Application Note

# Detecting Substrate Ubiquitination by the E3 Ligase, XIAP, Using Quantitative Assays


## Summary

Quantitative assays for investigating ubiquitination pathways are essential for advancing ubiquitin-related drug discovery research. To illustrate the utility of *in vitro* ubiquitination enzyme cascades, we quantify the ubiquitination of Smac/DIABLO by the E3 ligase XIAP using a functional enzymatic reaction coupled to multiple assay readouts. We compare different detection methods that can be used to test small molecules for their potential to modulate XIAP-mediated ubiquitination in the context of this assay. The data provide a basis for use of these recombinant E3 ligase cascades in various applications, including screening campaigns.


## Introduction

Ubiquitination is a reversible post-translational modification used extensively as a regulatory mechanism in eukaryotic cells. The attachment of ubiquitin to cellular proteins is a feature of numerous cell signaling pathways and influences activities such as protein localization, trafficking and degradation by the proteasome.

Misregulation of ubiquitin signaling is linked with a number of human diseases, including neurodegeneration, cancer, inflammation and muscle-wasting disorders, and there is growing interest in the ubiquitin system as a source of potential drug targets[1]. One such target of interest is the E3 ligase, X-linked inhibitor of apoptosis (XIAP), which acts as an endogenous inhibitor of caspase-3, -7 and -9. XIAP belongs to the RING family of ubiquitin E3 ligases and facilitates the ubiquitination of numerous substrates, including those not directly concerned with apoptotic suppression[2]. Among the pro-apoptotic substrates ubiquitinated by XIAP is the mitochondrial protein Smac/DIABLO. When released to the cytosol in response to apoptotic stimuli, Smac/DIABLO induces the release of XIAP from caspases, thus antagonizing XIAP's anti-apoptotic effect[3,4]. Ubiquitination of Smac/DIABLO by XIAP promotes its proteasomal degradation, adding a layer of regulatory complexity to the control of apoptosis[5]. Expression of XIAP is elevated in several malignancies, and the use of Smac/DIABLO mimetics is considered a promising strategy in anticancer therapies[6].

In this study, we show that quantitative detection of the ubiquitination of Smac/DIABLO by XIAP can now be achieved using recombinant proteins and robust, scalable assay technologies. Specifically, we compare Western blot, electrochemiluminescence and ELISA detection of ubiquitination and the modulation of ubiquitination cascades by small molecules.

## *In Vitro* Ubiquitination of the Smac/DIABLO Substrate by XIAP

Similar to other ubiquitination pathways, the ubiquitination of Smac/DIABLO by XIAP follows three major steps:

I. Activation of ubiquitin via a **ubiquitin activating enzyme (E1)**. A single ubiquitin is transferred onto the E1 in an ATP-dependent reaction.

II. Spontaneous transfer of the activated ubiquitin from the E1 enzyme to a **ubiquitin conjugating enzyme (E2)**.

III. Transfer of the ubiquitin to the substrate. This process is facilitated by an **E3 ligase**, which coordinates ubiquitination by providing a binding platform for ubiquitin-charged E2 and specific substrates. This process may be repeated multiple times to yield a poly-ubiquitinated substrate.

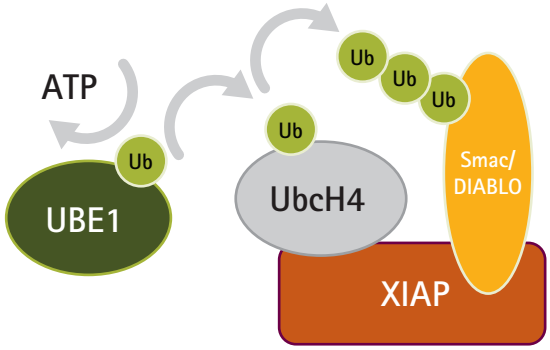


Figure 1. **Schematic representation of the XIAP ubiquitination cascade.** The three major steps leading to ubiquitination of Smac/DIABLO are indicated by the arrows (see text for details).

## Materials and Methods

### Generation of recombinant proteins

To reconstitute the ubiquitination of Smac/DIABLO by XIAP *in vitro*, UBE1 and UbcH4 were selected as the E1 and E2, respectively (Figure 1). Recombinant forms of the proteins comprising the cascade were expressed in either insect cells or *E.coli* and purified (>80% pure) via the affinity tag engineered into each protein (Figure 2). As shown in the table below, the components have been cloned with different epitope tags to allow them to be distinguished in downstream applications.

