**A High Sensitivity Assay of UBE3A Ubiquitin Ligase Activity**

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**Abstract**

UBE3A is an E3 ubiquitin ligase associated with several neurodevelopmental disorders. The development of several preclinical therapeutic approaches involving UBE3A, such as gene therapy, enzyme replacement therapy, and epigenetic reactivation, require the detection of its ubiquitin ligase activity. Prior commercial assays leveraged Western Blotting to detect shifts in substrate size due to ubiquitination, but these suffered from long assay times, noise, and have also been discontinued. Here we develop a new assay that quantifies UBE3A activity. It measures the fluorescence intensity of ubiquitinated substrates with a microplate reader, eliminating the need for Western Blot antibodies and instruments, and enables detection in just 1 hour. The assay is fast, cost-effective, low noise, and uses components with long shelf lives. Importantly, it is also highly sensitive, detecting UBE3A levels as low as 1nM, similar to that observed in human and mouse cerebrospinal fluid. It also differentiates the activity of wild-type UBE3A and a catalytic mutant. We also design a p53 substrate with a triple-epitope tag HIS-HA-CMYC on the N terminus, which allows for versatile detection of UBE3A activity from diverse natural and engineered sources. This new assay provides a timely solution for growing needs in preclinical validation, quality control, endpoint measurements for clinical trials, and downstream manufacturing testing and validation.

**Key words**

Biochemical assay; neurodevelopmental disorder; therapeutic endpoint; biomarker; Angelman Syndrome; Autism Spectrum Disorder; Dup15q Syndrome

**Introduction**

Ubiquitination is a post-translational modification with diverse regulatory functions ranging from protein degradation (Ciechanover, 1998) and gene expression (Yao, 2012) to subcellular localization (Xu, 2011) and kinase activation (Lu, 2009). Three key enzymes are involved in the ubiquitination cascade pathway: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3) (Ebner, 2017). E3 ubiquitin ligases, in particular, modulate various biological and developmental processes (Yang, 2021).

The *UBE3A* gene encodes a specific 100 kDa E3 ubiquitin ligase belonging to the HECT (homologous to E6-AP C-terminus) domain family. *UBE3A* is expressed from both the paternal and maternal alleles of chromosome 15 in most tissues. However, in neurons, paternal *UBE3A* is imprinted and silenced (Knoll et al, 1989). Deletion or loss of activity of the maternally inherited *UBE3A* results in an absence or reduction of UBE3A protein in neural cell types (Ehlen, 2015; Lee, 2018; Sen, 2020; Sirois, 2020; Pandya, 2022; Estridge, 2024), and is linked to autism spectrum disorders (ASDs) and Angelman syndrome (AS) (Vatsa, 2018; Greer, 2010). Its ubiquitin ligase activity has been found to be particularly important (Mishra et al, 2009; Sominsky et al, 2014; Kuslansky et al, 2016; Yi et al, 2017; Kühnle et al, 2018). These findings have motivated several preclinical therapeutic approaches currently being developed for Angelman Syndrome including enzyme replacement therapies (ERT), gene replacement, and epigenetic reactivation (Fink, 2017; Wolter, 2020; Daily, 2021; Judson, 2021; Schmid, 2021; [Nenninger](https://pubmed.ncbi.nlm.nih.gov/?term=Nenninger%20AW%5BAuthor%5D), 2022; Dindot, 2023; Li, 2023; O’Green, 2023).

Given the biological and therapeutic importance of UBE3A, there is a need for research tools to detect the ubiquitin ligase activity of UBE3A for mechanistic studies, as a biomarker, as well as for manufacturing and clinical endpoints measurements. Existing assays rely on Western Blot analysis of size shifts caused by ubiquitination of a substrate (Scott, 2019; [Müller](https://pubmed.ncbi.nlm.nih.gov/?sort=date&term=M%C3%BCller+L&cauthor_id=34057440), 2021; Passos, 2022; Brillada, 2023) or on intracellular luciferase assays (Major, 2007; Weston, 2021) performed in HEK cells. Our goal was to develop a highly controlled in vitro assay that avoids the use of cell-based measurements and Western Blotting, which can introduce substantial noise or pleiotropic effects, and that is fast, sensitive, and cost-effective. The resultant assay possesses high sensitivity that is capable of detecting UBE3A as low as 1 nM. This is at the expected levels of UBE3A found in cerebrospinal fluid where it is expected to be at lower levels than in cells and tissue but is accessible for biomarker and clinical sampling. The assay can also be completed in one hour with a simple plate reader and standard molecular biology equipment.

