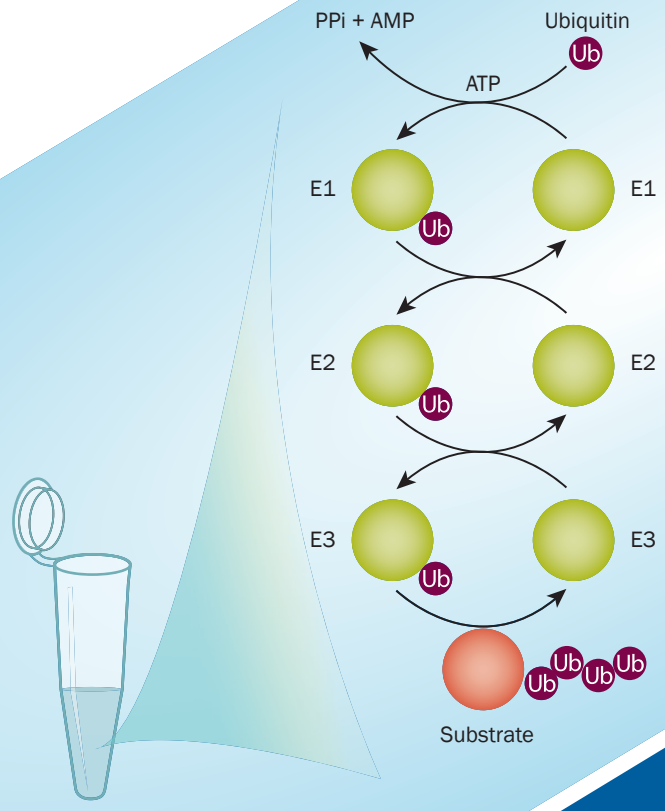
R&D SYSTEMS
a bio-techne brand
powered by
BostonBiochem

In Vitro Ubiquitin Conjugation Protocols



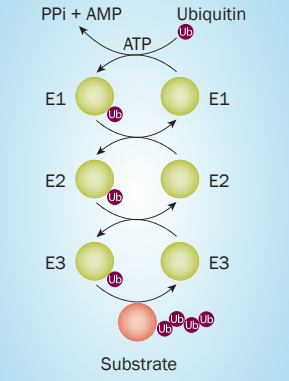
Protocols:

- *In Vitro* Ubiquitin Conjugation Reaction
- Distinguish Between Poly-ubiquitination and Multi-mono-ubiquitination
- Determine Ubiquitin Chain Linkage

In Vitro Ubiquitin Conjugation Reaction

In vitro Ubiquitin conjugation reactions are relatively quick and simple to carry out and they can answer several questions:

- Can your protein of interest be ubiquitinated?
- If so, is it mono-ubiquitinated, poly-ubiquitinated, or multi-mono-ubiquitinated?
- If it is poly-ubiquitinated, what is the chain linkage?
- Which E2 enzyme and E3 ligase are required?



Ubiquitination Pathway

The protocol below describes in detail how to carry out an *in vitro* ubiquitination reaction and analyze the results to determine if your protein of interest is ubiquitinated.

Materials and Reagents

Material or Reagent	Stock Concentration
E1 Enzyme	5 µM
E2 Enzyme[i]	25 µM
E3 Ligase[ii]	10 µM
10X E3 Ligase Reaction Buffer	10X
Ubiquitin	1.17 mM (10 mg/mL)
MgATP Solution	100 mM
SDS-PAGE sample buffer *if not using reaction products for downstream applications*	2X
EDTA or DTT *if using reaction products for downstream applications*	500 mM (EDTA); 1 M (DTT)
Microcentrifuge tubes	
Water Bath (37 °C)	
Western Blot Equipment	

[i]Each E2 enzyme functions with only a subset of E3 ligases and some E3's are more promiscuous than others. If you're not sure which E2 enzyme is involved in the ubiquitination of your substrate we recommend using our E2Select Ubiquitin Conjugation Kit (Catalog # K-982). This kit contains a panel of 26 E2 enzymes and allows for the rapid identification of the E2 enzyme(s) that functionally interact with an E3 Ubiquitin ligase *in vitro*.

[ii]The E3 ligase will likely need to be supplied by the user, but we do offer a small selection. We also offer E3 Ubiquitin Ligase Kits that include everything required to carry out the *in vitro* ubiquitination reaction.

