MANUAL

E2 SELECT™ Profiling & Selection Kit

Catalog Number UC104C

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BACKGROUND

Ubiquitin and Ubiquitin Conjugation Machinery

Ubiquitin is a small polypeptide that can be conjugated via its C-terminus to amine groups of lysine residues on target proteins. This conjugation is referred to as monoubiquitylation. Additional ubiquitin moieties can be conjugated to this initial ubiquitin utilizing any one of the seven lysine residues present in ubiquitin. The formation of these ubiquitin chains is referred to as polyubiquitylation. The most well characterized of this polyubiquitylation is chain formation via lysine at position 48 of ubiquitin (K48-linked chains). Monoubiquitylation has been shown to alter the localization, activity, and/or function of the target protein. The most prevalent consequence of polyubiquitylation is the proteasome-mediated degradation of the target protein.

The conjugation of ubiquitin to a target protein requires the co-ordinated function of three distinct enzymes, **E1** (ubiquitin activating enzyme), **E2** (ubiquitin conjugating enzyme), and **E3** (ubiquitin ligase) resulting in isopeptide bond formation between the C-terminus of ubiquitin and the ε-amino group of the lysine residue on target proteins. Ubiquitin E3 ligases act as scaffold proteins, providing docking sites for an ubiquitin-conjugating enzyme (E2), and a target substrate. Typically, E3 ligases mediate the transfer of ubiquitin from an E2 thioester intermediate to an amide linkage with a substrate protein (Hershko and Ciechanover, 1998). In addition to the ubiquitylation of substrates, E3 ligases can also "autoubiquitylate" themselves. There are two classes of E3 ligases: RING E3s, which act as scaffolds to bring the components of the ubiquitylation machinery together in close contact with the substrate, and HECT E3s that form intermediates with ubiquitin before transferring it to the substrate.

ABOUT THE ASSAY

The E2 SELECT™ Profiling & Selection Kit has been developed for the exploration of functional competency between E2's and E3's. At the core of the assay are microtiter plates pre-coated with a proprietary reagent that captures polyubiquitin chains formed in an E3 ligase-dependent reaction. For the assay, an E1, E2, Ubiquitin (Ub) cocktail is present in each well of the plate; different wells contain different E2's, with each of 24 human E2's being represented by triplicate wells (see map, page 5). An E3 ligase of interest, together with ATP, is then added directly to the wells. During the reaction, polyubiquitin chains generated by the E1-E2-E3 machinery are recognized and captured in the wells. Following the reaction and subsequent wash steps, the isolated polyubiquitylated product is incubated with Detection Reagent 1 and streptavidin-HRP allowing for detection by colorimetry. Thus, the signal generated by captured polyubiquitylated product in this "sandwich" ELISA-like assay is a quantitative measure of E2-E3 functionality. **Furthermore, this detection strategy does not require additional non-native tagging or labeling of ubiquitin, which could lead to experimental artifacts.**

BENEFITS

- Ready-to-assay. Contains 24 human E2's pre-plated in triplicate with E1 and Ubiquitin
- Just add an E3 and ATP, detect and read
- Detection system provides robust readout for E3 ligase activity
- E2 SELECT™ kit utilizes non-radioactive reporter substrates
- Uses native, rather than tagged, ubiquitin

SUGGESTED USES

1. Testing functionality of over 24 E2's with an E3 ligase of interest

COMPONENTS

Store Colorimetric Reagent and Stopping Reagent at 4ºC. All other materials should be stored at -80ºC. Avoid cycles of freezing and thawing. *For consistent and reproducible results, use the kit within one month.*

1. **Detection Reagent 1**

   Size:1 x 35 µl (1000X)

   Buffer:20 mM Tris (pH 8.0), 150 mM NaCl, 10% glycerol

2. **96-well microtiter plate (modular)**

   Plates are pre-coated with LifeSensors' proprietary polyubiquitin capture reagent, and pre-filled with solution containing E1, E2 (variable by position, see map page 5), and Ub. Reactions are initiated by adding E3 and ATP directly to plate wells.