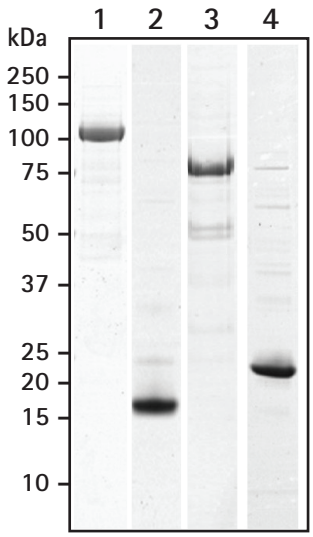


Figure 2. **SDS-PAGE gel showing recombinant proteins required to build the XIAP ubiquitination cascade.** Lane 1: UBE1 (E1); Lane 2: UbcH4 (E2); Lane 3: XIAP (E3); Lane 4: Smac/DIABLO (substrate).

| Product | Catalogue No |
|---|---|
| UBE1 (6His tag) | 23-021 |
| UbcH4 (HA and 6His tags) | 23-025 |
| XIAP (GST tag) | 23-056 |
| Smac/DIABLO (c-Myc and 6His tags) | 23-057 |

### Western blot analysis of *in vitro* ubiquitination

Following incubation of reaction components (50 nM UBE1 (E1), 1 µM UbcH4 (E2), 100 nM XIAP (E3), 1 µM c-Myc-tagged Smac/DIABLO (substrate), 20 µM ATP and 10 µM ubiquitin (Sigma-Aldrich) for 16 hours at room temperature, the reaction was stopped with sodium dodecyl sulfate (SDS) sample buffer. Samples were loaded onto an SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to nitrocellulose. The blot was probed with anti-c-Myc antibody (Cat. No. 05-724) and a corresponding secondary antibody.

### ELISA and electrochemiluminescence (ECL) detection of *in vitro* ubiquitination

Cascade components were incubated in a 20 µL reaction volume in 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$ for 30 minutes at room temperature. The reaction was stopped by addition of 25 µL of 25 mM MOPS pH 7.5, 125 mM EDTA, 150 mM NaCl, 0.05% Tween® 20 and a 10 µL aliquot was transferred to the appropriate capture plate (coated with anti-c-Myc antibody).

After washing in phosphate-buffered saline (PBS) with 0.5% Tween® 20, the signal was detected by addition of streptavidin-ruthenium (Meso Scale Discovery; for ECL) or streptavidin-HRP (GE Healthcare; for ELISA). ELISA signal was detected by addition of a suitable chemiluminescence reagent. Final assay concentrations were 10 nM UBE1 (E1), 50 nM UbcH4 (E2), 7.5 nM XIAP (E3), 12.5 nM Smac/DIABLO (substrate), 10 µM ATP and 2 µM biotinylated ubiquitin.

## Results

### Detection of substrate ubiquitination by Western blot

Detection of Smac/DIABLO ubiquitination can be performed in a qualitative manner by Western blot analysis. Incubation of the cascade components with ATP and ubiquitin allows the reaction to occur. Ubiquitination of c-Myc-tagged Smac/DIABLO can then be observed by probing an aliquot of the stopped reaction on a Western blot with an anti-c-Myc antibody that specifically detects the substrate (Figure 3).

A ladder of bands can be seen, corresponding to multiple poly-ubiquitinated forms of Smac/DIABLO (Lane 1). This experiment confirms that all reaction components are active, and also demonstrates the specificity of the ubiquitination reaction, which does not occur when key components are excluded (Lanes 2-4).

