

E2Select Ubiquitin Conjugation Kit

Catalog Number K-982

This kit contains reagents sufficient to perform 2 Ubiquitination reactions for each E2 included in the kit.

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 posttranslational modifications observed in eukaryotic organisms. Poly-Ubiquitination of proteins directs them to varied cellular fates such as altered subcellular localization, assembly into multi-subunit complexes, or degradation via the proteasome. Poly-Ubiquitin chains are typically formed using the E1, E2, E3 enzyme conjugation cascade via an isopeptide bond linkage between the C-terminal glycine of Ubiquitin and the ϵ -amino group of the appropriate lysine on a target protein.

E2 conjugating enzymes typically interact with one or two E1 activating enzymes (upstream) and potentially hundreds of E3 Ubiquitin ligase enzymes (downstream) to form these chains. They connect the activation of Ubiquitin to the covalent modification of select lysine residues (Ubiquitination) found on target proteins. While E3 ligases play the major role, E2's are also important determinants in substrate selection and which residues of those substrates become Mono- or Poly-Ubiquitinated. Therefore, they can directly control the cellular fate of the substrate. Mechanistically, E3 ligases bind a "charged" E2-Ubiquitin thioester conjugate and catalyze the transfer of Ubiquitin from the E2 active site cysteine to a substrate lysine (for RING E3s) or to a catalytic cysteine in the E3 itself (for HECT and RBR E3s).

Due to the complexity of this system, it can be challenging to determine which E2 conjugating enzyme(s) are utilized by a recently discovered or poorly characterized E3 ligase. The investigator may set up an *in vitro* Ubiquitination reaction using an E1 activating enzyme, Ubiquitin, ATP, and the E3 ligase of interest, but the appropriate E2 for the reaction may not be known. Considering there are approximately 35 active E2 enzymes in humans (15-35 in other eukaryotes), the cost of purchasing each recombinant E2 individually may be cost prohibitive.

We offer the E2Select kit as a practical solution to this problem by supplying two duplicate panels of 26 E2 conjugating enzymes contained in a 96 well plate. The kit is designed to help identify which E2 enzymes facilitate *in vitro* substrate Ubiquitination catalyzed by a recombinant E3 ligase and substrate of choice using SDS-PAGE with Coomassie or Western blot analysis.

TECHNICAL HINTS

- The E2Select kit was designed for the purpose of quickly screening a panel of E2 enzymes to identify which ones facilitate the Ubiquitination of proteins by an E3 ligase or Auto-Ubiquitination of the E3 ligase itself. Only the E3 of interest and a substrate (if testing one) need be provided by the user. In addition to the 26 E2 enzymes provided in duplicate wells on a 96 well plate, E1 enzyme, Ubiquitin, and ATP are provided. The reaction products may be analyzed by SDS-PAGE analysis or Western blotting using antibodies specific to the E3 Ligase or substrate of interest.
- If performing SDS-PAGE with Coomassie staining to analyze reactions, it should be noted that the following E2s can catalyze formation of free or anchored Poly-Ubiquitin chains independently of E3 Ligases: UBE2D1, UBE2D3, UBE2E1, UBE2E3, UBE2N/UBE2V1, UBE2N/UBE2V2, UBE2S, UBE2T, and UBE2W2. Visualization of these bands on a Coomassie gel may complicate interpretation of data.
- The positive control wells contain an E3 Ligase (Itch), substrate (S5a), and compatible E2. S5a Ubiquitination can be confirmed by Coomassie staining of an SDS-PAGE gel, anti-6His Western blot, or anti-S5a Western blot (using anti-S5a antibody, R&D Systems Catalog# AF5540).
- Guidelines for performing the Ubiquitination reactions, SDS-PAGE analysis, and Western blot analysis are provided. However, as user provided E3s and substrates vary, it is up to the end user to determine appropriate concentrations of these components.
- Note that *in vitro* assay results may not always provide definitive evidence of Ubiquitination of an E3 substrate *in vivo*. Conditions in the cell that promote Ubiquitination of a substrate or Auto-Ubiquitination of an E3 may not be replicated in a microplate well. 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

Excluding the E2's on the plate, this kit contains reagents sufficient for 125 x 20 μ L reactions.

