UbiCREST Deubiquitinase Enzyme Set

Catalog Number K-400

This kit contains reagents sufficient for 5 UbiCREST analysis experiments.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Covalent attachment of ubiquitin to target proteins is one of the most versatile and intriguing post-translational modifications observed in eukaryotic organisms. Because polyubiquitin chains may be generated by connecting adjacent ubiquitin molecules in one of eight different orientations, a large number of varied architectures may be generated. Polyubiquitination of proteins directs them to different cellular fates such as translocation to different areas within the cell, assembly into multi-subunit complexes, or degradation via the proteasome, depending on the type of linkage used in the polyubiquitin chain. Recent studies have begun to define the roles of these different polyubiquitin signals in physiology and disease, and it is obvious that identifying and understanding the uses of the various linkages will be important in understanding and treating human disease. Polyubiquitin chains are typically formed via the E1, E2, E3 enzyme conjugation cascade where the C-terminal glycine of one ubiquitin is linked to the ε-amino group of a lysine on another ubiquitin; this linkage is referred to as an isopeptide bond. Polyubiquitin chains of all possible linkages including K6-, K11-, K27-, K29-, K33-, K48-, K63-, and Met1-linked (or "linear") have been detected *in vivo*.

UbiCREST (Ubiquitin Chain Restriction Analysis) is designed to analyze the ubiquitin linkages present in free polyubiquitin chains or polyubiquitin chains conjugated to a substrate protein. Developed in the Komander lab, the UbiCREST approach utilizes the linkage specificity of the ovarian tumor domain (OTU) class of deubiquitinases (DUBs) to characterize the linkages present in a sample containing polyubiquitin. This kit provides DUBs that are useful for *in vitro* analysis of user supplied polyubiquitinated protein samples. The samples may be generated by *in vitro* ubiquitination reactions, or obtained from cell lysates or other complex mixtures following immunoprecipitation or other means of purification.

TECHNICAL HINTS

- UbiCREST Kit data output consists primarily of SDS-PAGE gel-based detection of monoubiquitin and short polyubiquitin chains that are generated by DUB treatment of a polyubiquitinated substrate(s). Therefore, when performing UbiCREST analysis the protein being analyzed should first be purified to remove contaminating DUB activity and free ubiquitin species that may be present in the sample. We recommend the use of immunoprecipitation (IP) to clean up the ubiquitinated sample prior to UbiCREST analysis. UbiCREST Kit components have been optimized for 50 µL reactions containing ubiquitinated substrate proteins immobilized to resin using an IP approach. Previously purified samples may be analyzed without using IP as long as the sample is devoid of free (unanchored) monoubiquitin. (Contact Boston Biochem for more details.)
- UbiCREST DUB digestion reactions should not exceed 30 minutes at 37 °C. Longer incubation times may result in a loss of DUB specificity that may complicate interpretation of results. It should be noted that if an IP isolates two or more proteins as part of a stable complex (co-IP), there may be multiple polyubiquitin chain types in the sample. The kit cannot differentiate if multiple polyubiquitin linkages in a sample are due to independent homotypic chains of different linkages, or branched- or mixed-linkages within one or more chains.
- Guidelines for sample immunoprecipitation, DUB digestions, and silver staining and Western Blot analysis of results are provided. It is up to the investigator to carefully consider the amount and purity of polyubiquitinated sample that can be obtained before deciding on which approach to follow. It is *strongly* recommended that the investigator read "OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis" by Mevissen, *et. al.* (2013, Cell **154**:169-84) prior to using this kit. The authors provide a comprehensive analysis and description of the substrate preferences of the majority of the UbiCREST Kit deubiquitinases that will undoubtedly be of use in interpreting results.
- Note that the kit may not always provide definitive evidence of all chain linkages present due to the physical limitations of silver stain and/or Western Blot techniques. The reagents and protocols provided or referenced in this kit have been chosen following careful testing at Boston Biochem. Modifications to the protocol or selection of alternative reagents will require assay optimization by the end-user. More information is available at techsupport@bostonbiochem.com.

MATERIALS PROVIDED & STORAGE CONDITIONS

Kit contains reagents sufficient for 5 UbiCREST analysis experiments.

COMPONENT	VOLUME	STORAGE OF COMPONENTS
10X Otubain 1 (OTUB1)	25 µL	Store at -80 °C.* Avoid multiple freeze-thaw cycles.
10X GST-AMSH	25 µL	
10X OTUD3$_{CD}$	25 µL	
10X His$_6$-Trabid	25 µL	
10X Cezanne$_{CD}$	25 µL	
10X YOD1	25 µL	
10X Otulin$_{FL}$	25 µL	
10X USP2$_{CD}$	25 µL	
10X DUB Reaction Buffer	1.5 mL	Store at -20 °C.*

* Provided this is within the expiration date of the kit.