Procedure for a 25 µL reaction (scale as needed)

1. In a microcentrifuge tube, combine the indicated volume of each component listed in Table 1 in the order shown. For a negative control reaction replace the MgATP Solution with dH_2O.
2. Incubate in a 37 °C water bath for 30–60 minutes.
3. Terminate the reaction. See Table 2 for the appropriate method of termination.
4. Analyze Ubiquitin conjugation reaction products by SDS-PAGE and Western blot.
   a. Determine if ubiquitination products have been generated: separate the reaction products by SDS-PAGE and stain the resulting gel with Coomassie blue. See Figure 1 for a graphical data example and how to interpret the data.*
   b. Verify which bands represent ubiquitinated substrate: follow up with a Western blot using anti-Ubiquitin and/or anti-substrate antibodies. All bands on the anti-substrate blot should correlate to the anti-Ubiquitin blot, but the opposite is not true since other proteins in the reaction can often be ubiquitinated. See Figures 2 and 3 for graphical data examples and how to interpret the data.*
   c. Identify E3 ligase autoubiquitination: follow up with a Western blot using anti-E3 ligase antibody. An anti-Ubiquitin Western blot alone cannot distinguish between ubiquitinated substrate and autoubiquitinated E3 ligase. See Figure 4 for a graphical data example and how to interpret the data.*

Table 1

Reagent	Volume	Working Concentration
dH_2O	X µL (to 25 µL; dependent on volume of substrate and E3 ligase)	N/A
10X E3 Ligase Reaction Buffer	2.5 µL	1X
Ubiquitin	1 µL	Approximately 100 µM
MgATP Solution	2.5 µL	10 mM
Substrate	X µL[iii]	5–10 µM
E1 Enzyme	0.5 µL	100 nM
E2 Enzyme	1 µL	1 µM
E3 Ligase	X µL[iv]	1 µM

[iii]The volume needed will depend on the stock concentration of your substrate.
[iv]The volume needed will depend on the stock concentration of your E3 ligase.

Table 2

Are you using reaction products for downstream enzymatic applications?	Termination method	Volume (final concentration)
No	SDS-PAGE sample buffer	25 µL (1X)
Yes	EDTA or DTT[v]	0.5 µL EDTA (20 mM) or 1 µL DTT (100 mM)

[v]EDTA and DTT are equally effective at terminating the reaction; determining which to use will depend on the intended downstream enzymatic application of the reaction products.

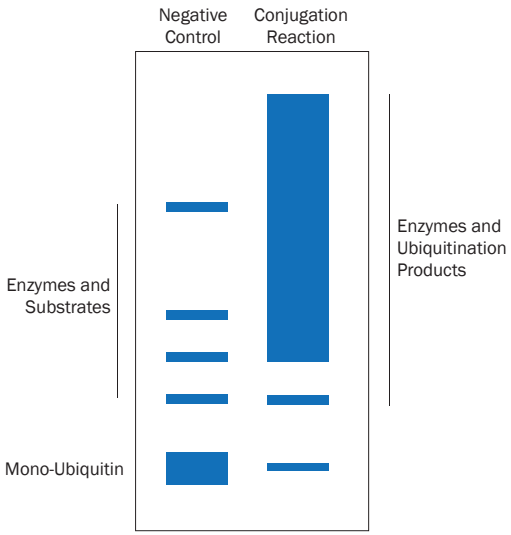


Figure 1. Graphical Representation of Ubiquitin Conjugation Reaction Products Separated by SDS-PAGE and Visualized with Coomassie Stain. Coomassie stain will bind all of the protein species in the reaction. Ubiquitination products are not able to be generated in a negative control reaction lacking MgATP solution (left lane) so all that should appear is a band for each protein used in the reaction. The conjugation reaction (right lane) yields a large number of ubiquitination products that generally appear as a smear of staining. Note also that the band representing mono-Ubiquitin at the bottom of the gel may be significantly reduced or absent in the Conjugation Reaction lane if the ubiquitination reaction is very efficient. If the conjugation reaction does not generate a smear or ladder of additional bands then either something was missing from the reaction or the substrate of interest is not ubiquitinated by the specific set of enzymes in the reaction.