3. **Colorimetric Reagent (CR)**

   Size: 1 x 5 mL

4. **Stopping Reagent (SR)**

   Size: 1 x 5 mL

ADDITIONAL ITEMS REQUIRED

1. **Wash Buffer(s)**

   Phosphate Buffered Saline, 0.1% Tween (PBST)

   5% Bovine Serum Albumin (BSA) in PBST

2. **Absorbance capable plate reader**

   After adding the Colorimetric reagent, the solution will change to blue color and can be detected at Absorbance of 650 nm. After adding the Stop solution, it will change color from blue to yellow and can be measured at Absorbance 450 nm.

3. **Streptavidin Secondary Detection Reagent**

   The selection of appropriate streptavidin reagent is critical to generating a robust signal. We do not recommend using avidin-horseradish peroxidase (HRP) conjugates. We strongly recommend using one of the following streptavidin-HRP reagents which have been used successfully with the assay:

   Anaspec (catalog # 60668)

   Jackson ImmunoResearch Laboratories, Inc. (catalog # 016-030-084)

   Rockland, Inc. (catalog # S000-03)

   Sigma-Aldrich (catalog # S-2438)

4. **Adenosine triphospate (ATP)**

   Recommended: 0.1M stock.

5. **1.5 ml snap cap tubes**
6. **15 and 50 ml centrifuge tubes**

SOLUTIONS FOR E3 LIGASE REACTION

E3 Ligase, ATP Solution (10x): 1ml

In a final volume of 1ml, dilute ATP to 2mM and E3 ligase of interest to (10x) optimized concentration in PBS. If optimal concentration is unknown, 10nM final (10x = 0.1μM) is a recommended starting point.

PBST with 5% BSA: 20ml

Add 1g of BSA to 20ml of PBST.

Detection Solution 1: 10 ml

Add 10 µl **Detection Reagent 1** to 10ml of PBST with BSA **immediately before use (Step 9)**.

Streptavidin Secondary Solution: 10 ml

Dilute **Streptavidin Secondary Detection Reagent** into 10ml of PBST with BSA. **Use immediately (Step 11)**.

A dilution of 1:10,000 is recommended for Streptavidin-HRP.

E3 Ligase Activity Assay (Suggested Protocol)

1. As directed in previous sections, prepare all reagents and samples.
2. Remove aluminum seal from plate and allow coated plate wells to equilibrate to room temperature. Removing seal from frozen plate will prevent cross-contamination of wells.
3. Add 10μl of **E3 Ligase, ATP Solution (10x)** to each well of the plate. **NOTE: When washing the plate wells and adding reagents, be sure to pipet onto the side of the well to avoid contact with the bottom.**
4. Incubate for approximately 60 minutes at room temperature.
5. Remove and discard well contents and wash each well, repeating the process for a total of three washes. Wash each well by filling each well with **PBST** (200 µl).
6. Add 100 µl of **Detection Solution 1** (1:1000 in PBST + 5% BSA) to each well. Incubate for 1 hour at room temperature.
7. Repeat the removal/wash as in step 8.
8. Add 100 µl of **Streptavidin Secondary Solution** (1:10,000 in PBST + 5% BSA) to each well. Incubate for 1 hour at room temperature.
9. Repeat the removal/wash, for a total of four washes.
10. For colorimetric detection, add 50 µl of Colorimetric Reagent to each well, and incubate for 1-30 minutes. A blue color will develop and can be read at Absorbance 650 nm using the plate reader. When the solution has turned to a bright blue color (absorbance 650 = ~1.0) proceed to step 11.

    Note: prolonged incubation which makes the color darker blue might saturate the signal after adding stop solution. Do not let the signal saturate.
11. To stop the reaction, add 50 µl/well of Stopping Reagent on top of colorimetric reagent. The solution will change color from blue to yellow. Measured the Absorbance at 450 nm within 30 minutes of adding the stopping reagent.

