Protocol Guide

Ubiquitin Solutions

Simplifying Ubiquitination Drug Discovery

Merck Millipore has the first commercially-available panel of active ubiquitination enzyme cascades and services for small molecule screening. Based on the assays used in our UbiquitinProfiler™ service lab, this manual provides researchers with screening conditions for the E1, E2, and E3 enzymes and substrates available in our catalog (www.millipore.com/ubiquitin). These assay protocols have been optimized for robustness, sensitivity, and specificity by our in-house enzymologists and assay developers to allow for rapid adoption in the hands of users.

Contact us at UK.project.manager@merckgroup.com for further information about these protocols or about our enzyme services, such as our FlexLab℠ services for extensive mechanism of action studies, custom assay development or protein production.

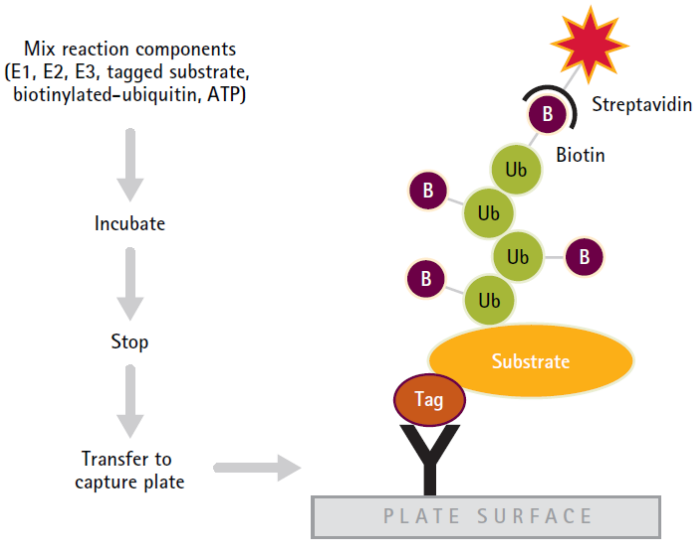
Merck Millipore offers industry leading profiling services for:

- Ubiquitin E1, E2, and E3 enzymes
- Kinases
- Phosphatases
- GPCRs
- Ion Channels

Ubiquitin Enzyme Assays
Overview

Each activity assay comprises a member of each of the three key families involved in a physiological ubiquitination reaction - an E1 activating enzyme, an E2 conjugating enzyme and an E3 ligase - plus a substrate. Where indicated, the substrate is the E3 ligase itself and thus in these cases, the assay measures auto-ubiquitination of the E3 ligase.

Reaction Overview:



- 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 which is coated with anti-c-Myc antibody to capture the substrate, for an enzyme-linked immunosorbant assay (ELISA) or electrochemiluminescence assay (ECL).
- Bound ubiquitin is detected by addition of labelled streptavidin.
- The red symbol in the schematic shown above represents the appropriate label: ruthenium bipyridine (for ECL) or horseradish peroxidase (for ELISA).

Ubiquitin Enzyme Assays
Cascade Reactions

This document contains reaction protocols for the following cascades:

	E1	E2	E3	Substrate
Cascade 1	UBE1	UbcH3	$SCF^{Skp2/Cks1}$	p27
Cascade 2	UBE1	UbcH3	SCF^{Fbw7}	Cyclin E1
Cascade 3	UBE1	UbcH5a	SCF^{Fbw7}	Cyclin E1
Cascade 4	UBE1	UbcH3	$SCF^{\beta TrCP1}$	IκBα
Cascade 5	UBE1	UbcH4	$SCF^{\beta TrCP1}$	IκBα
Cascade 6	UBE1	UbcH3	$SCF^{\beta TrCP1}$	β-catenin
Cascade 7	UBE1	UbcH4	$SCF^{\beta TrCP1}$	β-catenin
Cascade 8	UBE1	UbcH4	MDM2/CK1δ	p53
Cascade 9	UBE1	UbcH5c	MDM2/CK1δ	p53
Cascade 10	UBE1	UbcH4	MDM2	auto-ubiquitination
Cascade 11	UBE1	UbcH5c	MDM2	auto-ubiquitination
Cascade 12	UBE1	UbcH4	c-Cbl	Src
Cascade 13	UBE1	UbcH4	c-Cbl	Kit
Cascade 14	UBE1	UbcH4	VHL	HIF-1α
Cascade 15	UBE1	UbcH5a	VHL	HIF-1α
Cascade 16	UBE1	UbcH5c	VHL	HIF-1α
Cascade 17	UBE1	UbcH7	Parkin	auto-ubiquitination
Cascade 18	UBE1	UbcH7	Parkin	p38/JTV-1
Cascade 19	UBE1	UbcH13/Uev1A	CHIP	p53
Cascade 20	UBE1	UbcH13/Uev1A	CHIP	Hsp70
Cascade 21	UBE1	UbcH13/Uev1A	CHIP	auto-ubiquitination
Cascade 22	UBE1	UbcH5c	MuRF1	Cardiac Troponin
Cascade 23	UBE1	UbcH6	Parkin	auto-ubiquitination
Cascade 24	UBE1	UbcH6	Parkin	p38/JTV-1
Cascade 25	UBE1	UbcH4	XIAP	Smac/DIABLO
Cascade 26	UBE1	UbcH5a	TRAF6	IKKγ
Cascade 27	UBE1	UbcH5c	Smurf1	Smad7