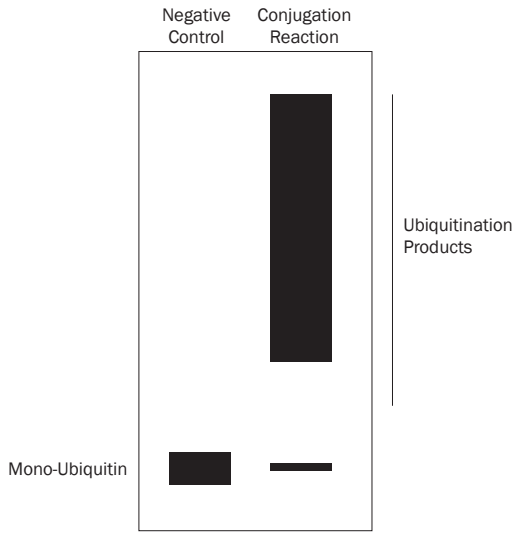


Figure 2. Graphical Representation of Ubiquitin Conjugation Reaction Products Separated by SDS-PAGE and Analyzed by Western Blot with anti-Ubiquitin Antibody. Western blot analysis with an anti-Ubiquitin antibody reveals only Ubiquitin and substrates modified with Ubiquitin. Ubiquitination products are not able to be generated in a negative control reaction lacking MgATP solution (left lane) so all that should appear is a single band representing mono-Ubiquitin. The conjugation reaction (right lane) yields a large number of ubiquitination products that generally appear as a smear or ladder of bands. Note also that the band representing mono-Ubiquitin at the bottom of the gel may be significantly reduced or absent in the conjugation reaction lane if the ubiquitination reaction is very efficient. Unlike the Coomassie stained gel, only ubiquitination products can be seen in the right lane, not any unmodified proteins. This analysis verifies that the protein species in the smear are ubiquitinated.

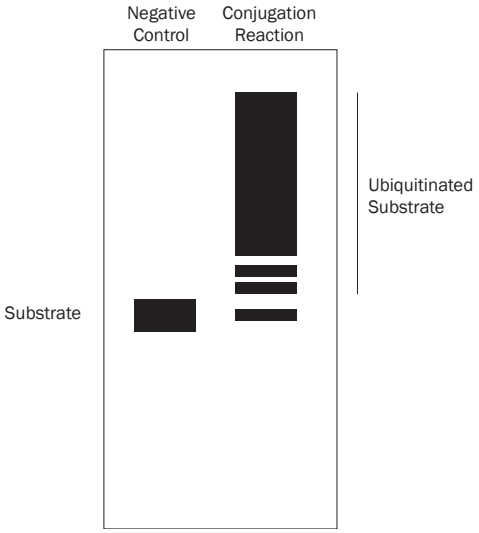


Figure 3. Graphical Representation of Ubiquitin Conjugation Reaction Products Separated by SDS-PAGE and Analyzed by Western Blot with anti-Substrate Antibody. Western blot analysis with an anti-substrate antibody reveals only the substrate and ubiquitinated substrate. Ubiquitination products are not able to be generated in a negative control reaction lacking MgATP solution (left lane) so all that should appear is a single band representing the substrate. The conjugation reaction (right lane) yields a large number of ubiquitination products that generally appear as a smear or ladder. Note also that the band representing the substrate might either mostly or completely disappear depending on how much of it becomes ubiquitinated. This analysis verifies that the substrate of interest is ubiquitinated.

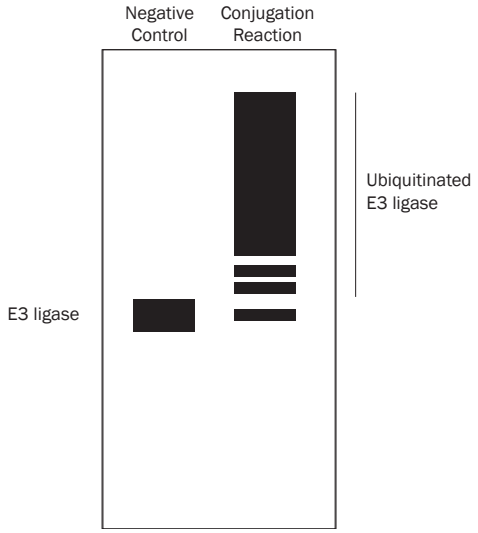


Figure 4. Graphical Representation of Ubiquitin Conjugation Reaction Products Separated by SDS-PAGE and Analyzed by Western Blot with anti-E3 Ligase Antibody. Western blot analysis with an anti-E3 ligase antibody reveals only the E3 ligaes and autoubiquitinated E3 ligase. Ubiquitination products are not able to be generated in a negative control reaction lacking MgATP solution (left lane) so all that should appear is a single band representing the E3 ligase. The conjugation reaction (right lane) yields a large number of ubiquitination products that generally appear as a smear of staining. Note also that the band representing the E3 ligase might either mostly or completely disappear depending on how much of it becomes autoubiquitinated. This analysis verifies that autoubiquitination of the E3 ligase has occurred.

