) and Ubi^{select}-MEF_Bio-Ub (P0) cell lines have been derived

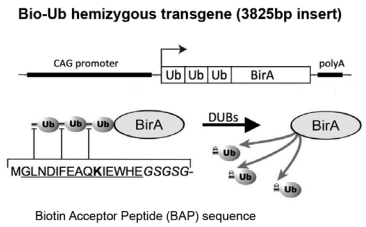


Figure 1a: Ubi^{select}-MEF_Bio-Ub (P0): A hemizygous cell line which expresses three moieties of biotin-accepting ubiquitin plus the *E. coli* enzyme BirA. (Lectez *et al.*, 2014)

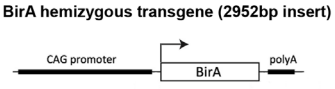


Figure 1b: Ubi^{select}-MEF_BirA (P0): A hemizygous cell line which expresses the *E. coli* enzyme BirA. (Lectez *et al.*, 2014)

Continued on page 2

INSIDE:

Authors: Dr. Jane Hilton and Dr. Jason Brown.
Ubiquigent Ltd.
Sir James Black Centre
Dow Street, Dundee, DD1 5EH
Scotland, UK

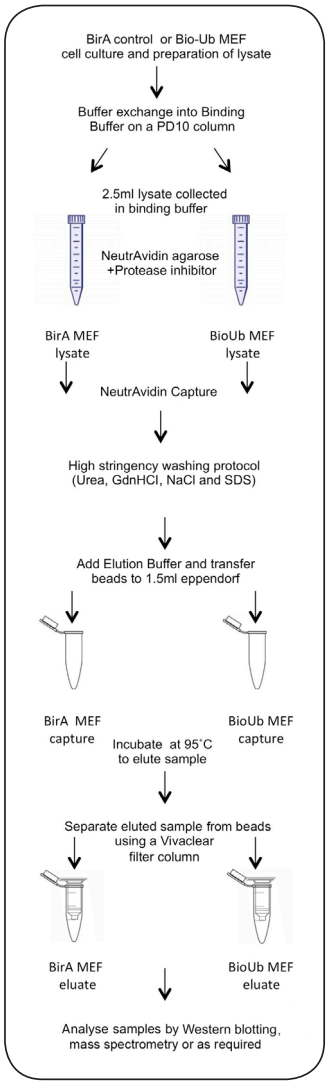
Correspondence:
tech.support@ubiquigent.com
Version 1.0 2 July 2014
www.ubiquigent.com

Why use the Ubi*select* KIT?

The Ubi*select* Kit is designed to facilitate the isolation and identification of your ubiquitylated proteins of interest from an embryonic fibroblast (MEF) cell line derived from a hemizygous transgenic mouse expressing ubiquitin N-terminally tagged with a Biotin Acceptor Peptide (BAP tag) sequence. By employing a powerful capture system which utilises the high affinity biotin-avidin interaction alongside a high stringency washing protocol you can capture your ubiquitylated target of interest without any associated ubiquitin proteins and/or ubiquitylated protein binding proteins. Thus one may be confident that the proteins analysed are ubiquitylated proteins. The Ubi*select* Kit contains control Ubi*select*-MEF_BirA (P0), Ubi*select*-MEF_Bio-Ub (P0) cell lines, and a ubiquitin conjugate antibody for detection of ubiquitylated proteins. Also included in the kit is a protocol insert which provides you with culture conditions for the Ubi*select*-MEF_BirA and Ubi*select*-MEF_Bio-Ub cell lines plus a detailed capture protocol for the isolation of ubiquitylated proteins from the Bio-Ub cell line. Eluted 'captured' products may be analysed by Western Blotting using the provided ubiquitin conjugate specific antibody, a biotin antibody or an antibody specific to your protein of interest. Captured proteins may also be analysed by other methods such as mass spectrometry.

What does the Ubi*select* Kit contain?

The kit contains control Ubi*select*-MEF_BirA (P0) cell line (Cat# 66-5010-001), Ubi*select*-MEF_Bio-Ub (P0) cell line (Cat# 66-5011-001), and an anti mono and polyubiquitylated conjugates antibody (Cat# 68-0122-025) for detection of your ubiquitylated protein. Also included in the kit is a protocol insert which provides you with culture conditions and a detailed capture protocol for the isolation of ubiquitylated proteins from the cell lines.



Ubi*select* Experimental Procedure

Figure 2: Ubi*select* experimental procedure: Summary of the Ubi*select* protocol for capturing and isolating ubiquitylated proteins from Ubi*select*-MEF_Bio-Ub cell lysates. Reaction products can be analysed by SDS-PAGE/Western Blotting or if you wish to analyse your reaction products by mass spectrometry, bands may be excised from SDS page gels and subjected to standard tryptic digestion and subsequent analysis procedures. Full experimental protocols are provided with the kit (Cat# 67-0018-001).

For the isolation of thioester conjugates, elute under non-reducing conditions (i.e. minus DTT).

Background, continued from page 1

isolate ubiquitylated proteins only (Lectez *et al.*, 2014). The specific isolation of ubiquitylated proteins is achieved through the high affinity interaction between biotin and avidin and high stringency washing which allows for the removal of interacting partners of ubiquitylated proteins which may otherwise predominate under the milder washing conditions of alternative ubiquitin capture systems (such as with ubiquitin chain binding proteins). By utilising liver lysates derived from the BAP tagged-Ub expressing transgenic mouse, the isolation of ubiquitin conjugated proteins (both thioester and isopeptide linked) has been achieved demonstrating this technology as the first mammalian system for ubiquitin proteomics which allows for the direct detection of ubiquitin conjugates by MS and Western Blotting. Isolation of ubiquitylated proteins from Ubi*select*-MEF_Bio-Ub has also been demonstrated (see below and data not published). It is anticipated that this technology will enable researchers to gain a better understanding of ubiquitin signalling under unperturbed as well as user modified cellular environments (such as in models of disease).