OTHER MATERIALS REQUIRED

- **Deionized water (dH$_2$O)** - Sterile
- **PBST** - 1X PBS (Calbiochem # 524650) + 0.05% Tween®-20 (Sigma # P1379) or equivalent.
- **SDS-PAGE Gels** - 10-20% gradient, 16%, and others. See Protocols for guidance.
- **SDS-PAGE Sample Buffer** - Reducing. Compatible with SDS-PAGE gels.
- **Lysis Buffer** - SDS-free RIPA Buffer for solubilizing target proteins.
- **Sample For Analysis** - Consists of a polyubiquitinated Target Protein purified from a cell lysate (via IP or other means), or protein ubiquitinated with an *in vitro* conjugation reaction.
- **Silver Stain Reagents (optional)** - (BioRad #161-0449) or equivalent.
- **Immunoprecipitation Kit (optional)** - (Pierce # 26146) or equivalent. Alternatively, commonly used IP reagents may be used to purify the ubiquitinated protein prior to analysis.
- **Western Blot Reagents** - Including primary and secondary antibodies, nitrocellulose or PVDF membranes, and ECL reagents.

PROTOCOL OVERVIEW

UbiCREST Analysis provides a rapid and economical means of identifying the types of linkages present in polyubiquitin chains. Briefly, the method consists of purifying a polyubiquitinated substrate, treating it with a panel of linkage-specific deubiquitinases (8 reactions in parallel), then analyzing the data to determine which of the DUBs were active against the substrate (See Figure 1). Data output may consist of silver stain or Western Blot visualization of liberated ubiquitin and polyubiquitin chains ("Primary analysis"), or Western Blot analysis of the Target Protein ("Secondary analysis"). While the methods are straightforward, it is often useful to generate two readouts (ubiquitin-based and Target-based) in order to obtain a more comprehensive picture of the linkage(s) present in a ubiquitinated substrate. Both types of data may be generated from a single experiment.

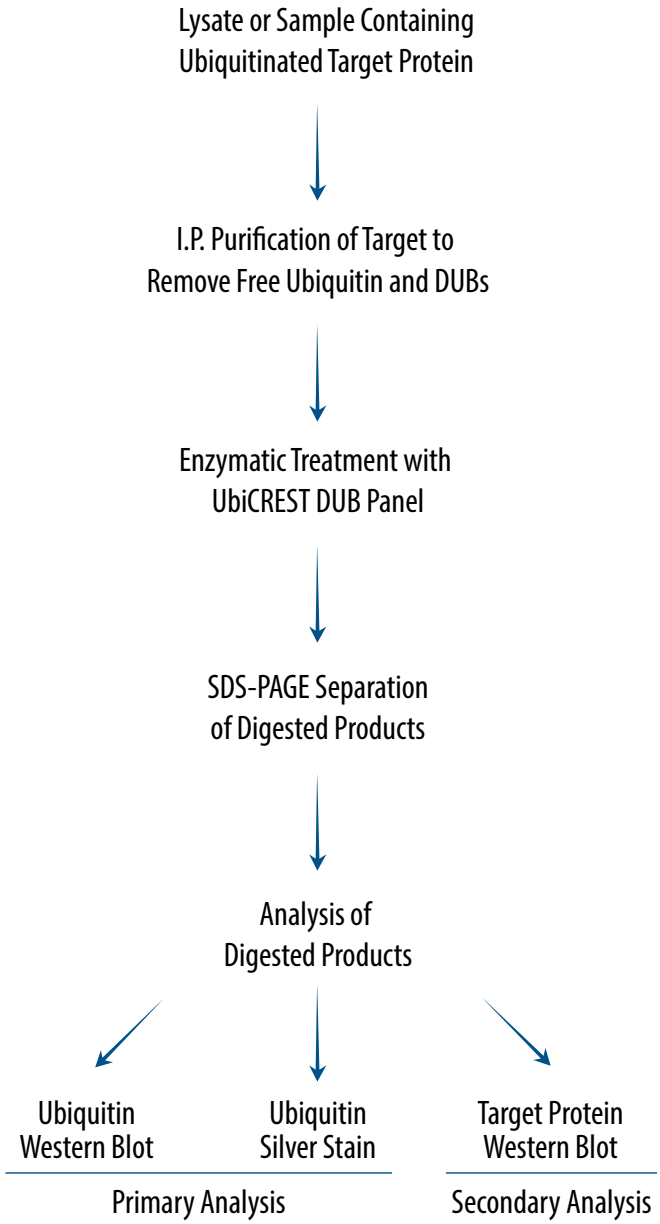

Figure 1: Overview of the workflow for UbiCREST Analysis. Refer to this overview throughout the protocol as needed.