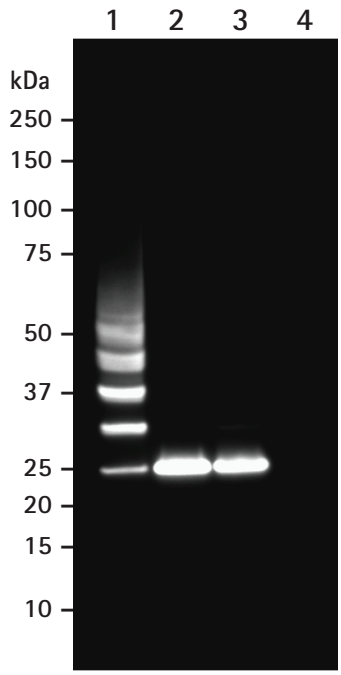


Figure 3. **Western blot shows that *in vitro* Smac/DIABLO ubiquitination by XIAP requires addition of ubiquitin, E1 and E2 proteins.** Lane 1: all reaction components (E1, E2, E3, substrate, ATP, ubiquitin); Lane 2: no ubiquitin; Lane 3: no E2; Lane 4: no substrate.

Similar analysis can be conducted to reveal ubiquitination of other reaction components (data not shown). To probe for ubiquitination of the E2, UbcH4, an anti-HA tag antibody may be used, while auto-ubiquitination of the E3, XIAP (in the absence of Smac/DIABLO) can be detected using an anti-GST antibody. Charging of the E1 with ubiquitin, in the absence of the E2, E3 and substrate, can be detected using an anti-His antibody.

The use of an anti-ubiquitin antibody as the primary detection reagent in these Western blots is not recommended, as several of the protein components may become ubiquitinated during the reaction, making the results difficult to interpret.

## Quantitation of substrate ubiquitination by ECL and ELISA

Western blot analysis provides an informative qualitative indication of the activity of a given ubiquitination cascade. However, for a quantitative measurement of ubiquitination, an alternative format is required. In particular, robust, highly sensitive assays are required by research and screening laboratories for testing the ability of small molecules to modulate the activity of ubiquitination cascades. For these applications, we used an electrochemiluminescence (ECL) assay (available from Merck Millipore as a service, UbiquitinProfiler™), and an enzyme-linked immunosorbent assay (ELISA).

To investigate the performance of these two assays, we compared the ECL method with the ELISA format. Both of these methods rely on biotinylated ubiquitin in the reaction and subsequent capture of the ubiquitinated substrate via its c-Myc tag. After washing, bound ubiquitin can be detected using a modified form of streptavidin appropriate for the assay format (Figure 4).

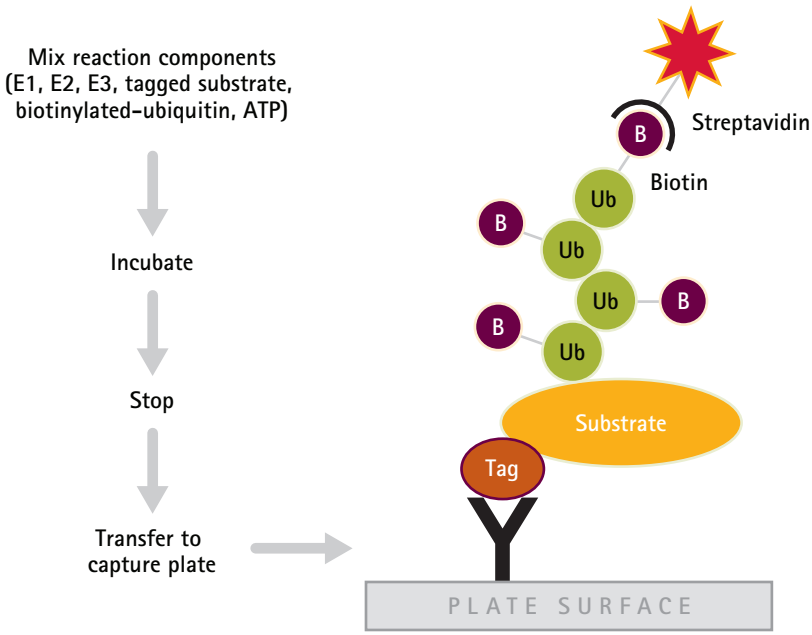


**Figure 4. Reaction outline for the ECL and ELISA assay formats.** During the reaction, the substrate is modified with biotinylated ubiquitin (B-Ub). An aliquot of the stopped reaction is transferred to a suitable capture plate for ECL or ELISA, which is coated with anti-c-Myc antibody to capture the substrate. Bound ubiquitin is detected by addition of labeled streptavidin. The red symbol represents the appropriate label: ruthenium bipyridine (in the case of the ECL assay) or HRP (in the ELISA).