**Results**

**A plate-reader based assay detects ubiquitin ligase activity of UBE3A**

A central aim of this work was to eliminate the use of Western Blot analysis and replace it with commonly used plate readers. Towards this goal, we developed an initial assay where fluorescent FITC-conjugated ubiquitin is combined with an epitope-tagged substrate, UBE1, UBE2, Mg2+, ATP, and an experimental sample containing UBE3A or no UBE3A (Table 1). If UBE3A is present, it will ligate the FITC-ubiquitin to the substrate. Subsequently, a magnetic bead coated with an antibody against the epitope tag is introduced to pull down the substrate and separate it from free FITC-ubiquitin (Figure 1a). ATP is included, as E1 enzymes activate ubiquitin in an ATP dependent mechanism. p53 is used as the substrate (Scheffner et al., 1993) and is HIS-tagged to facilitate magnetic pulldown.

**Table 1 Assay Components**

|  |  |  |  |
| --- | --- | --- | --- |
|  | Sample (μL) | Negative Control (μL) | Working Concentration or Amount |
| dH2O | 8.8 | 28.8 |  |
| 10\*Rx Buffer | 5 | 5 |  |
| Mg2+ | 5 | 5 | 10 mM |
| ATP | 5 | 5 | 10 mM |
| UBE1 | 1.2 | 1.2 | 100 nM |
| UBE2 | 0.5 | 0.5 | 1 μM |
| UBE3A | 20 | / | 200 nM |
| HIS-FLAG-p53 | 4 | 4 | 5 μg |
| Ubiquitin-fluorescein | 0.5 | 0.5 | 1 μM |

To test this assay, samples with or without 200 nM UBE3A were incubated in the reaction mixture at 37 °C for 60 minutes followed by addition of anti-HIS magnetic beads to pull out p53. The fluorescence intensity of the supernatant, as well as the subsequent elution of the p53 off the magnetic bead, were quantified by a fluorescence plate reader (Fig. 1b). As expected, when UBE3A was present, most of the FITC-ubiquitin was ligated to p53 and appeared in the elution while there was little free FITC-ubiquitin remaining in the supernatant. Both the reaction and elution buffers exhibited minimal background fluorescence. We also verified the pull-down assay with an SDS-PAGE analysis visualizing the FITC-ubiquitinated p53 only in elution of the sample containing UBE3A (Fig. 1c).

**Inclusion of HPV-E6 and reducing reaction volumes improves assay efficiency**

Biochemical assays are often expensive due to the use of recombinant proteins and specialty chemicals. We therefore sought to increase the efficiency of the assay through three modifications. First, we asked if HPV-E6 could increase the sensitivity of the assay. E6 (Early protein 6) is a viral protein produced in cells infected with the Human Papillomavirus. E6 forms a complex with the host cell E6AP generating a ligase activity that polyubiquitinates target substrates (Masuda et al, 2019). When included in the reaction, HPV-E6 did indeed boost the assay signal by nearly fivefold compared to a sample with the same concentration of UBE3A but lacking HPV-E6 (Fig. 2a). Second, we then asked if the volume of the reaction could be scaled down to preserve reagents. We found that the reaction could be scaled down from 50 μL to 25 μL or 10 μL, both preserving the sensitivity of the assay (Fig. 2b). Finally, we asked if another substrate could provide sensitivity to UBE3A activity. As FADD is another known substrate of the E6/E6AP complex, we compared its level of ubiquitination to p53 but found it was substantially weaker (Fig. 2c). Consequently, we employed p53 as the substrate for all subsequent assays.

**p53 with multiple epitope tags provides assay versatility**

In the assay, we used commercially available HIS-tagged p53, and the HIS tag to pull out the substrate after the ubiquitination reaction. However, UBE3A protein generated by different therapeutic modalities including for enzyme replacement therapy or gene therapy, may include epitope tags as well which could cross react with an identical epitope used for pulldown of the substrate. We therefore designed a custom p53 substrate with three epitope tags, HIS-HA-CMYC, appended to the N-terminus of p53. This version of substrate could be universally used by researchers by choosing antibodies and magnetic beads that bind an epitope present on p53 but not on any engineered UBE3A of interest. We tested that this substrate maintains activity in the assay when using anti-HIS, anti-HA, or anti-CMYC magnetic beads to pull out p53 following a ubiquitination reaction. All three epitope tags worked well (Fig. 3). The anti-CMYC assay yielded slightly better signal but costs three times that of anti-HIS beads. We therefore used anti-HIS beads for most of the remaining work except in cases where UBE3A contained a HIS tag.