COMPONENT	VOLUME	STORAGE OF COMPONENTS
96 well plate	N/A	Store at -80 °C.* Avoid multiple freeze-thaw cycles.
10X Reaction Buffer	0.5 mL	
10X E1 Enzyme	0.25 mL	
20X Ubiquitin	0.15 mL	Store at -20 °C.*
4X Mg ²⁺ -ATP	0.75 mL	
5X SDS-PAGE Buffer	1 mL	Store at room temperature.
Foil Plate Sealer	N/A	

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

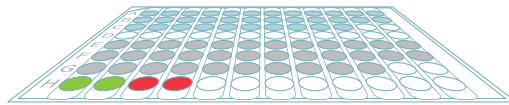
OTHER MATERIALS REQUIRED

- Deionized water (dH₂O) - Sterile
- 1M Dithiothreitol (DTT) - to add to sample buffer when running reducing SDS-PAGE gels
- SDS-PAGE Gels - 10-20% gradient or others. See protocols for guidance
- E3 Ubiquitin Ligase - recombinant or purified from cellular lysates
- Substrate (optional) - not required for analyzing E3 Auto-Ubiquitination
- Coomassie stain reagent (optional)
- Western Blot Reagents – Includes transfer buffer, primary and secondary antibodies, nitrocellulose or PVDF membranes, and ECL reagents
- Centrifuge with 96-well plate adapter
- 37 °C incubator

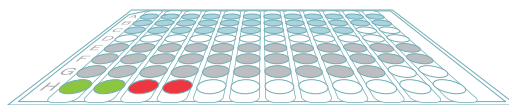
PROTOCOL OVERVIEW

The E2Select kit contains a panel of 26 E2 conjugating enzymes arrayed in duplicate on a 96 well plate (See Figure 1). This configuration allows an experiment to be performed in duplicate, or the testing of two E3s against a single substrate, or the testing of two substrates with a single E3. Two steps are required to initiate reactions. The first is to assemble all the non-E2 reagents except ATP into a Master Mix and add it to each E2 containing well of the plate (except the positive control wells which already contain these reagents). The second is to add 5 μ L ATP to all wells (except the negative control well) to initiate the Ubiquitination reactions. After a 60 minute incubation, the reactions are quenched with 5 μ L of 5X Sample Buffer for subsequent analysis by SDS-PAGE/Coomassie or Western blot analysis. Refer to this overview throughout the protocol as needed.

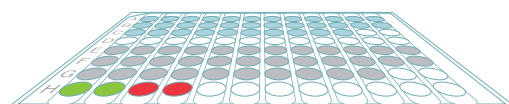
Add 11 μ L of a Ubiquitination Master Mix
to 4 μ L of E2 on the plate



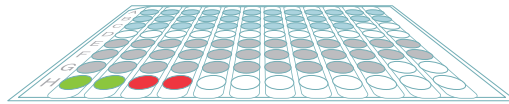
Add 5 μ L of 4X ATP



Incubate at 37°C for 60 minutes



Add 5 μ L of 5X SDS-PAGE Buffer



Analyze by SDS-PAGE and Coomassie
or Western blotting

REAGENT PREPARATION

1. Quickly thaw all protein reagents and buffers by gently and continuously swirling in a lukewarm water bath ($\leq 30\text{ }^{\circ}\text{C}$). Alternatively, tubes may be thawed with a rapid back-and-forth rolling motion between palms of the hands. Do not heat tubes for an extended period of time. Do not vortex.
2. When all reagents are thawed, gently tap tubes to ensure components are well mixed (SDS-PAGE sample buffer may be inverted to mix), then spin tubes briefly in a microfuge to collect contents at the bottom.
3. Immediately place contents in an ice bucket (SDS-PAGE sample buffer can be kept at room temperature) until ready to use. The above steps should be completed in approximately 5 minutes.
4. Centrifuge the E2 plate for 2 minutes at 1000xg to collect contents in the bottom of the wells. Do not remove foil cover at this time. Visually inspect the plate to make certain contents have thawed. The plate may be placed briefly at $37\text{ }^{\circ}\text{C}$ to help thaw if needed.

REACTION ASSEMBLY

Prepare Master Mixes at room temperature. Note that indicated reagent concentrations are relative to the final 20 μL reaction volume in each well.