Ubiquitin Enzyme Assays
Reaction Protocols

Remarks:

- Component concentrations in the following pages are provided as a guide. 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 and plate reader.
- Further optimization may be possible by conducting a reaction time course. An end-point should be chosen such that the ubiquitination reaction does not reach a saturating level.
- A reaction volume of 25 µl/well is suggested for 96-well plates. Recommended Stop solution volumes are 5 µl/well for all cascades except Cascades 17, 18, 23, 24 and 25 (25 µl/well).

Suggested Materials for ELISA and ECL

- Capture antibody
  - c-Myc antibody: Merck Millipore cat # 05-724, used at 1/250 in PBS. For coating plates, the antibody solution is added to a Costar plate cat # 3693 (25 µl/well) and the sealed plate is incubated overnight at 4ºC.
- Blocking buffer
  - PBS + 10% BSA (w/v)
- Detection reagents
  - Streptavidin-horseradish peroxidase (for ELISA): HRP-conjugated streptavidin, GE Healthcare cat # RPN1231, used at 1/5000 in PBS-Tween 20 + 10% BSA (w/v)
  - ELISA detection reagent: Thermo Scientific SuperSignal ELISA Pico ECL Substrate cat # 37070, used as per manufacturer's instructions.
  - Streptavidin-ruthenium (for ECL): Sulfo-tag conjugated streptavidin, Mesoscale Discovery cat # R32AD1, used at 1/500 in PBS

Cascade 1
(UBE1-UbcH3-SCF$^{Skp2/Cks1}$-p27)

SCF$^{Skp2/Cks1}$ complex is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH3, 20 nM p27 complex, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF$^{Skp2/Cks1}$ complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 2
(UBE1-UbcH3-SCF^{Fbw7}-Cyclin E)

SCF^{Fbw7} complex is incubated with 25 mM MOPS pH7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH3, 100 nM Cyclin E1 complex, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF^{Fbw7} complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 3
(UBE1-UbcH5a-SCF^{Fbw7}-Cyclin E)

SCF^{Fbw7} complex is incubated with 25 mM MOPS pH7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5a, 100 nM Cyclin E1 complex, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF^{Fbw7} complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 4
(UBE1-UbcH3-SCF$^{\beta TrCP1}$-IκBα)

SCF$^{\beta TrCP1}$ complex is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH3, 25 nM IκBα and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF$^{\beta TrCP1}$ complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 5
(UBE1-UbcH4- SCF$^{\beta TrCP1}$-IκBα)

SCF$^{\beta TrCP1}$ complex is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH4, 25 nM IκBα and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF$^{\beta TrCP1}$ complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 6
(UBE1-UbcH3-SCF$^{\beta TrCP1}$-β-catenin)

SCF$^{\beta TrCP1}$ complex is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH3, 100 nM β-catenin and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF$^{\beta TrCP1}$ complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 7
(UBE1-UbcH4-SCF$^{\beta TrCP1}$-β-catenin)

SCF$^{\beta TrCP1}$ complex is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH4, 100 nM β-catenin and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. SCF$^{\beta TrCP1}$ complex activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 8
(UBE1-UbcH4-MDM2/CK1δ-p53)