*These figures are designed to represent ideal data to serve as a teaching tool; actual data may not be as easy to analyze.

Distinguish Between Poly-ubiquitination and Multi-mono-ubiquitination

Ubiquitin can be attached to a protein substrate via two distinct mechanisms (Figure 1). Poly-ubiquitination occurs when Ubiquitin molecules are attached end-to-end to a single lysine residue on a substrate protein to form a poly-Ubiquitin chain (Figure 1A). Alternatively, multi-mono-ubiquitination is the attachment of a single Ubiquitin molecule to multiple lysine residues on a substrate protein (Figure 1B). It is important to distinguish between poly-ubiquitination and multi-mono-ubiquitination because these different types of ubiquitination lead to different functions of the substrate protein. To complicate matters, a lightly poly-ubiquitinated protein and a multi-mono-ubiquitinated protein may look very similar by SDS-PAGE and Western blot. Fortunately, it is relatively simple to differentiate between poly-ubiquitination and multi-mono-ubiquitination by performing *in vitro* Ubiquitin conjugation reactions. The protocol below describes in detail how to determine if your protein of interest is poly-ubiquitinated or multi-mono-ubiquitinated.

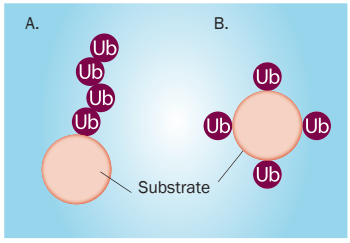


Figure 1. Poly-ubiquitination vs Multi-mono-ubiquitination

Materials and Reagents

Material or Reagent	Stock Concentration
E1 Enzyme	5 µM
E2 Enzyme[i]	25 µM
E3 Ligase[ii]	10 µM
10X E3 Ligase Reaction Buffer	10X
Ubiquitin	1.17 mM (10 mg/mL)
Ubiquitin No K	1.17 mM (10 mg/mL)
MgATP Solution	100 mM
SDS-PAGE sample buffer *if not using reaction products for downstream applications*	2X
EDTA or DTT *if using reaction products for downstream applications*	500 mM (EDTA); 1 M (DTT)
Microcentrifuge tubes	
Water Bath (37 °C)	
Western Blot Equipment	

[i]Each E2 enzyme functions with only a subset of E3 ligases and some E3's are more promiscuous than others. If you're not sure which E2 enzyme is involved in the ubiquitination of your substrate we recommend using our E2Select Ubiquitin Conjugation Kit (Catalog # K-982). This kit contains a panel of 26 E2 enzymes and allows for the rapid identification of the E2 enzyme(s) that functionally interact with an E3 Ubiquitin ligase *in vitro*.

[ii]The E3 ligase will likely need to be supplied by the user, but we do offer a small selection. We also offer E3 Ubiquitin Ligase Kits that include everything required to carry out the *in vitro* Ubiquitination reaction.

Procedure for distinguishing between poly-ubiquitination and multi-mono-ubiquitination

Two *in vitro* Ubiquitin conjugation reactions will need to be performed: 1) one that contains wild type Ubiquitin and 2) one that contains Ubiquitin No K, a mutant in which all 7 lysines have been mutated to arginines. Wild type Ubiquitin can be conjugated to substrate proteins and is capable of forming chains (Figure 2; left). Ubiquitin No K can also be conjugated to substrate proteins, but it is unable to form chains due to its lack of lysine residues (Figure 2; right). Therefore, if your substrate is poly-ubiquitinated, high molecular weight bands will be generated in reaction 1, but not reaction 2 (Figure 3A). Conversely, if your substrate is multi-mono-ubiquitinated, high molecular weight bands will be generated in both reaction 1 and reaction 2 (Figure 3B). Note that reaction 1 will look similar for both poly-ubiquitinated and multi-mono-ubiquitinated substrates.