LYSATE PREPARATION

Buffer Composition

Procedures should be done on ice or at 4 °C to minimize degradation of polyubiquitinated Target Protein. Cell lysates may be generated using commonly used, immunoprecipitation-compatible lysis buffers described in the literature. When possible, we recommend that SDS not be included in the lysis buffer as it may interfere with downstream UbiCREST DUB treatment.

Buffer Additions

Complex lysates from eukaryotic cells contain DUB's that can quickly remove ubiquitin from a Target Protein of interest. Adding the non-specific cysteine protease inhibitor N-ethylmaleimide (NEM, 5-10 mM final) to lysis buffers helps mitigate unwanted DUB activity in the lysates during purification. Alternatively, adding 2-10 µM ubiquitin-aldehyde, ubiquitin-vinyl sulfone, ubiquitin-vinyl methyl ester, or ubiquitin-propargyl amide to lysis buffers provides excellent protection against most DUBs. Addition of EDTA to lysis buffers may also be helpful to inhibit unwanted metallo-protease degradation of substrates. While these additives provide protection from unwanted DUB activity during the purification procedure, they will also inhibit the UbiCREST Panel of DUBs used in subsequent steps. It is therefore essential that they be washed away at the end of the purification procedure.

IMMUNOPRECIPITATION OF UBIQUITINATED TARGET

Immunoprecipitation (IP) Considerations

It is left to the investigator to determine how best to perform IP reactions. Enough Target Protein should be IP'd for nine reactions and subsequent gel analysis (eight different UbiCREST DUB treatments plus an untreated control). We recommend running a single, larger "batch" IP process using a polyclonal antibody (previously validated by the investigator) against the ubiquitinated Target Protein. Protein A/G Sepharose (approximately 200 µL packed volume or 400 µL 50% slurry) can be used to collect immunocomplexes for washing and then later divided into 9 x ~20 µL aliquots of packed resin for UbiCREST DUB treatment as described in the following section.

Suggested Workflow

1. Prepare 3 mL of 1X DUB Reaction Buffer by diluting 300 µL of 10X DUB Reaction Buffer (supplied) with 2.7 mL of dH_2O. Store remaining 10X DUB Reaction Buffer at -20 °C in convenient aliquots.
2. Obtain polyubiquitinated Target Protein of interest from cultured cell lysate, tissue lysates, or in vitro ubiquitination reactions. (Refer to Lysate Preparation). We recommend diluting sample to a volume of 500-1000 µL for easy handling (when necessary).
3. Add an appropriate amount of antibody to the sample and incubate for 1-4 hours at 4 °C, typically in a 1.5 mL microcentrifuge tube.
4. Add 200 µL (packed volume) of Protein A/G Sepharose ("resin") to the sample and place on rocker for 1-2 hours at 4 °C.
5. Pellet resin by brief low-speed centrifugation (1 minute at 1000 x *g*) and carefully remove supernatant (lysate) by aspiration.
6. Wash resin by adding 1 mL cold PBST (Phosphate-Buffered Saline + 0.05% Tween-20) or similar wash buffer (a higher stringency wash may be used as necessary). Invert tube for 2-3 minutes then briefly centrifuge to pellet resin. Carefully aspirate wash supernatant. Repeat twice for a total of three washes. Washes are essential to remove contaminating DUBs, DUB inhibitors, and free (unanchored) ubiquitin prior to UbiCREST reactions.
7. Wash resin with 1 mL 1X DUB Reaction Buffer (prepared previously). Invert tube for 5 minutes then briefly centrifuge at low speed to pellet resin. Carefully aspirate wash supernatant. Repeat once for a total of two washes.
8. Distribute the resin equally among 9 microcentrifuge tubes. We recommend doing this by adding 250 µL of 1X DUB Reaction Buffer to the 200 µL of resin, gently vortexing to suspend beads, then immediately pipetting out 50 µL of the bead slurry with a wide-pore pipette tip (made by cutting off the end of a 200-250 µL pipette tip). This is repeated 8 more times to generate 9 tubes, each containing approximately 20 µL of resin and 30 µL of supernatant. **Note:** *Some resins adhere to pipette tips and may be difficult to manipulate*. The use of silanized pipette tips may help alleviate this problem. Additional manipulation may be required to uniformly distribute the resin among the 9 tubes.
9. Briefly centrifuge the tubes to pellet resin. Carefully aspirate excess supernatant from the 9 tubes of resin. Label the top of each tube with one of the UbiCREST enzyme names: OTUB1, AMSH, OTUD3, Trabid, Cezanne, Yod1, Otulin, or USP2. The remaining tube will be used for a no-treatment control and should be labeled as such.
10. Samples (9 tubes with approximately 20 µL of resin) are now ready for UbiCREST DUB additions.