	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.
A.	Empty	Empty	Empty	UBE2D3	UBE2D3	UBE2D3	UBE2L3	UBE2L3	UBE2L3	UBE2T	UBE2T	UBE2T
B.	No E2	No E2	No E2	UBE2E2	UBE2E2	UBE2E2	UBE2L6	UBE2L6	UBEL6	UBE2W	UBE2W	UBE2W
C.	Complete	Complete	Complete	UBE2E3	UBE2E3	UBE2E3	UBE2M	UBE2M	UBE2M	UBE2Z	UBE2Z	UBE2Z
D.	3-Linear	3-Linear	3-Linear	UBE2F	UBE2F	UBE2F	UBE2N/ UBE2V2	UBE2N/ UBE2V2	UBE2N/ UBE2V2	No E2	No E2	No E2
E.	UBE2A	UBE2A	UBE2A	UBE2G2	UBE2G2	UBE2G2	UBE2Q2	UBE2Q2	UBE2Q2	Complete	Complete	Complete
F.	UBE2C	UBE2C	UBE2C	UBE2H	UBE2H	UBE2H	UBE2R1	UBE2R1	UBE2R1	3-Linear	3-Linear	3-Linear
G.	UBE2D1	UBE2D1	UBE2D1	UBE2I	UBE2I	UBE2I	UBE2R2	UBE2R2	UBE2R2	Empty	Empty	Empty
H.	UBE2D2	UBE2D2	UBE2D2	UBE2K	UBE2K	UBE2K	UBE2S	UBE2S	UBE2S	Empty	Empty	Empty

Map of E2 SELECT™ plate showing spatial arrangement of E2's (above). Note that UBE2N and UBE2V2 are known to function as a heterodimer and therefore were combined in wells D7-D9. Empty wells contain reaction buffer but no enzymes or Ub. Wells labeled "No E2" contain E1 and Ub only (negative control). Complete reactions contain E1, E2, E3, and Ub, but no ATP (positive control for enzymes). Wells labeled "3-linear" contain tri-ubiquitin chains linked through the N-terminal amine, and serve as a control for the plate's ability to capture formed chains. Addition of **E3 Ligase, ATP Solution (10x)** to each well will not compromise the intended controls in any way, and is required in the case of "complete" wells which lack their own ATP. Below is a list of the individual components, their alternative names, and the catalog numbers (LifeSensors, Inc.) for ordering additional supply (as desired; not necessary for this kit).

Name	Alt.	Cat#
UBE2A	Rad6A	UB226H
UBE2C	Ubc10	UB222
UBE2D1	UbcH5	UB210
UBE2D2	UbcH5b	UB207H
UBE2D3	UbcH5c	UB201
UBE2E2	UbcH8	UB212
UBE2E3	UbcH9	UB213
UBE2F	NCE2	UB216
UBE2G2	Ubc7	UB227
UBE2H	E2-20K	UB215T
UBE2I	Ubc9	UB228H
UBE2K	UbcH1	UB204
UBE2L3	UbcH7	UB202
UBE2L6	UbcH8	UB229
UBE2M	Ubc12	UB217H
UBE2N	Ubc13	UB218
UBE2Q2	-	UB225
UBE2R1	CDC34	UB208H
UBE2R2	Ubc3B	UB209
UBE2S	E2EPF	UB221
UBE2T	PIG50	UB219
UBE2V2	MMS2	UB220
UBE2W	Ubc16	UB223H
UBE2Z	Use1	UB230

REFERENCES

1. Hershko, A. and A. Ciechanover, The ubiquitin-proteasome pathway. *Annu Rev Biochem*, 1998. **67**: p. 425-479.
2. Welchman, R.L., C. Gordon, and R.J. Mayer, Ubiquitin and ubiquitin-like proteins as multifunctional signals. **Nat Rev Mol Cell Biol**, 2005. **6**(8): p. 599-609.
3. Ciechanover, A., The ubiquitin-proteasomal pathway: on protein death and cell life. Embo J, 1998. **17**(24): p. 7151-7160.
4. Raasi, S., *et al.*, Diverse polyubiquitin interaction properties of ubiquitin-associated domains. Nat Struct Mol Biol, 2005. **12**(8): p. 708-14.

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