MDM2/CK1δ is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH4, 125 nM p53, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. MDM2/CK1δ activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 9
(UBE1-UbcH5c-MDM2/CK1δ-p53)

MDM2/CK1δ is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5c, 125 nM p53, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. MDM2/CK1δ activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 10
(UBE1-UbcH4-MDM2-MDM2(Auto))

MDM2 is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH4 and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. MDM2 auto-ubiquitination activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 11
(UBE1-UbcH5c-MDM2-MDM2(Auto))

MDM2 is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5c and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. MDM2 auto-ubiquitination activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 12
(UBE1-UbcH4-c-Cbl-Src)

c-Cbl is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 100 nM UbcH4, 200 nM Src and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. c-Cbl activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 13
(UBE1-UbcH4-c-Cbl-Kit)

c-Cbl is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 100 nM UbcH4, 200 nM Kit and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. c-Cbl activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 14
(UBE1-UbcH4-VHL-HIF-1α)

VHL is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 1000 nM UbcH4, 50 nM HIF-1α and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes

at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. VHL activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 15 (UBE1-UbcH5a-VHL-HIF-1α)

VHL is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5a, 50 nM HIF-1α and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. VHL activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 16 (UBE1-UbcH5c-VHL-HIF-1α)

VHL is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5c, 50 nM HIF-1α and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. VHL activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 17 (UBE1-UbcH7-Parkin-Parkin(Auto))

Parkin is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH7, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. Parkin activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 18 (UBE1-UbcH7-Parkin-p38/JTV-1)

Parkin is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 1000 nM UbcH7, 50 nM p38/JTV-1, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. Parkin activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 19
(UBE1-UbcH13/Uev1A-CHIP-p53)

CHIP is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 265 nM UbcH13/Uev1a, 100 nM p53 and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. CHIP activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 20
(UBE1-UbcH13/Uev1A-CHIP-Hsp70)

CHIP is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 529 nM UbcH13/Uev1a, 200 nM Hsp70, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. CHIP activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 21
(UBE1-UbcH13/Uev1A-CHIP-CHIP(Auto))

CHIP is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 529 nM UbcH13/Uev1a, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. CHIP activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 22
(UBE1-UbcH5c-MuRF1-Cardiac Troponin)

MuRF1 is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH5c, 100 nM cardiac troponin I,and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. MuRF1 activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 23
(UBE1-UbcH6-Parkin-Parkin(Auto))

Parkin is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 500 nM UbcH6, and

2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. Parkin activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 24 (UBE1-UbcH6-Parkin-p38/JTV-1)

Parkin is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 1000 nM UbcH6, 50 nM p38/JTV-1, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. Parkin activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 25 (UBE1-UbcH4-XIAP-Smac/DIABLO)

XIAP is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 50 nM UbcH4, 12.5 nM Smac/DIABLO, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. XIAP activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 26 (UBE1-UbcH5a-TRAF6-IKKγ)

TRAF6 is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 1000 nM UbcH5a, 25 nM IKKγ, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 150 mM NaCl, and 0.05% Tween 20. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. TRAF6 activity is measured by detection of bound ubiquitin via horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

Cascade 27 (UBE1-UbcH5c-Smurf1-Smad7)

Smurf1 is incubated with 25 mM MOPS pH 7.5, 0.01% Tween 20, 5 mM $MgCl_2$, 10 µM ATP, 10 nM UBE1, 1000 nM UbcH5c, 50 nM Smad7, and 2 µM biotinylated-ubiquitin. The reaction is initiated with the addition of biotinylated-ubiquitin. After 30 minutes at room temperature the reaction is terminated by the addition of 25 mM MOPS pH 7.5 containing 125 mM EDTA, 400 mM NaCl and 1% Triton-X-100. Reaction products are separated by capture onto a microplate coated with anti-c-Myc antibody and washing with PBS containing 0.05% Tween 20. Smurf1 activity is measured by detection of bound ubiquitin via

horseradish peroxidase-labelled streptavidin (ELISA) or ruthenium bipyridine-labeled streptavidin (ECL). See suggested ELISA and ECL materials above.

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