TARGET SAMPLE TREATMENT

1. Quickly thaw all UbiCREST enzymes by gently swirling tubes in a lukewarm water bath (≤ 30 °C). **Note:** *Do not leave tubes in water bath for an extended period of time. Do not vortex or shake vigorously.*
2. When completely thawed, briefly spin tubes in a microcentrifuge (5 seconds) to collect components in bottom of tubes.
3. Gently tap tubes to make sure components are well mixed. Store enzymes on ice.
4. It is recommended that each enzyme be divided into smaller aliquots to minimize the number of freeze-thaw cycles of kit components. Avoid multiple freeze-thaw cycles to maximize kit performance. Rapidly re-freeze any unused aliquoted materials in dry ice bath.
5. Add 45 µL of 1X DUB Reaction Buffer (previously prepared) to each of the 8 tubes labeled with an UbiCREST enzyme name. Add 50 µL of 1X DUB Reaction Buffer to the control tube.
6. Add 5 µL of each UbiCREST DUB enzyme to the appropriate tube. For example, the sample tube labeled "Cezanne" receives 5 µL of 10X Cezanne enzyme, while the sample tube labeled "Yod1" receives 5 µL of 10X Yod1, and so on. Gently mix the tubes to uniformly disperse the enzymes throughout the supernatant and resin. This may be done by gently flicking the tubes for 10-15 seconds. Use brief centrifugation if needed to collect the resin at the bottom of the tube.
7. Incubate all nine tubes at 37 ºC for 30 minutes. **Note:** *Longer reaction times are not recommended.*
8. After incubation, mix samples again by flicking tubes 5-10 times, then pellet resin by low-speed centrifugation for 1-2 minutes.
9. Carefully withdraw and save reaction supernatants (~50 µL each) into a second set of microfuge tubes labeled appropriately with the name of the enzyme used in each treatment. At this point you will have 18 tubes (9 pairs) containing either the supernatant or the resin from each enzyme treatment (and the untreated control).
10. Add 10 µL of 6X SDS-PAGE Buffer (reducing) to the 9 tubes containing the reaction supernatants. Mix well and heat at 80-90 °C for 5 minutes. These samples are for "Primary analysis".
11. Add 50 µL of 1X SDS-PAGE Buffer (reducing) to the 9 tubes containing resin. Mix well and heat at 80-90 °C for 5 minutes. These samples are for "Secondary analysis".
12. Samples may now be analyzed or stored at -80 °C for future analysis.

SDS-PAGE SEPARATION

It is left to the investigator to choose appropriate SDS-PAGE gels for UbiCREST Analysis. Assemble and run gels according to manufacturer's instructions using the following guidelines:

1. For "Primary analysis" (if analyzing supernatants for liberated ubiquitin, see Target Sample Treatment section steps 9 and 10) a higher percentage SDS-PAGE gel should be chosen. We recommend gels with a 10-20% gradient, or a uniform 16% acrylamide concentration. The gel should be chosen so that ubiquitin (8.6 kDa, ~ 6 kDa apparent) and shorter polyubiquitin chains are well resolved from other proteins and the dye-front.

2. For "Secondary analysis" (if analyzing resins for Target Protein deubiquitination, see Target Sample Treatment section step 11) the choice of SDS-PAGE gel will depend on the size of the Target Protein being studied. A uniform percentage or gradient gel should be chosen so that polyubiquitinated and deubiquitinated forms of the Target Protein can be clearly resolved as visualized by Western Blot.

3. Once the type of analysis and gel format are chosen, load at least 10 µL of the appropriate samples onto the gel. The load volume may be increased depending on the type of gel used. It is imperative that the order of sample loading is carefully recorded and saved for later reference. (*e.g.* MW markers, untreated control, USP2, AMSH, Cezanne, OTUB1, OTUD3, OTULIN, Trabid, YOD1).

4. Run gel just until dye-front reaches bottom of gel. In the case of Primary analysis, do not run the gel too long as monoubiquitin migrates closely behind the dye-front in many gel formats. Over-running the gel may result in loss of the monoubiquitin signal.

5. Once the gels are complete, proceed to Primary Analysis or Secondary Analysis section, depending on which analysis type you are performing.