To compare the two formats, the UBE1-UbcH4-XIAP-Smac/DIABLO cascade was used. As an initial indication of ubiquitination activity and the specificity of the signal, a series of 'knock-out' experiments was performed, involving systematic omission of individual components. As shown in Figure 5, omitting a single element from the reaction resulted in significant signal loss. Both assay formats gave comparable results.

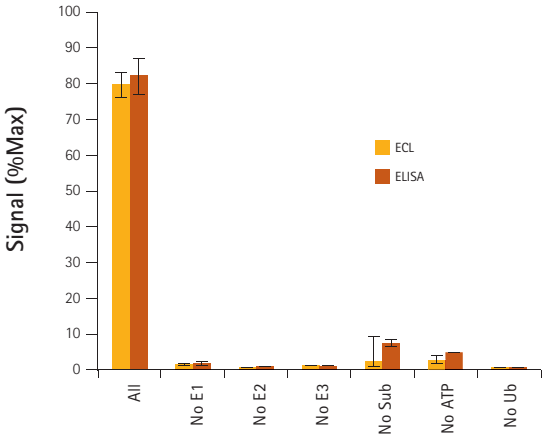


**Figure 5. Comparison of component omission in the ECL and ELISA formats.** The omitted component is indicated on the x-axis. Both ECL and ELISA results show that all components (E1, E2, E3, substrate, ATP, ubiquitin) are required for Smac/DIABLO ubiquitination by XIAP.

Further optimization of the assay conditions was performed by running a series of iterative titrations. The results are expressed as a percentage of the maximum signal obtained in each particular assay format (Figure 6). Excellent overlap was observed between the data obtained from the two formats, indicating that both detection methods are suitable for performing component titrations during functional assay development. This observation was also made for other ubiquitination cascades tested side-by-side in the ECL and ELISA formats (data not shown).

Merck Millipore's UbiquitinProfiler™ service is primarily designed for detecting modulators of E3 ligase activity. For this purpose, the concentrations of all components are set at saturating levels with the exception of the E3. It is recommended that researchers wishing to employ the ECL or ELISA format in their own laboratories should perform their own titrations, and set final assay concentrations based on their own requirements. Appropriate concentrations may also depend upon the selected plate reader and the achievable signal. It may be possible to optimize this further by conducting a reaction time course (ensuring that the response has not reached a saturating level at the selected endpoint).