Ubi^{select}: Comparison of target capture using Ubi^{select} or Ubiquitin chain Binding Domain technologies.

Experiments conducted at Ubiquigent comparing the isolation of ubiquitin conjugates with Ubi^{select} alongside Ubiquitin Binding Domains (UBDs) are presented in Figure 3. Ubiquitin conjugates were captured in parallel using Ubi^{select} or UBDs and both capture techniques were subjected to either high stringency washing conditions designed for Ubi^{select} or the milder conditions required for UBD-based capture experiments.

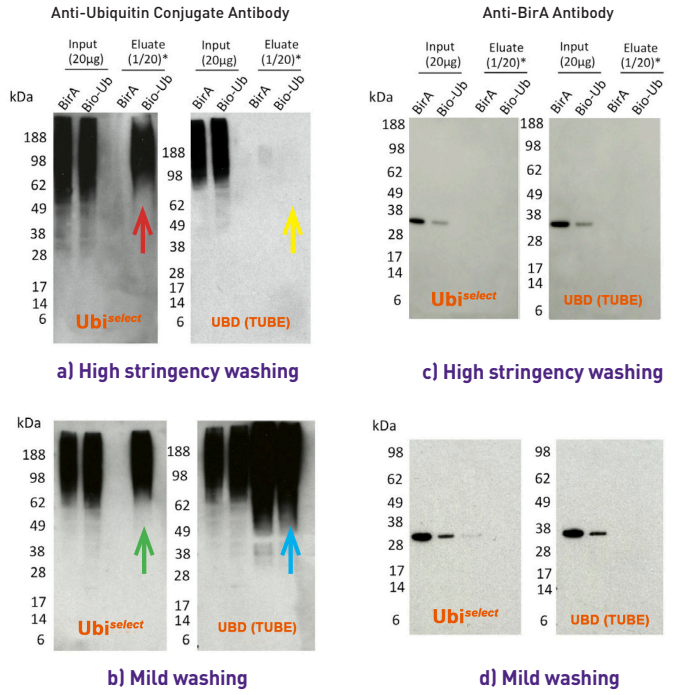


Figure 3: Ubi^{select} Results: Data shown in a) demonstrates that ubiquitin conjugates are still captured under the high stringency washing conditions of Ubi^{select} (red arrow) compared to UBD capture (yellow arrow). While data shown in b) demonstrates that under milder washing conditions proteins are now captured by both Ubi^{select} (green arrow) and UBDs (blue arrow). Data shown in c) and d) shows the presence of the BirA enzyme, detected using the anti-BirA antibody, in the input lanes only for the Ubi^{select}-MEF_BirA control and Ubi^{select}-MEF_Bio-Ub cell lysate capture experiments.

The Ubiquitin Binding Domain (UBD) evaluated in this experiment by way of example was a Tandem Ubiquitin Binding Entity (TUBE) however it is predicted that the Ubi^{select} system will also provide similar advantages over other UBD based ubiquitin conjugate capture systems.

*(1/20) One twentieth of the recovered sample analysed.

DISCUSSION

Comparison of Ubi^{select} and UBD capture methods demonstrated that under highly stringent washing conditions the capture of biotinylated ubiquitin conjugates from the Ubi^{select}-MEF_Bio-Ub cell lysates was possible while ubiquitin conjugates were lost using the UBD capture system. Thus the high stringency washing protocol of Ubi^{select} allows for the isolation of only those proteins that are conjugated to ubiquitin (either by a thioester bond or isopeptide linkage) and not associated ubiquitin binding proteins or ubiquitylated protein binding proteins (of course by altering the stringency of the wash conditions one can also capture these proteins if so desired; for example for comparative purposes). Whereas the UBD capture protocol was able to capture ubiquitylated conjugates *only* under the milder wash conditions thus risking the co-capture of ubiquitylated protein or ubiquitin chain binding proteins or indeed other interacting proteins.

References:

Franco M, (2011) A novel strategy to isolate ubiquitin conjugates reveals wide role for ubiquitination during neural development. *Mol Cell Proteomics* **10**, M110 002188.

Kirkin V and Dikic I. (2007) Role of ubiquitin- and Ubl-binding proteins in cell signaling. *Curr Opin Cell Biol* **19**, 199-205.

Lectez B, Migotti R, Lee SY, Ramirez J, Beraza N, Mansfield B, Sutherland JD, Martinez-Chantar ML, Dittmar G, Mayor U. (2014) Ubiquitin Profiling in Liver Using a Transgenic Mouse with Biotinylated Ubiquitin. *J Proteome Res* Apr 30 [Epub ahead of print].

Peng J, Schwartz D, Elias J E, Thoreen C C, Cheng D, Marsischky G, Roelofs J, Finley D, Gygi SP (2003) A proteomics approach to understanding protein ubiquitination. *Nat Biotechnol* **21**, 921-926.

Sylvestersen K B, Young C, Nielsen M L. (2013) Advances in characterizing ubiquitylation sites by mass spectrometry. Curr Opin Chem Biol **17**, 49–58.

Tirard M, Hsiao H H, Nikolov M, Urlaub H, Melchior F, Brose N. (2012) In vivo localization and identification of SUMOylated proteins in the brain of His6-HA-SUMO1 knock-in mice. *Proc Natl Acad Sci U S A* **109**, 21122-21127.

Tsirigotis M, Thurig S, Dube M, Vanderhyden BC, Zhang M, Gray DA (2001) Analysis of ubiquitination in vivo using a transgenic mouse model. *Biotechniques* **31**, 120-126.