PRIMARY ANALYSIS

Primary Analysis of UbiCREST Reactions consists of visualizing monoubiquitin and short polyubiquitin chains that have been liberated (deconjugated) from the Target Protein by one or more of the UbiCREST DUBs. Any ubiquitin that was released from the Target Protein into the reaction supernatants was separated using SDS-PAGE in the previous steps. Ubiquitin may be visualized by either silver staining the gel, or performing Western Blot analysis of the gel using an anti-ubiquitin antibody. The choice is left to the investigator.

Option 1: Analysis of UbiCREST supernatant samples; Silver Staining for detection of deconjugated ubiquitin. **Note:** *If using silver-stain analysis, wear gloves throughout the procedure to minimize the detection of fingerprints on gels.*

1. Carefully disassemble the gel and prepare for silver staining. It is left to the researcher to choose a protocol for silver staining the gel. We routinely use the *Silver Stain Plus Kit* (Biorad #161-0449) for visualizing the ubiquitin banding pattern readout.
2. When using a silver stain procedure, the UbiCREST DUBs will be seen in addition to ubiquitin. Refer to **Figure 2** for help in identifying these protein signatures.
3. See **Results Interpretation** for more information.

Option 2: Analysis of UbiCREST supernatant samples; Western Blot for detection of deconjugated ubiquitin.

1. Carefully disassemble the gel and prepare for Western Blot procedure. It is left to the researcher to choose a protocol for routine Western Blot analysis. We routinely utilize a BioRad Trans® Blot Semi Dry Transfer Cell and Towbin Buffer to transfer proteins to PVDF membranes. Membrane blocking is done in PBST solution containing non-fat milk (details available upon request).
2. Western Blot analysis should utilize a primary antibody against ubiquitin. This detection antibody is typically of a different species than the antibody that was used in the Target Protein IP procedure to prevent potential interference in the blot.
3. Enhanced Chemiluminescence (E.C.L.) exposure times will need to be determined experimentally to ensure visualization of ubiquitin and polyubiquitin species in the Western Blot.
4. See **Results Interpretation** for more information.

SECONDARY ANALYSIS

Secondary Analysis of UbiCREST Reactions consists of visualizing the Target Protein for changes in apparent molecular weight that result from the removal of ubiquitin (deubiquitination) by one or more of the UbiCREST DUBs. The majority of the Target Protein remained associated with the IP resin during the DUB treatment, and was recovered by denaturing the resin with 1X SDS-PAGE Sample Buffer and heating (done previously). Target Protein may be visualized by performing Western Blot analysis using an anti-Target Protein primary antibody.

1. Carefully disassemble the gel and prepare for Western Blot procedure. It is left to the researcher to choose a protocol for routine Western Blot analysis. We routinely utilize a BioRad Trans® Blot Semi Dry Transfer Cell and Towbin Buffer to transfer proteins to PVDF membranes. Membrane blocking is done in PBST solution containing either BSA or non-fat milk (details available upon request).
2. Western Blot analysis should be carried out using a primary antibody against the Target Protein being analyzed. This detection antibody is typically of a different species than the antibody that was used in the IP procedure to prevent interference in the blot.
3. E.C.L. exposure times will need to be determined experimentally so that visualization of ubiquitinated and non-ubiquitinated forms of the Target Protein are observed in the Western Blot.
4. See **Results Interpretation** below for more information.

LINKAGE SPECIFICITY

The enzymes provided in this kit have been determined to have the following linkage specificity when used as described.

Enzyme	Linkage
$OTUD3_{CD}$	K6 and K11-linked polyubiquitin
$Cezanne_{CD}$	K11-linked polyubiquitin only
His_6-Trabid	K29, K33, and K63-linked polyubiquitin
Otubain1 (OTUB1)	K48-linked polyubiquitin only
GST-AMSH	K63-linked polyubiquitin only
YOD1	K6, K11, K27, K29, and K33 linked polyubiquitin
$Otulin_{FL}$	Linear polyubiquitin only
$USP2_{CD}$	Control, Cleaves most ubiquitin linkages studied

RESULTS INTERPRETATION

UbiCREST data can include silver stain or Western Blot visualization of liberated ubiquitin and polyubiquitin chains, or Western Blot analysis of the ubiquitinated substrate. While the methods are straightforward, it is often useful to generate two readouts (anti-ubiquitin *and* anti-substrate) in order to obtain a more comprehensive picture of the linkage(s) present in a ubiquitinated substrate. It is *strongly recommended* that the investigator read "OTU deubiquitinases reveal mechanisms of linkage specificity and enable ubiquitin chain restriction analysis" by Mevissen, *et.al.* (Cell, 2013, **154**:169-84) prior to using this kit.