1. Assemble a **Master Mix A** (enough for 30 wells) in a polypropylene tube using the following volumes and order of addition. Note that these volumes are sufficient for one of the two E2 panels, control wells, and some pipetting error.
 - a. 60 μL of 10X Reaction Buffer
 - b. 30 μL of 20X Ubiquitin
 - c. 60 μL of 10X E1 Enzyme
 - d. 180 μL of user designed combination of E3 Ubiquitin Ligase, Substrate, and dH_2O (see note below)
2. Mix gently but thoroughly, then centrifuge briefly to collect contents in bottom of tube.
3. Master Mix A is sufficient to test one of the two duplicate series of E2 enzymes on the plate. A second mix **Master Mix B** may be put together using the same technique outlined in step 1. Master Mix B may utilize a different E3 Ligase (or Ligase concentration) than Master Mix A. Alternatively, Master Mix B might use a different substrate (or concentration) than Mix A. If the end-user prefers to run duplicate reactions using the same conditions, then Master Mix A and Master Mix B will be identical. It is up to the investigator to choose which approach to use.

Note: As the investigator is providing the E3 and/or substrate when using the E2Select plate, they must determine first if they are using an E3 Ligase alone or an E3 with a test substrate. They must also decide on a final concentration of E3 (and substrate) to use in each 20 μL reaction and then add the appropriate amount to each Master Mix. We suggest an initial E3 Ubiquitin Ligase concentration of 0.2-2 μM , and an initial substrate concentration (if used) of 1-5 μM . Contact techsupport@bostonbiochem.com for assistance.

REACTION ASSEMBLY *CONTINUED*

- Carefully remove foil seal from E2 plate, ensuring that all E2 aliquots remain in the bottom of the wells. Placing the plate in a 96-well thermocycler block helps to hold the plate steady while removing foil.
- Transfer 11 μL of Master Mix A to all wells in Rows A and B, plus wells C1 and C2, and the negative control well, H1 (see Figure 1). Use of a repeat pipettor can help to facilitate the process. Do not cross-contaminate wells.
- Transfer 11 μL of Master Mix B to all wells in Rows E and F, plus wells G1 and G2, and the negative control well, H2 (see Figure 1). Again, avoid cross-contamination during transfers.
- Initiate reactions by adding 5 μL of 4X ATP to all wells except negative control wells (H1 and H2). Add 5 μL of dH₂O to negative control wells. Make sure to add ATP to positive control wells, H3 and H4. Mix contents of all wells gently but thoroughly. This is easily achieved with a multichannel pipette.
- Reseal plate with the provided foil seal. Centrifuge plate for 1-2 minutes at 1000 x g to collect contents in bottom of wells. Place the plate in a 37 °C incubator for 60 minutes.

REACTION ANALYSIS

- Remove plate from the incubator and carefully remove the foil seal.
- Terminate the reactions by adding 5 μL of the 5X SDS-PAGE Buffer plus 1 μL of fresh, 1M DTT (user supplied). Heat samples (in plate) to 75-95 °C for 5 minutes prior to SDS-PAGE analysis.

SDS-PAGE SEPARATION

It is left to the end user to choose an appropriate SDS-PAGE gel for analysis of the E2 reactions. The choice will depend on the size of the E3 or substrate being analyzed. We recommend using either 4-20% or 10-20% gradient gels. Assemble SDS-PAGE gel according to manufacturer's instructions using the following guidelines:

- If Coomassie stain will be used to analyze results, load 15 μL or more of each reaction to the appropriate well on a the gel. If Western Blot Analysis will be used to analyze results, then less sample (as little as 5 μL) may be used depending on the quality of the primary antibody against the E3 Ligase or substrate. It is essential that the order of sample loading on the gel is recorded and saved for later reference to avoid confusion during data interpretation.
- Run gel until dye-front just reaches bottom of gel to ensure that no proteins are run off the gel.

DATA GENERATION

E2Select Reaction products consist of Mono-Ubiquitin or Poly-Ubiquitin chains attached to the E3 ligase or substrate of interest. Visualization of the products is achieved by resolving the samples on an SDS-PAGE gel followed by either 1) staining the gel with Coomassie, or 2) performing Western blot analysis of the gel using an antibody specific to the E3 ligase or substrate of interest. The choice is left to the end user.

Option 1: Coomassie staining for visualization of Ubiquitinated proteins

1. Carefully disassemble the gel and stain with Coomassie Blue following manufacturers suggestions.
2. As mentioned in the Technical Hints section, some of the E2s are capable of producing free or anchored Poly-Ubiquitin chains in an E3-independent manner. These will also be visualized by Coomassie staining, so care must be taken when interpreting results.
3. See Results Interpretation and Figure 3 for more information.