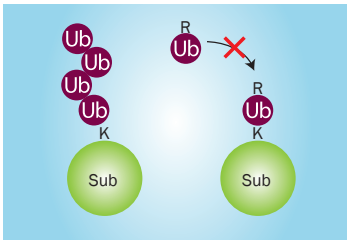


Figure 2. Wild Type Ubiquitin vs Ubiquitin No K

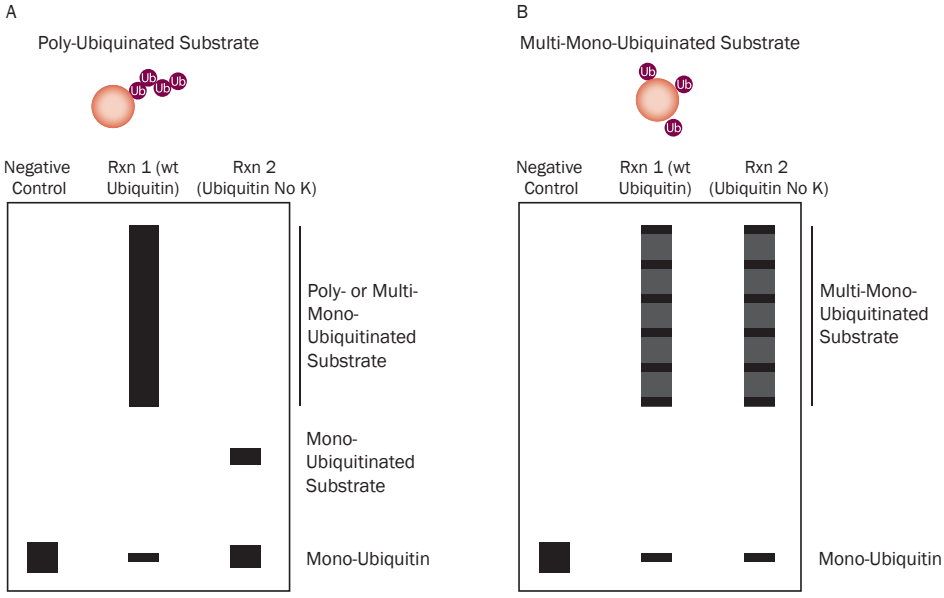


Figure 3. Differentiation Between Poly-Ubiquitination and Multi-Mono-Ubiquitination Using Ubiquitin No K

Procedure for a 25 µL reaction (scale as needed)

1. For reaction 1, combine the indicated volume of each component listed in Table 1, in the order shown, in a microcentrifuge tube. For a negative control reaction replace the MgATP Solution with dH_2O.
2. For reaction 2, combine the indicated volume of each component listed in Table 2, in the order shown, in a microcentrifuge tube. The only difference between reaction 1 and reaction 2 is the substitution of Ubiquitin No K for wild type Ubiquitin.
3. Incubate reaction 1 and reaction 2 in a 37 °C water bath for 30–60 minutes.
4. Terminate the reactions. See Table 3 for the appropriate method of termination.
5. Analyze Ubiquitin conjugation reactions.
   a. Separate the reaction products by SDS-PAGE and transfer them to a PVDF or nitrocellulose membrane.
   b. Perform a Western blot using an anti-Ubiquitin antibody.
   c. Compare your Western blot to figure 3 to determine if your substrate is poly-ubiquitinated or multi-mono-ubiquitinated.*

*It is possible for a substrate to be both poly-ubiquitinated and multi-mono-ubiquitinated. If this is the case the Ubiquitin No K will still prevent poly-ubiquitination and the highest molecular weight protein species should disappear from reaction 2.

Contact us for technical assistance!

Table 1

Reagent	Volume	Working Concentration
dH_2O	X µL (to 25 mL; dependent on volume of substrate and E3 ligase)	N/A
10X E3 Ligase Reaction Buffer	2.5 µL	1X
Ubiquitin	1 µL	Approximately 100 µM
MgATP Solution	2.5 µL	10 mM
Substrate	X µL[iii]	5-10 µM
E1 Enzyme	0.5 µL	100 nM
E2 Enzyme	1 µL	1 µM
E3 Ligase	X µL[iv]	1 µM

[iii]The volume needed will depend on the stock concentration of your substrate.
[iv]The volume needed will depend on the stock concentration of your E3 ligase.

