# Ubi$^{select}$™ Kit

Cat. No. 67-0018-001
Lot. No. 30348

Storage: cryopreserved / -20˚C

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## Ubi$^{select}$ Kit Utility

The Ubi$^{select}$ Kit is designed to facilitate the isolation and identification of your ubiquitylated proteins of interest from a transgenic mouse-derived embryonic fibroblast (MEF) cell line expressing ubiquitin tagged with an N-terminal biotinylation signal (which is biotinylated by co-expression of the BirA enzyme). By employing a powerful capture system which utilises the transgenic MEF cell line expressing ubiquitin - which is biotinylated in the cell - and the high affinity biotin-avidin interaction alongside a high stringency washing protocol you can capture your ubiquitylated target of interest without any associated ubiquitin binding proteins and/or ubiquitylated protein binding proteins. The Ubi$^{select}$ Kit contains an aliquot of each of the control Ubi$^{select}$-MEF_BirA (P0) and the Ubi$^{select}$-MEF_Bio-Ub (P0) cell lines and an anti-ubiquitin conjugate antibody for the detection of ubiquitylated proteins. Eluted captured products may be analysed by Western blotting using the ubiquitin conjugate specific antibody, an anti-biotin antibody or an antibody specific to your protein of interest.

### 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. Ubiq-

## Kit Components

| Product | Cat. No. | Lot No. | Amount | Storage |
|---|---|---|---|---|
| **Ubi$^{select}$™-MEF_BirA (P0)** | 66-5010-001 | 30346 | 2.5x10$^6$ cells/ml | cryopreserved |
| **Ubi$^{select}$™-MEF_Bio-Ub (P0)** | 66-5011-001 | 30347 | 2.5x10$^6$ cells/ml | cryopreserved |
| **Mono and Polyubiquitylated conjugates, mAb (FK2) HRP linked** | 68-0122-025 | 30125 | 25 µg | -20˚C |

## Cell Culture Characteristics

**Species:** mouse

**Source:** mouse embryonic fibroblasts

**Culture Medium:** DMEM (HG), 200mM L-glutamine, 100mM sodium pyruvate, 10% fetal bovine serum (FBS), biotin 0.2 µg/mL, 1x pen/strep

**Growth Mode:** adherent

**Passage Number:** passage 0

**Storage:** cryopreserved

**Quality Assurance:**
Mycoplasma: not detected
Morphology: fibroblast
Viability (%): ≥95

## Ubi$^{select}$ Cell Culture Protocol

**Cell culture procedure for Ubi$^{select}$-MEF_BirA (P0) and Ubi$^{select}$-MEF_Bio-Ub (P0)**

Thaw the cells by swirling the vial in a 37°C waterbath. Place 5 ml of media into a T25 flask and pipette cells into the flask containing the media. Place the T25 flask containing the cells into a 37°C 5% $CO_2$ incubator overnight and re-feed the next morning with fresh media.

When BirA and Bio-Ub MEF cells reach confluency, passage as follows.

1. Aspirate culture media from the T25 flasks and rinse cells with 5 ml PBS.
2. Aspirate PBS from the T25 flask and add 1 ml 0.05% Trypsin-EDTA (1x). Incubate the cells in a 37°C 5% $CO_2$ incubator for a few minutes until the MEF cells begin to detach then knock the side of the flask to fully detach the cells.
3. Add 4 ml of media, collect in a 15 ml tube and count a sample of the cells. Seed MEF cells in T75 flasks at 1.2 x 10$^4$ cells/cm$^2$ (i.e. 9x10$^5$ cells per T75 flask) then place in a 37°C 5% $CO_2$ incubator and grow until confluency (P1).
4. Re-feed MEF cells every 3-4 days where necessary. When the BirA and BioUb MEFs have reached confluency, passage for a second time (P2) as above into T75 flasks, seeding at 1.2 x 10$^4$ cells/cm$^2$ (i.e. 9 x 10$^5$ cells per T75 flask).



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Lot-specific COA version tracker: v1.0.0

uitin 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 UPS substrates 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 which express either three moieties of biotin-accepting ubiquitin plus the *E. coli* enzyme BirA or the *E.coli* enzyme BirA enzyme alone (Lectez *et al.*, 2014). The

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## Ubi$^{select}$ Cell Culture and Capture Protocol

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5. At passage 3 (P3), plate BirA and Bio-Ub MEFs onto 100 mm dishes at a seeding density of 6 x $10^5$ cells per dish. Harvest BirA and Bio-Ub MEFs when they reach confluency or as necessary.