Option 2: Western Blot analysis for visualization of Ubiquitinated proteins

1. Carefully disassemble the gel and prepare for Western blot analysis. It is left to the end user to choose an appropriate protocol for 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, and primary antibody staining should be done in accordance with the primary antibody manufacturer's recommendations.
2. The primary antibody chosen is left to the investigator. We recommend a high-quality, affinity-purified polyclonal antibody targeting the E3 Ligase or substrate of interest.
3. Enhanced Chemiluminescence (ECL) exposure times will need to be determined experimentally to ensure visualization of the Ubiquitinated E3 Ligase or Substrate of interest.
4. See Results Interpretation for more information.

RESULTS INTERPRETATION

While E2Select data may include Coomassie stain or Western blot visualization of the results, Western Blot analysis is the preferred means, as the data is less ambiguous. In some reactions Coomassie staining will reveal Ubiquitination patterns that are independent of the E3 Ligase or substrate chosen. These bands consist of anchored and/or unanchored Poly-Ubiquitin chains and will not be visualized using the suggested Western Blotting guidelines provided above.

The Sample Data (see Figure 2) shows the Western blot analysis results from an E2Select kit when used to analyze the Ubiquitination of RPN10/S5a substrate using the E3 ligase AIP4/Itch. The Western blot analysis was performed using an anti-S5a primary antibody (R&D Systems, Catalog # AF5540). In Figure 2, the positive control lane ("P") clearly shows significant Ubiquitination of S5a, While the negative control lane has only the unmodified substrate band. When the rest of the reactions are subjected to Western blot analysis, the E2s UBE2D1, UBE2D2, UBE2D3, UBE2D4, UBE2E1, UBE2E3, UBE2L3, and UBE2W2 are also able to promote significant Ubiquitination of S5a by Itch. None of the other E2s from the E2Select kit were able to do this as only unmodified S5a is detected when analyzing those reactions by Western blot. Note: in this example a single antibody (anti-S5a) was used to detect both the experimental and control results, since the same enzyme/substrate pair was used. It is unlikely that the investigator will be able to analyze both unknowns and controls on a single Western Blot unless S5a is chosen as the substrate in the sample reactions.