**Optimization of assay conditions for different target UBE3A concentrations**

Users may have different applications ranging from testing recombinantly produced UBE3A to assaying UBE3A activity within biological samples. These applications could have substantially different demands on assay sensitivity. We therefore first profiled the assay at four different reaction incubation times (5, 30, 60, 90 minutes) and at four concentrations of UBE3A (1, 10, 25, 50 nM). The reaction was stopped by putting reaction tubes on the ice after the ubiquitination reaction and incubating with the magnetic beads at 4 °C. The signal from 1, 10 and 25 nM UBE3A increased with extended incubation time, while 50nM UBE3A saturated at 60 minutes (Fig. 4a).

In some applications like assaying recombinant UBE3A protein, speed would be preferable and there would not be a substantial limit on the amount of UBE3A available for testing. We tested what concentrations could be detected by the assay with only a 5 minute reaction incubation and found 50 nM UBE3A and higher could be detected as statistically different from the negative control (Fig. 4b). Including the other steps, the entire assay could then be completed in 1 hour. In other applications, the amount of UBE3A protein may be low. We therefore performed a series of optimizations, finding that a 60 minute incubation was only able to distinguish 50 nM UBE3A and higher (Fig. 4c) and a 90 minute incubation could detect 10 nM UBE3A (Fig. 4d). We further extended the incubation time to 120 minutes and bound the magnetic beads at room temperature for 60 minutes instead of at 4 °C for 30 minutes (Fig. 4e); these two changes enabled detection of 1 nM UBE3A. Lastly, we tested whether the assay could detect a mutant of UBE3A with impaired catalytic activity (R482P) (Weston et al, 2021) using one of these conditions. Using a 5 minute incubation with 1 μM UBE3A, the assay was able to distinguish the wildtype from R482P mutant.

**Discussion**

In this study, we developed a new UBE3A detection assay that is quantitative, versatile to diverse sensitivity ranges and engineered UBE3A proteins, and rapid and facile to perform. It uses widely available microplate readers to detect fluorescence signals. It aims to replace Western Blot assays that require more equipment and multiple lengthy processing steps, and that yield often noisy results. This assay is also fast and cost-effective (Table 2).

**Table 2 Technical Differentiators**

|  |  |  |
| --- | --- | --- |
|  | **Previous Assays** | **This Assay** |
| **Separation Method** | Western Blot | Magnetic Pull-down |
| **Detection Method** | SDS-PAGE, Gel transfer, Membrane blocking, Antibody staining, ECL detection | Standard magnetic rack, microplate reader |
| **Time Needed** | >20 h | 2-3 h |
| **Sensitivity** | Not specified, but typically poor and variable | 1 nM UBE3A |
| **Resolution** | Not specified, but typically poor and variable | Can distinguish WT and catalytically-impaired mutant |
| **Cost per reaction** | $50 | $10 |
| **Shelf Life** | ~ 6 months | ~ 12 months |

Another highlight of this assay is its exceptional sensitivity. Following optimization, it can detect UBE3A levels as low as 1 nM, the concentration expected in human cerebrospinal fluid CSF. Dodge and colleagues estimated the amount of UBE3A protein in human CSF to be ~100 ± 7 ng/mL (Dodge, 2021), which corresponds approximately to 1 nM. CSF sampling is of potential importance, as it represents the only widely applicable methodology for obtaining information on free drug concentrations within the individual human brain (Lange, 2013) and could therefore be used for assaying therapeutic responses and biomarker levels in clinical studies related to dysregulated UBE3A including for Angelman Syndrome, Dup15Q, and Autism Spectrum Disorder. The versatility of the system across low and high concentration ranges, and the ability to work with various engineered UBE3A proteins also provides potential utility for research in cell biology, neuropharmacology, and neuroscience.