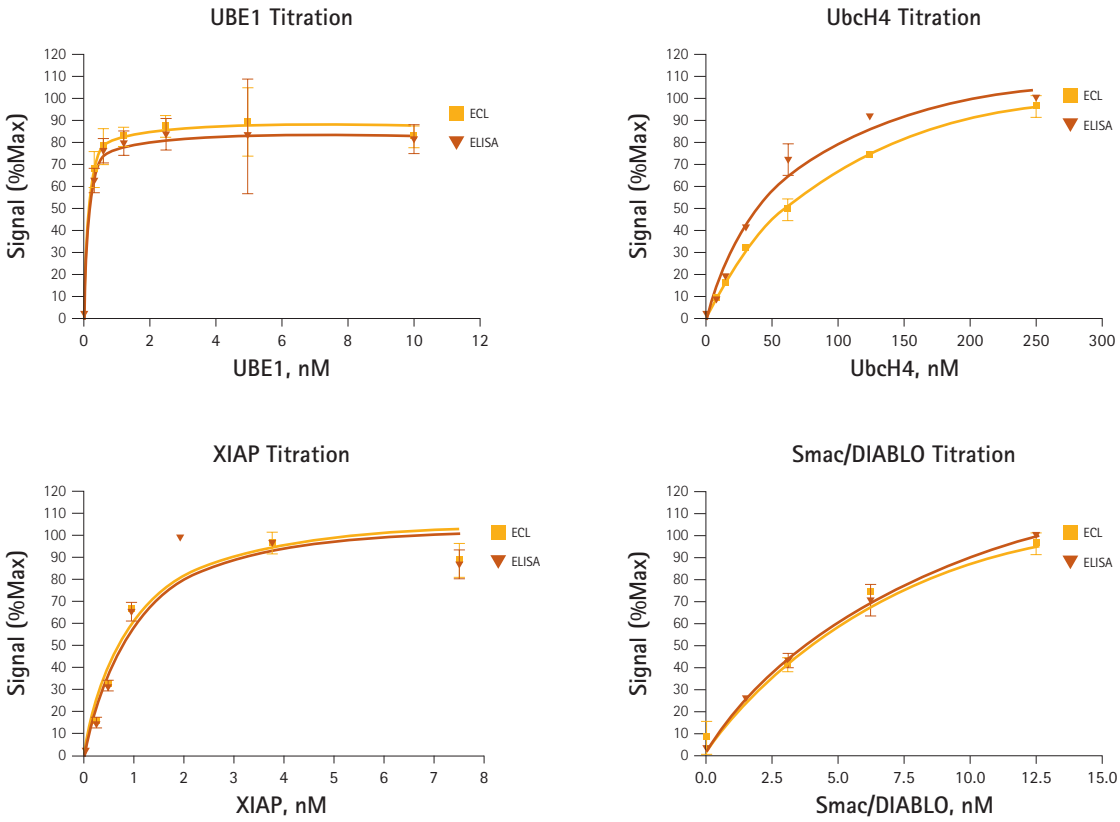


**Figure 6. Comparison of component titrations for the UBE1–UbcH4–XIAP–Smac/DIABLO cascade in the ECL and ELISA formats.** The selected component was titrated as shown on each x-axis, with all other components held at fixed concentrations. Aliquots of the stopped reaction were transferred to capture plates for processing by both the ECL and ELISA methods to ensure a direct comparison between the two. Similar titrations were also conducted for ATP and biotinylated ubiquitin (data not shown).

## Testing small molecule inhibitors of ubiquitination

Once optimal concentrations had been established for all cascade components, the ECL and ELISA formats were compared for their response to inhibitory compounds (Figure 7). Ro-106-9920 is reported to inhibit several E3 ligases and displays inhibition of the cascade of interest here, UBE1-UbcH4-XIAP-Smac/DIABLO, in the micromolar range. GW5074 is a characterised kinase inhibitor, which also behaves as an inhibitor towards the XIAP cascade as well as a number of other E3 ligase cascades (data not shown). PYR-41 is a known E1 inhibitor and as expected, also reduces the activity of the cascade UBE1-UbcH4-XIAP-Smac/DIABLO.

Importantly, all three compounds showed comparable $IC_{50}$ values in the two assay formats. This supports the interchangeability between the ECL format, used regularly compound screening by the UbiquitinProfiler™ service, and the ELISA format for the screening of small molecule modulators of the ubiquitin ligase cascades. With robust signal:noise ratios and Z' values routinely in excess of 0.6, the ELISA should readily lend itself to screening campaigns and may represent a practical choice for those researchers who do not have access to ECL capabilities in-house.