E2SELECT PLATE CONFIGURATION

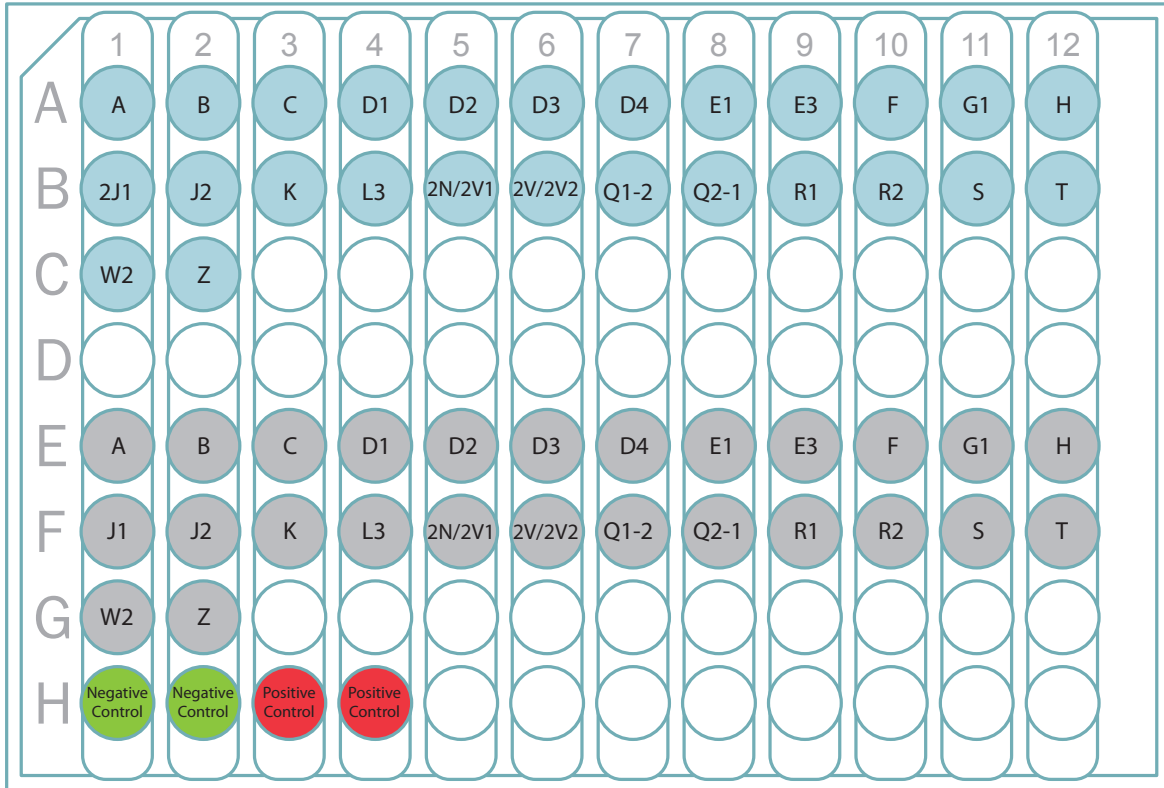


Figure 1: E2 well layout with duplicate wells for each E2, Negative control wells (green), and positive control wells (red). 4 μ L of the indicated E2 enzyme is present in each well as indicated (for example: wells A1 and E1 contain UBE2A, wells B10 and F10 contain UBE2R, etc).

SAMPLE DATA

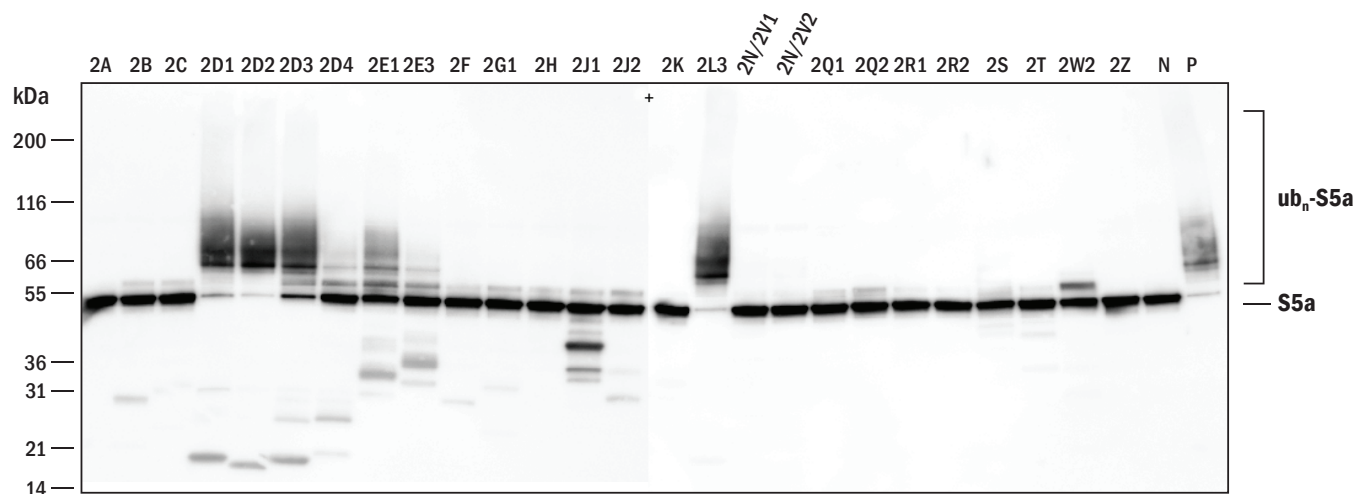


Figure 2: Western blot analysis of E2Select plate reactions looking at Ubiquitination of RPN10/S5a mediated by the E3 ligase AIP4/Itch. *In vitro* reactions using purified E3 Ligase Itch and S5a substrate. Reactions were analyzed by Western Blot Analysis using an anti-S5a primary antibody. **N:** Control lane with S5a, but no ATP. **P:** Positive control reaction showing Ubiquitination of S5a. Note the depletion of the unmodified S5a band and the presence of the high molecular weight smear that is Ubiquitinated S5a. Lanes containing a smear or banding pattern above the unmodified S5a band indicate that the E2 is used by Itch in the Ubiquitination process. In this experiment Itch interacted with UBE2D1, -2D2, -2D3, -2D4, UBE2E1, UBE2E3, UBE2L3, and UBE2W2 to promote S5a Ubiquitination to various extents. In some instances cross-reactivity between the antibody and lower molecular species was observed. This can be minimized by using a lower primary antibody concentration.