UbiCREST enzymes have a strong preference for specific ubiquitin-ubiquitin linkages when used as directed (See Linkage Specificity of UbiCREST Deubiquitinases). When monoubiquitin or small polyubiquitin chains are released from a polyubiquitinated substrate by an UbiCREST DUB it indicates that the ubiquitin linkage(s) that the DUB prefers is present within the sample. By comparing the readouts across the DUB panel it is possible to deduce which linkages are prevalent in the sample. For example, if ubiquitin is liberated from a sample by OTUD3 and Yod1, but not Cezanne, then it is probable that K6-linked ubiquitin is present in the sample.

In the Example Data (see below) a highly polyubiquitinated RPN10/S5a protein was generated *in vitro* using purified UBE1, UBE2L3, ubiquitin, ATP, and the ubiquitin ligase AIP4/Itch. Itch has been reported to catalyze the addition of K29-, K48-, and K63-linked polyubiquitin chains to substrates. We used immunoprecipitation to purify the S5a away from other components, most importantly free ubiquitin which complicates the assay readout.

In **Figure 2**, the supernatants from UbiCREST reactions were analyzed by SDS-PAGE and silver stain (anti-ubiquitin Western Blot could also have been used with equal or better sensitivity) for the presence of free (unanchored) ubiquitin. A strong monoubiquitin signal was observed in the USP2 positive control lane as well as in the Trabid and AMSH lanes, corroborating the report of K63- and possibly K29- isopeptide linkages. However, no detectable signal was present in the OTUB1 lane, signifying that K48-linked polyubiquitin was not generated by Itch under these conditions. Similarly, OTULIN produced no detectable mono- or polyubiquitin chains in the analysis, demonstrating that Itch did not ubiquitinate S5a with linear (Met1) linkages.

Yod1, Cezanne and OTUD3 did not generate monoubiquitin in the **Figure 2** analysis, but all three lanes had detectable di-, tri-, and tetraubiquitin (stronger in the Yod1 and Cezanne) suggesting that linkages other than K63 were present at lower levels. Yod1 will digest a number of ubiquitin linkages, but Cezanne has a strong K11 specificity when using the kit conditions. Together these suggest that K11 chains are present in the ubiquitinated S5a, and this is supported by the limited but detectable signal generated by OTUD3, an enzyme that is also capable of digesting K11 linkages.

RESULTS INTERPRETATION *CONTINUED*

In **Figure 3**, anti-S5a Western Blot was used as a readout for the UbiCREST analysis. The undigested control lane ("Con") demonstrates that ubiquitinated S5a migrates as a smear from 66 kDa to greater than 200 kDa, with a prevalent band at approximately 220 kDa. Digestion with the USP2 positive control enzyme results in a collapse of most immunoreactive material to a molecular weight of approximately 44 kDa, the weight of non-ubiquitinated S5a. After the USP2 control, Yod1, Trabid, and AMSH produced the next largest reduction in the intensity of the S5a-ubiquitin band at 220 kDa. Of these three enzymes, only Yod1 generated a significant increase in completely deubiquitinated S5a as judged by the intensity of the signal at 44 kDa. This may suggest that in addition to cleaving various polyubiquitin linkages (listed above), Yod1 is also capable of cleaving chains off one or more of the S5a lysine anchor points as well. In fact some of the polyubiquitin chains visualized in the Yod1 lane in **Figure 2** may originate from this activity. Trabid and AMSH generated large amounts of monoubiquitin in the **Figure 2** analysis, but almost no increase in unmodified S5a in the **Figure 3** analysis. This implies that these two enzymes cleaved ubiquitin off the S5a substrate protein at distal and/or internal sites in the polyubiquitin chains (primarily at K63-linkages). This activity leaves one or more ubiquitins within a chain (proximal to the point of cleavage) attached to the S5a; this is supported by the appearance of S5a-ubiquitin adducts in the 55-66 kDa range in the Trabid and AMSH lanes. Yod1 also generated enhanced signals in the 55-66 kDa area relative to the untreated control, consistent with its ability to convert some (but not all) of the ubiquitinated S5a to an unmodified form. It is difficult to draw useful conclusions from the Cezanne and OTUD3 data in **Figure 3**, highlighting the importance of the silver stain or anti-ubiquitin Western Blot (Primary analysis) approach shown in **Figure 2.**

Taken together the data presented in Figures 1 and 2 point to K63 as the primary linkage present in Itch-catalyzed polyubiquitination of S5a, with other linkages including K11 and/or K29 present as well.