### Ubi$^{select}$ Capture Protocol*

1. To harvest BirA and Bio-Ub MEFs, remove media from the 100 mm BirA MEF and Bio-Ub MEF cell dishes, wash with 5 ml ice cold PBS and aspirate.
2. Collect the cells from each 100 mm dish by adding 200 µl of Cell Lysis Buffer to each dish, using a cell scraper as necessary.
3. Prepare separate MEF cell lysates from P3 BirA and Bio-Ub MEF cells. Homogenise using a needle and syringe and clarify the lysate by centrifuging at 4000 rpm for 10 minutes. Collect the supernatant and determine the protein concentration using standard methods (Input Sample). In the protocol below, a final volume of 2.5 ml of Input Sample derived from each cell line has been assumed.
4. Keep aside Input Sample for both BirA and Bio-Ub MEF cell lysates to run on your SDS-Page gels for Western blotting.
5. Equilibrate two PD10 columns by allowing 25 ml of Binding Buffer (-NEM) to flow through.
6. Add 2.5 ml of P3 BirA and Bio-Ub MEF Input Samples separately to the two equilibrated PD10 columns.
7. Add 3.5 ml of Binding Buffer (+NEM) to the two PD10 columns and separately collect the eluate for the BirA and Bio-Ub MEF cell lysates into 15 ml falcon tubes containing 250 µl of 25x protease inhibitor (PI) mixture (prepare 25x PI by adding one Protease Inhibitor tablet (Roche Cat# 11836170001) to 0.4ml of Binding Buffer (-NEM)) and 250 µl of NeutrAvidin agarose bead slurry. To prepare the NeutrAvidin bead slurry, wash the required amount of beads three times in Binding Buffer (-NEM) then generate a 50:50 bead:Binding Buffer (-NEM) slurry.
8. Incubate the 4 ml of eluate plus PI mix and NeutrAvidin agarose bead suspension on a rolling platform for 40 minutes at RT then a further 2 hrs and 20 mins at 4˚C.
9. Wash beads in 10 ml WB1 x2 washes, centrifuge for 1 minute at 4000 rpm, discarding the supernatant after each wash.

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Ubi$^{select}$ kit includes an aliquot of both the Ubi$^{select}$-MEF_Bio-Ub (P0) (Cat# 66-5011-001) and the control Ubi$^{select}$-MEF_BirA (P0) (Cat# 66-5010-001) cell lines and an anti-ubiquitin conjugate antibody for the detection of ubiquitylated proteins; see Application Note 004 for details. Ubi$^{select}$-MEF_Bio-Ub (P0) and Ubi$^{select}$-MEF_BirA (P0) have been derived from 13.5 day old embryos from these transgenic mouse lines by 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 has been possible to 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 would otherwise predominate under the milder washing conditions of alternative ubiquitin capture systems. By utilising Bio-Ub expressing liver lysates, the isolation of ubiquitin conjugated proteins (both thioester and isopeptide linked) has been demonstrated, therefore this is 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 Bio-Ub Mouse Embryonic Fibroblast (MEF) cell lines has also been demonstrated (See Application Note 004)

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## Ubi$^{select}$ Cell Culture and Capture Protocol

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Continue with the following wash steps centrifuging and carefully discarding the supernatant as above in-between each wash step.

10. 10 ml WB2 x3 washes
11. 10 ml WB3 x1 wash
12. 10 ml WB4 x3 washes
13. 10 ml WB1 x1 wash
14. 10 ml WB5 x1 wash
15. 10 ml WB6 x3 washes
16. Add 100 µl of Elution Buffer to each lot of beads, transfer to a 1.5 ml tube and incubate for 5 min at 95 °C.
17. Add the sample to a Vivaclear Mini 0.8-µm PES microcentrifuge filter unit and centrifuge for 1 minute at 6000 rpm. The recovered sample is the Output Sample.
18. The Output Sample (captured biotinylated ubiquitin - both in its free form and conjugated to ubiquitylated proteins) is now ready for analysis. For analysis by Western Blotting you may wish to run 1/20 of the Output Sample per lane by SDS-PAGE and an aliquot of the Input Sample alongside (1/20 or an amount to be determined empirically) for comparison (pre and post-purification). Analyse both your BirA and Bio-Ub MEF Input and Output Samples for comparisons (see Application Note 004 for an example).
19. For the detection of biotinylated ubiquitin conjugates by Western blotting use the Mono and Polyubiquitylated conjugates, mAb (FK2) HRP linked antibody supplied with the Ubi$^{select}$ kit. The incubation conditions for this antibody are as follows: Block the Western blot membrane in 5% Milk/TBST for 1 hour at room temperature then rinse the membrane in TSBT to remove any residual milk. Then incubate the Western blot membrane with Mono and Polyubiquitylated conjugates, mAb (FK2) HRP linked antibody (1:4000) in 1% BSA/TBST for 2 hours at room temperature, wash for 5 minutes in TBST, repeat three times and develop the Western Blot membrane accordingly (for example by ECL).