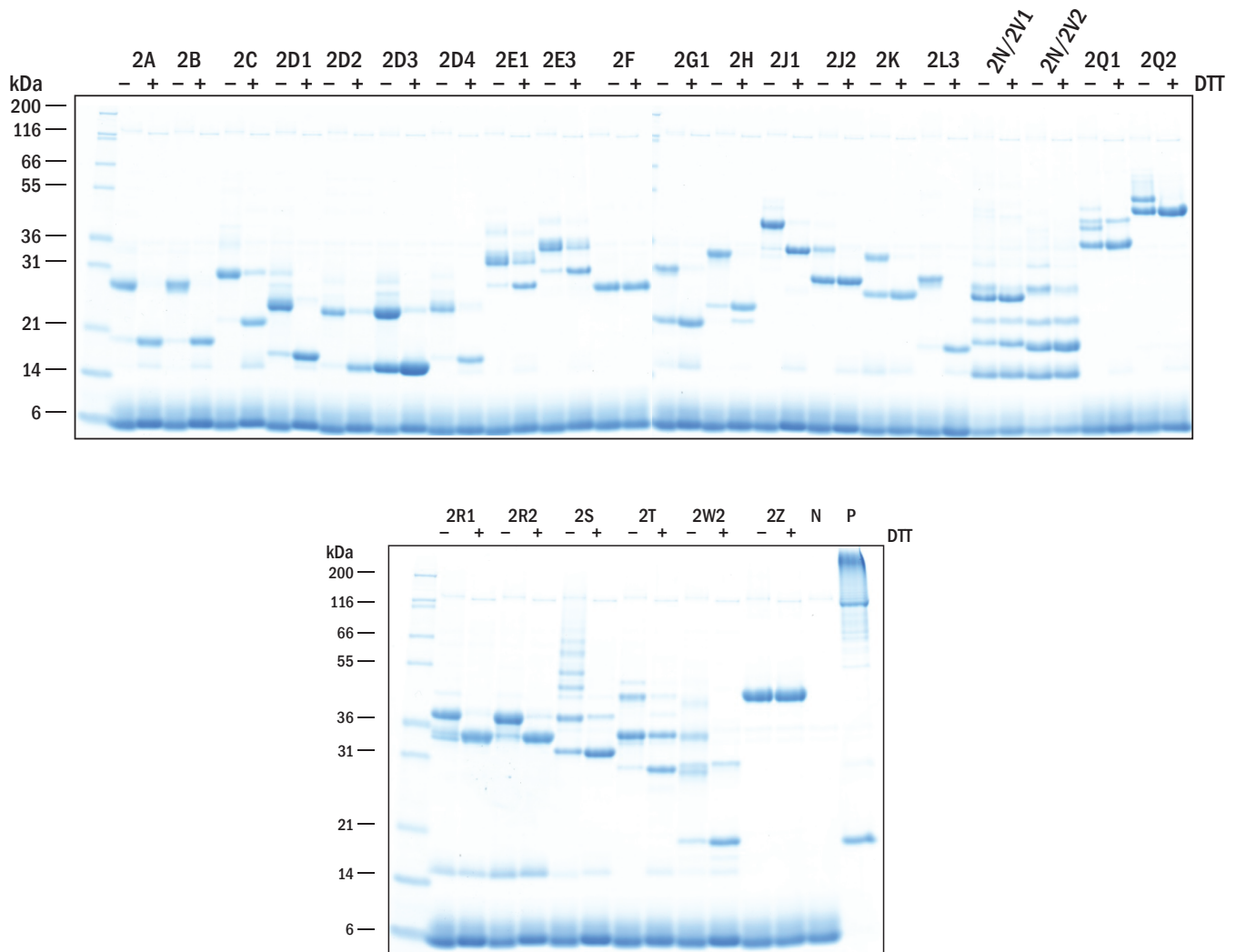


Figure 3: Coomassie analysis of E2Select plate E2s demonstrating their activity by thioester assay. One set of wells on the E2Select plate was used where the Master Mix was added and then initiated with ATP as described in the protocol. No E3 or substrate was added to the Master Mix. For the second set of wells on the same plate, DTT was added to the Master Mix so the final concentration during the reaction was 10 mM. This prevents thioester formation. Coomassie staining as described in option 1 was used to analyze these reactions. All E2s show a thioester shift indicating active E2 enzyme except UBE2F and UBE2Z which generally do not use Ubiquitin as a substrate. The presence of DTT significantly or completely inhibits that thioester shift. Also, as noted in the Technical hints, the following E2s promote free Poly-Ubiquitin chain formation or attach Poly-Ubiquitin chains to themselves: UBE2D1, UBE2D3, UBE2E1, UBE2E3, UBE2N/UBE2V1, UBE2N/UBE2V2, UBE2S, UBE2T, and UBE2W2.

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