SAMPLE DATA

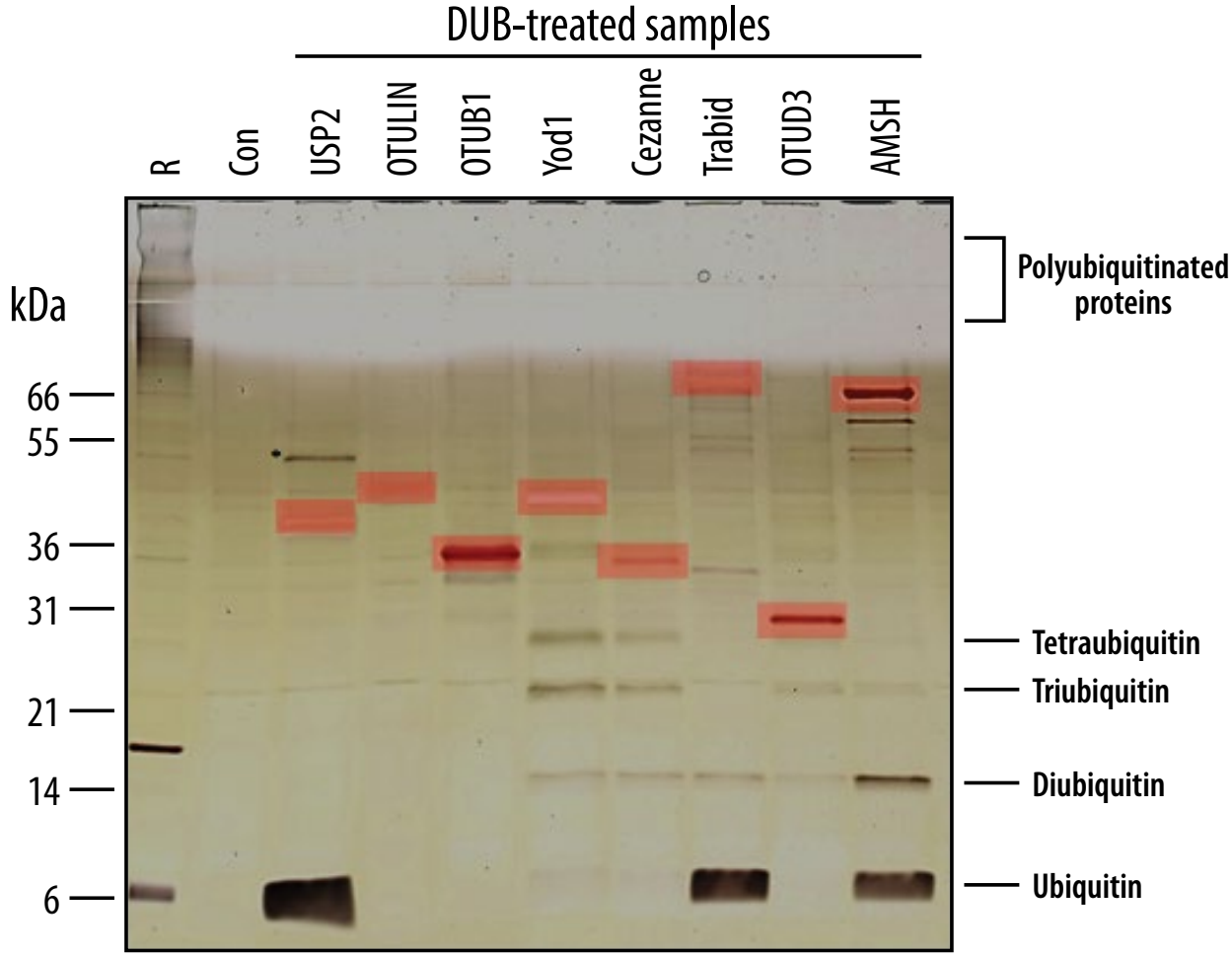

Figure 2: UbiCREST Analysis of polyubiquitinated RPN10/S5a-mediated by the ubiquitin ligase AIP4/Itch. *In vitro* reactions using purified ubiquitin ligase Itch, E1, UBE2L3, and other components were used to produce polyubiquitinated S5a substrate protein. UbiCREST analysis using an immunoprecipitation purification strategy plus an SDS-PAGE/silver stain readout is shown. **R:** Control lane showing Itch/S5a ubiquitination reaction without IP or DUB treatment. Polyubiquitinated proteins are seen as a high molecular weight smear, and UBEL3 (18 kDa) and trace residual ubiquitin (~ 6 kDa apparent) are evident. **Con:** No DUB control demonstrating free ubiquitin and UBE2L3 are removed by IP/wash treatment. **Note:** *Polyubiquitinated S5a is not observed in the gel (other than in lane **R**) because this is an analysis of the IP resin supernatant, not the proteins that bound to the resin.* **DUB-treated samples:** The highlighted boxes in each lane indicate the migration of the UbiCREST DUB enzymes used in the assays. The band marked with an asterisk in the USP2 lane is an unknown artifact that wasn't present in duplicate assays (data not shown). Strong monoubiquitin signals are evident in the USP2 positive control lane, and in the Trabid and AMSH lanes. The Trabid lane implies the majority of the S5a-polyubiquitin adducts are K29-, K33-, and/or K63-linked, while the AMSH lane corroborates a prevalence of K63 linkages. Lighter staining di-, tri-, and tetraubiquitin species in the Yod1 (K6-, K11-, K27-, K29-, and K33-specific) and Cezanne (K11-specific) suggests that non-K63 isopeptide bonds are present at lower levels.