Table 2

Reagent	Volume	Working Concentration
dH_2O	X µL (to 25 µL; dependent on volume of substrate and E3 ligase)	N/A
10X E3 Ligase Reaction Buffer	2.5 µL	1X
Ubiquitin No K	1 µL	Approximately 100 µM
MgATP Solution	2.5 µL	10 mM
Substrate	X µL[iii]	5–10 µM
E1 Enzyme	0.5 µL	100 nM
E2 Enzyme	1 µL	1 µM
E3 Ligase	X µL[iv]	1 µM

[iii]The volume needed will depend on the stock concentration of your substrate.
[iv]The volume needed will depend on the stock concentration of your E3 ligase.

Table 3

Are you using reaction products for downstream enzymatic applications?	Termination method	Volume (final concentration)
No	SDS-PAGE sample buffer	25 µL (1X)
Yes	EDTA or DTT[v]	0.5 µL EDTA (20 mM) or 1 µL DTT (100 mM)

[v]EDTA and DTT are equally effective at terminating the reaction; determining which to use will depend on the intended downstream enzymatic application of the reaction products.

Determine Ubiquitin Chain Linkage

Eight residues within Ubiquitin can be utilized to form poly-Ubiquitin chains [K6, K11, K27, K29, K33, K48, K63, and Met1 (aka linear)] and linkage type directs the modified proteins to different cellular fates. Poly-Ubiquitin chains of all linkages listed above have been detected *in vivo* and have been shown to differentially affect many cellular processes, signaling pathways, and disease states. Ubiquitin chain linkage can be determined with *in vitro* Ubiquitin conjugation reactions by utilizing Ubiquitin lysine mutants. The protocol below describes in detail how to determine the linkage of Ubiquitin chains on your substrate of interest.

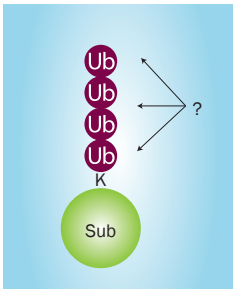


Figure 1. Eight Ubiquitin Chain Linkages are Possible

Procedure for determining Ubiquitin chain linkage

Two sets of nine *in vitro* Ubiquitin conjugation reactions will need to be performed: one set utilizing seven Ubiquitin lysine to arginine (K to R) Mutants followed by another set utilizing seven Ubiquitin K Only Mutants. The seven Ubiquitin K to R Mutants are used to identify the lysine or lysines being utilized for Ubiquitin chain linkage. The conjugation reaction containing the Ubiquitin K to R Mutant lacking the lysine required for chain linkage will not be able to form chains and only mono-ubiquitination will be observed by Western blot (Figure 2). For example, if Ubiquitin chains are linked via K63 then all of the conjugation reactions except the reaction containing the Ubiquitin K63R Mutant should yield Ubiquitin chains (Figure 3). If all of the conjugation reactions yield ubiquitin chains then the chains are either linked via Met1 (linear) or they contain a mixture of linkages.*

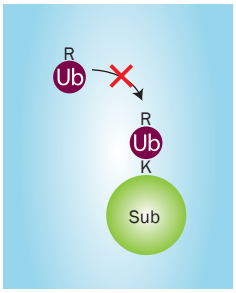


Figure 2. Lysine to Arginine Mutations Prevent Ubiquitin Chain Formation.

The seven Ubiquitin K only Mutants can then be used to verify Ubiquitin chain linkage. These Ubiquitin Mutants contain only one lysine, with the remaining six mutated to arginine. Therefore, Ubiquitin chains formed with a Ubiquitin K Only Mutant must be utilizing the single lysine available for linkage. Again using Ubiquitin chains linked via K63 as an example, only the conjugation reactions containing wild type Ubiquitin and the Ubiquitin K63 Only Mutant will yield Ubiquitin chains (Figure 4).

*It is possible for a single Ubiquitin chain to contain multiple linkages. In this case analysis will be more complex.

Contact us for technical assistance!