*NB: This protocol has been optimised to enable the selective capture and purification of ubiquitylated proteins *only* away from ubiquitylated protein binding proteins and ubiquitin and ubiquitin chain binding proteins. If you wish to also capture such binding proteins then adjust the formulation of the Cell Lysis, Binding Buffers and Wash Buffers (WB) and the protocol according to your stringency requirements (eg through the adjustment in the binding and wash step configurations including the concentration or removal of SDS, guanidine and/or urea).

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and it is anticipated that this technology will enable researchers to gain a better understanding of ubiquitin system signalling.

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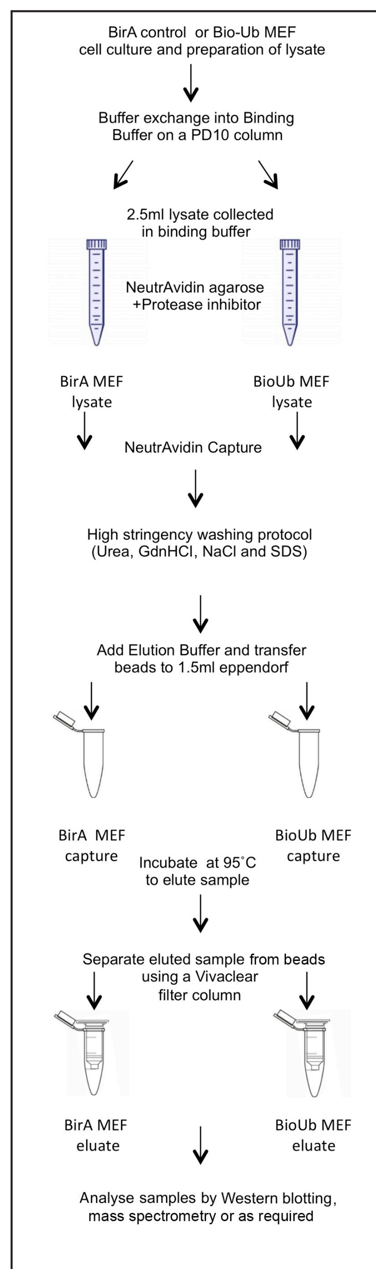
Tsirigotis M, Thurig S, Dube M, Vanderhyden BC, Zhang M, Gray DA (2001) Analysis of ubiquitination in vivo using a transgenic mouse model *Biotechniques* **31**, 120-126.

## Capture Protocol Flow Diagram



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## Buffers and Reagents

### Binding Buffer (+NEM)
3M urea, 1M NaCl, 0.25% SDS and 50 mM N-ethylmaleimide in PBS

### Binding Buffer (-NEM)
3M urea, 1M NaCl and 0.25% SDS in PBS.

### Cell Lysis Buffer
8M urea, 1% SDS and 50mM N-ethylmaleimide in PBS, (add one protease inhibitor tablet (Roche Cat# 11836170001) to 10ml of Cell Lysis Buffer).

### Elution Buffer
4× Laemmli buffer including 100 mM DTT for reducing Elution Buffer and minus DTT for non-reducing Elution Buffer

### TBS Tween (TBST)
50mM Tris (pH7.6), 150mM NaCl, 0.05% Tween 20

### WB1
8M urea and 0.25% SDS in PBS

### WB2
6M guanidine HCl in PBS

### WB3
6.4M urea, 1M NaCl and 0.2% SDS in PBS

### WB4
4M urea, 1M NaCl, 10% isopropanol, 10% ethanol and 0.2% SDS in PBS

### WB5
8M urea and 1% SDS in PBS

### WB6
2% SDS in PBS

### Reagents not included in this kit:

- High capacity NeutrAvidin agarose (Thermo Scientific Cat# 29202)
- Anti-BirA antibody (Sigma Cat# GW20013F)
- Anti-biotin (D5A7) rabbit mAb; HRP conjugate (Cell Signalling Cat# 5571)
- Urea (Sigma, Cat# U0631)
- Sodium Dodecyl Sulfate (SDS) (Sigma, Cat# L6026)
- Guanidine HCl (Sigma, Cat# G3272)
- N-Ethylmaleimide (NEM) (Thermo Fisher, Cat# 23030)
- Protease inhibitor cocktail-cOmplete mini-EDTA free (Roche, Cat# 11836170001)
- Phosphate Buffered Saline (Sigma, Cat# P4417)
- PD-10 Columns (GE Healthcare Cat# 17-0851-01)
- Vivaclear Mini 0.8-µm PES microcentrifuge

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