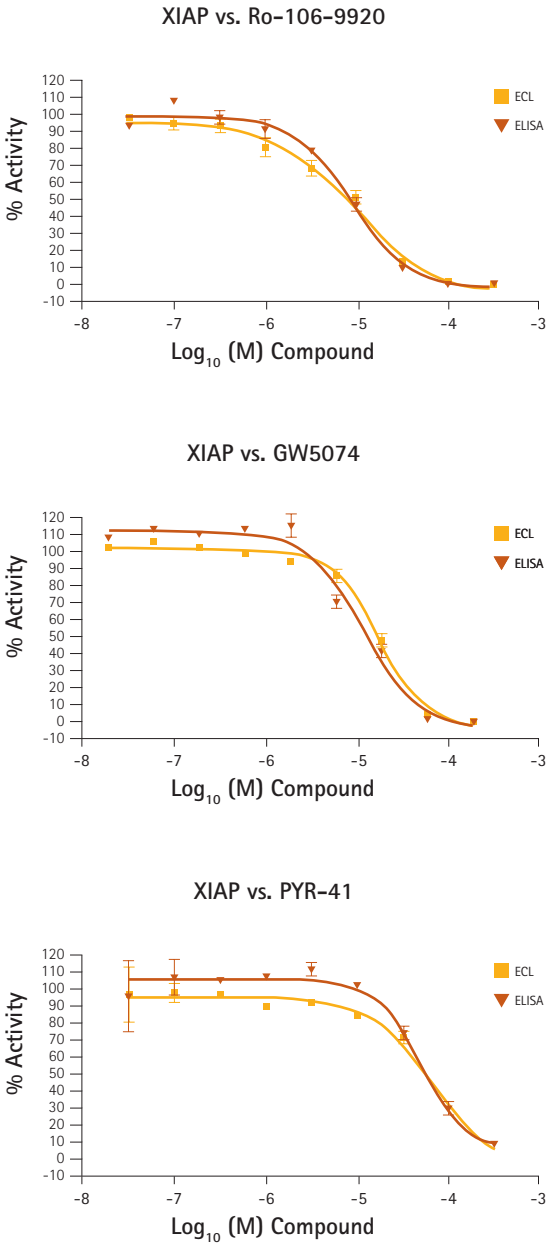


**Figure 7. Comparison of compound $IC_{50}$ determinations using the ECL and ELISA formats.** Cascade reactions were conducted as in Figure 5 but with the inclusion of test compound. The final concentration of DMSO solvent was 2%. Each data point represents the mean of two replicates, with results expressed as % activity of the XIAP cascade relative to control wells containing 2% DMSO. $IC_{50}$ values in the ECL and ELISA formats, respectively, were: Ro-106-9920, 9.3 µM and 8.8 µM; GW5074, 19 µM and 12 µM; PYR-41, 66 µM and 49 µM.

## Conclusions

Development of epitope-tagged proteins allows interrogation of the therapeutically-relevant UBE1-UbcH4-XIAP-Smac/DIABLO ubiquitination cascade. These proteins are amenable to use in multiple assay formats, and can be used to investigate the potential of small molecules to modulate the E3 ligase activity of XIAP, as well as other cascade components. An ELISA format performs comparably to the well-characterized ECL format and should be adaptable for compound screening campaigns. Similar principles to those outlined here can be used to investigate other ubiquitination cascades, using recombinant proteins available from Merck Millipore. Many of these cascades are also available for screening through the UbiquitinProfiler™ service.

The utility of these proteins extends beyond the functional assays described above, to use in applications such as binding assays and crystallography for the further investigation of protein-protein interactions. These products and associated services provide a complete solution to support researchers in the field of ubiquitin-related drug discovery.

## References


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| E3 Ligase or Complex | Cat. No. | E2 Conjugating Enzymes | Cat. No. | E1 Activating Enzyme | Cat. No. | Substrates | Cat. No. |
|---|---|---|---|---|---|---|---|
| XIAP **New!** | 23-056 | UbcH4 | 23-025 | | | Smac/DIABLO | 23-057 |
| MuRF1 **New!** | 23-054 | UbcH5c | 23-035 | | | Cardiac Troponin I | 23-055 |
| VHL Penta-complex | 23-044 | UbcH4<br>UbcH5a<br>UbcH5c | 23-025<br>23-029<br>23-035 | | | HIF-1α | 23-045 |
| MDM2/CK1δ | 23-032 | UbcH4<br>UbcH5c | 23-025<br>23-035 | | | p53 | 23-034 |
| MDM2(c-Myc) | 23-033 | UbcH4<br>UbcH5c | 23-025<br>23-035 | | | auto-ubiquitination | |
| Parkin<br>Parkin(c-Myc) | 23-048<br>23-046 | UbcH6<br>UbcH7 | 23-036<br>23-047 | UBE1 | 23-021 | p38/JTV-1<br>auto-ubiquitination | 23-049 |
| c-Cbl | 23-041 | UbcH4 | 23-025 | | | Kit<br>Src | 23-043<br>23-042 |
| CHIP<br>CHIP(c-Myc) | 23-050<br>23-053 | UbcH13/Uev1A | 23-051 | | | Hsp70<br>p53<br>auto-ubiquitination | 23-052<br>23-034 |
| SCF-βTrCP1 Quad-complex | 23-026 | UbcH3<br>UbcH4 | 23-022<br>23-025 | | | IκBα<br>β-Catenin | 23-028<br>23-027 |
| SCF-Fbw7 Quad-complex | 23-030 | UbcH3<br>UbcH5a | 23-022<br>23-029 | | | Cyclin E1 Complex | 23-031 |
| SCF-Skp2/Cks1 Penta-complex | 23-023 | UbcH3 | 23-022 | | | p27 Complex | 23-024 |

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