Materials and Reagents

Material or Reagent	Stock Concentration
E1 Enzyme	5 µM
E2 Enzyme[i]	25 µM
E3 Ligase[ii]	10 µM
10X E3 Ligase Reaction Buffer	10X
Ubiquitin	1.17 mM (10 mg/mL)
Ubiquitin Mutants – Single Lysine	1.17 mM (10 mg/mL)
Ubiquitin Mutants – Lysine to Arginine	1.17 mM (10 mg/mL)
MgATP Solution	100 mM
SDS-PAGE sample buffer *if not using reaction products for downstream applications*	2X
EDTA or DTT *if using reaction products for downstream applications*	500 mM (EDTA); 1 M (DTT)
Microcentrifuge tubes	
Water Bath (37 °C)	
Western Blot Equipment	

[i]Each E2 enzyme functions with only a subset of E3 ligases and some E3's are more promiscuous than others. If you're not sure which E2 enzyme is involved in the ubiquitination of your substrate we recommend using our E2Select Ubiquitin Conjugation Kit (Catalog # K-982). This kit contains a panel of 26 E2 enzymes and allows for the rapid identification of the E2 enzyme(s) that functionally interact with an E3 Ubiquitin ligase *in vitro*.

[ii]The E3 ligase will likely need to be supplied by the user, but we do offer a small selection. We also offer E3 Ubiquitin Ligase Kits that include everything required to carry out the *in vitro* ubiquitination reaction.

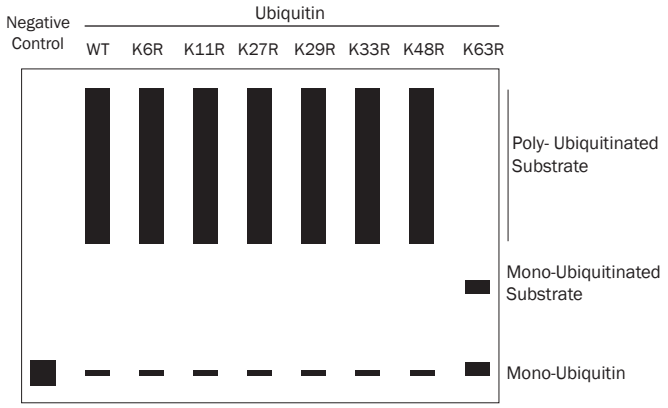


Figure 3. Determining Ubiquitin Chain Linkage Using Ubiquitin K to R Mutants.

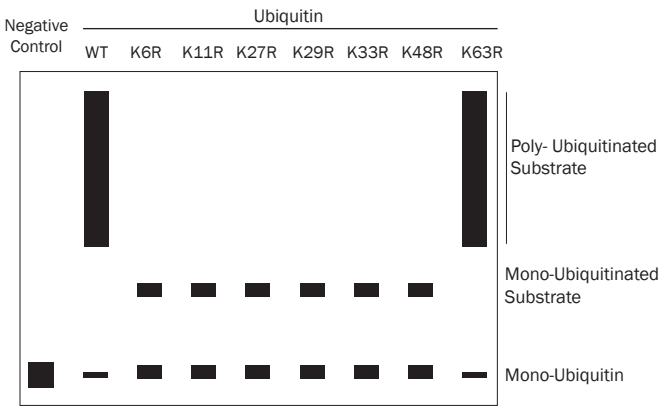


Figure 4. Verifying Ubiquitin Chain Linkage Using Ubiquitin K Only Mutants.

Procedure for 25 µL reactions (scale as needed)