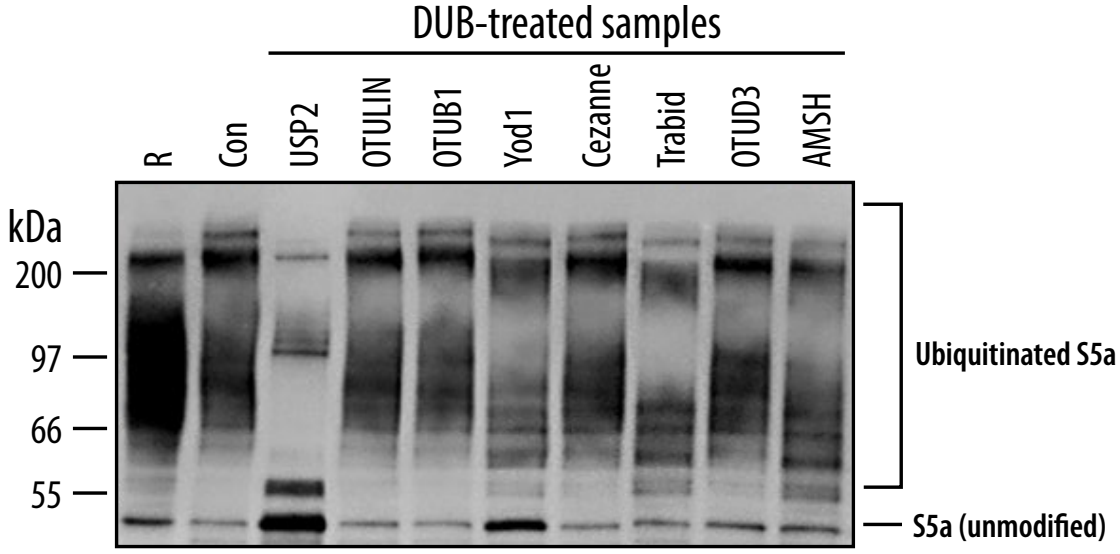

Figure 3: UbiCREST Analysis of polyubiquitinated RPN10/S5a-mediated by the ubiquitin ligase AIP4/Itch. *In vitro* reactions using purified ubiquitin ligase Itch, E1, UBE2L3, and other components were used to produce highly polyubiquitinated S5a substrate protein. UbiCREST analysis using an immunoprecipitation purification strategy plus an SDS-PAGE/anti-S5a Western Blot is shown. **R:** Control lane showing original ubiquitination reaction without IP or DUB treatment. **Con:** No DUB control providing a "baseline appearance" for IP'd S5a, including a trace of unmodified S5a (44 kDa apparent), and polyubiquitinated S5a (66-220 kDa apparent). **DUB-treated samples:** In the USP2 control the majority of S5a is detected in a band that migrates at the apparent molecular weight of unmodified protein, indicating that the resin-bound, polyubiquitinated S5a can be successfully deubiquitinated. A band in the 55 kDa region may represent a DUB-resistant species. A high molecular weight band of ubiquitinated-S5a (~ 220 kDa apparent MW) present in the control is noticeably reduced in the USP2, Yod1, Trabid, and AMSH lanes, with a concomitant increase in S5a-reactive bands in the 55-66 kDa range. Yod1 (K6-, K11-, K27-, K29-, and K33-specific) produced more fully deubiquitinated S5a than any other DUB except USP2. While Trabid (K29-, K33-, and K63-specific) and AMSH (K63-specific) clearly reduced the degree of S5a ubiquitination, neither of these enzymes was capable of removing all ubiquitin from this substrate.

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