1. Determine Ubiquitin chain linkage. Set up nine Ubiquitin conjugation reactions: one containing wild type Ubiquitin, seven containing Ubiquitin K to R Mutants, and one negative control reaction. Combine the indicated volume of each component listed in Table 1, in the order shown, in a microcentrifuge tube. Reactions 1–8 will be identical except for the Ubiquitin included:
   a. Reaction 1 – wild type Ubiquitin
   b. Reaction 2 – Ubiquitin K6R Mutant
   c. Reaction 3 – Ubiquitin K11R Mutant
   d. Reaction 4 – Ubiquitin K27R Mutant
   e. Reaction 5 – Ubiquitin K29R Mutant
   f. Reaction 6 – Ubiquitin K33R Mutant
   g. Reaction 7 – Ubiquitin K48R Mutant
   h. Reaction 8 – Ubiquitin K63R Mutant
   i. Negative control – replace the MgATP Solution with dH2O.
2. Incubate reactions 1–8 and the negative control reaction in a 37 °C water bath for 30–60 minutes.
3. Terminate the reactions. See Table 3 for the appropriate method of termination.
4. Analyze Ubiquitin conjugation reactions by Western blot.
   a. Separate the reaction products by SDS-PAGE and transfer them to a PVDF or nitrocellulose membrane.
   b. Perform a Western blot using an anti-Ubiquitin antibody.
   c. Use figure 3 as a guide to determine which lysine or lysines are required for Ubiquitin chain linkage.*
5. Verify Ubiquitin chain linkage. Set up nine Ubiquitin conjugation reactions: one containing wild type Ubiquitin, seven containing Ubiquitin K Only Mutants, and one negative control reaction. Combine the indicated volume of each component listed in the Table 2, in the order shown, in a microcentrifuge tube. Reactions 1–8 will be identical except for the Ubiquitin included:
   a. Reaction 1 – wild type Ubiquitin
   b. Reaction 2 – Ubiquitin K6 Only Mutant
   c. Reaction 3 – Ubiquitin K11 Only Mutant
   d. Reaction 4 – Ubiquitin K27 Only Mutant
   e. Reaction 5 – Ubiquitin K29 Only Mutant
   f. Reaction 6 – Ubiquitin K33 Only Mutant
   g. Reaction 7 – Ubiquitin K48 Only Mutant
   h. Reaction 8 – Ubiquitin K63 Only Mutant
   i. Negative control – replace the MgATP Solution with dH_2O.
6. Incubate reactions 1–8 and the negative control reaction in a 37 °C water bath for 30–60 minutes.
7. Terminate the reactions. See Table 3 for the appropriate method of termination.
8. Analyze Ubiquitin conjugation reactions by Western blot.
   a. Separate the reaction products by SDS-PAGE and transfer them to a PVDF or nitrocellulose membrane.
   b. Perform a Western blot using an anti-Ubiquitin antibody.
   c. Use figure 4 as a guide to verify which lysine ar lysines are required for Ubiquitin chain linkage.*

Table 1

Reagent	Volume	Working Concentration
dH_2O	X µL (to 25 µL; dependent on volume of substrate and E3 ligase)	N/A
10X E3 Ligase Reaction Buffer	2.5 µL	1X
Ubiquitin or Ubiquitin K to R Mutant	1 µL	Approximately 100 µM
MgATP Solution	2.5 µL	10 mM
Substrate	X µL[iii]	5–10 µM
E1 Enzyme	0.5 µL	100 nM
E2 Enzyme	1 µL	1 µM
E3 Ligase	X µL[iv]	1 µM

[iii]The volume needed will depend on the stock concentration of your substrate.
[iv]The volume needed will depend on the stock concentration of your E3 ligase.

Table 2

Reagent	Volume	Working Concentration
dH_2O	X µL (to 25 µL; dependent on volume of substrate and E3 ligase)	N/A
10X E3 Ligase Reaction Buffer	2.5 µL	1X
Ubiquitin or Ubiquitin K Only Mutants	1 µL	Approximately 100 µM
MgATP Solution	2.5 µL	10 mM
Substrate	X µL[iii]	5–10 µM
E1 Enzyme	0.5 µL	100 nM
E2 Enzyme	1 µL	1 µM
E3 Ligase	X µL[iv]	1 µM

[iii]The volume needed will depend on the stock concentration of your substrate.
[iv]The volume needed will depend on the stock concentration of your E3 ligase.

Table 3

Are you using reaction products for downstream enzymatic applications?	Termination method	Volume (final concentration)
No	SDS-PAGE sample buffer	25 µL (1X)
Yes	EDTA or DTT[v]	0.5 µL EDTA (20 mM) or 1 µL DTT (100 mM)

[v]EDTA and DTT are equally effective at terminating the reaction; determining which to use will depend on the intended downstream enzymatic application of the reaction products.

Having trouble analyzing the data?

Contact us for technical assistance!

*This approach to determining chain linkage using Ubiquitin mutants is powerful, but complimentary approaches may be required in certain cases.

R&D SYSTEMS NOVUS BIOLOGICALS TOCRIS proteinsimple

bio-techne®
Global info@bio-techne.com bio-techne.com/find-us/distributors TEL +1 612 379 2956
North America TEL 800 343 7475 **Europe | Middle East | Africa** TEL +44 (0)1235 529449
China info.cn@bio-techne.com TEL +86 (21) 52380373
bio-techne.com