**Materials and Methods**

**Cloning, expression and purification of recombinant proteins**

The E.coli codon-optimized gene coding for HIS-HA-CMYC-P53 was ordered from Twist and subcloned into pET (Addgene 29711) via ligation. Two gene fragments of wild type UBE3A isoform I were assembled into the pET vector with N-terminal Sumo and 6xHIS tags. UBE3A(R482P) was created using site-directed mutagenesis. Proteins were produced in BL21(DE3) cells and purified by FPLC IMAC cartridges. The eluted proteins were concentrated using Amicon Ultra filters (30 kDa and 100 kDa MW cutoff separately) and stored at -80°C. Proteins were analyzed via SDS-PAGE.

**Ubiquitin conjugation and pull-down assay**

In low-bind microcentrifuge tubes, each component was added in the indicated order and concentration listed in Table 1. 1 μM HPV-E6 was added after UBE3A. Negative control reactions were prepared by replacing UBE3A with dH2O. After a brief centrifugation at 2000g, the mixture was incubated in a 37 °C water bath for 5 minutes to 2 hours. The reaction was terminated by placing the reaction samples on ice unless otherwise described. Reaction mixtures were then incubated with pre-washed HisPur Ni-NTA magnetic beads (Fisher 88831) or anti-CMYC (Fisher 88842) or anti-HA (Fisher 88836) beads in Equilibration Buffer (25 mM HEPES, 0.15M NaCl, 0.25% Tween-20 Detergent) at 4 °C for 30 min with mixing, except where specified in the text at room temperature for 60 min. Supernatants were removed and saved for subsequent fluorescence detection. The beads were washed twice with Wash Buffer (25 mM HEPES, 0.15M NaCl, 0.25% Tween-20 Detergent, 20 mM Imidazole), and ubiquitinated p53 was eluted off the beads with Elution Buffer (25 mM HEPES, 0.15M NaCl, 0.25% Tween-20 Detergent, 200 mM Imidazole). The fluorescence intensity of the supernatants and elutions were quantified on a TECAN plate reader. Anti-HA and anti-CMYC magnetic beads used 1 x TBS-T (25 mM Tris, 0.15M NaCl, 0.25% Tween-20 Detergent) as the Equilibration Buffer, 5 x TBS-T (125 mM Tris, 0.75M NaCl, 1.25% Tween-20 Detergent) as the Wash Buffer, and 50 mM NaOH as the Elution Buffer.

**SDS-PAGE**

Proteins were mixed with reducing reagent and sample loading buffer. After incubation at 95 °C for 5 minutes, the samples were loaded into a Bis-Tris gel for SDS-PAGE analysis. Gel staining followed the Imperial Protein Stain protocol (Fisher PI24615). The elution and supernatant samples were mixed with reducing reagent and sample loading buffer. The mixtures were incubated at 95 °C for 5 minutes, after which they were loaded onto a Bis-Tris gel for SDS-PAGE analysis. The gel was inspected with a UV transilluminator.

**Competing Interests**

A technology disclosure to North Carolina State University has been filed listing LH and AJK as inventors.

**Funding**

Research reported in this publication was supported by the Foundation for Angelman Syndrome Therapeutics (FAST) under Award Numbers FT2022-008, FT2020-003, and FT2021-002.

**Acknowledgements**

We thank Allyson Berent and Jennifer Panagoulias for helpful insights into the assay needs of putative therapies.

**Figure Captions**

**Figure 1. A plate-reader based assay detects ubiquitin ligase activity of UBE3A**

(a)Schematic of assay. (b) Plate reader detection of FITC fluorescence for the elution and supernatant from the assay using 200 nM UBE3A. (c) SDS-PAGE visualization of FITC-ubiquitin conjugated to p53 appearing only in the elution of the sample with UBE3A added. N=4 separate replicate reactions. Error bars represent 95% confidence intervals. \*\*p<0.01 relative to sample without UBE3A. ANOVA with Tukey-Kramer post-hoc.

**Figure 2. Inclusion of HPV-E6 and reducing reaction volumes improves assay efficiency**

(a) 1 μM HPV-E6 boosts ubiquitination by almost five folds. (b) Reduction in reaction volume maintains assay sensitivity. (c) FADD presents inferior sensitivity as a substrate to p53. UBE3A levels were 200 nM for all experiments in this figure. N=3 separate replicate reactions. Error bars represent 95% confidence intervals. \*\*p<0.01, \*p<0.05 relative to sample without UBE3A. ANOVA with Tukey-Kramer post-hoc in panel (a). Two-tailed t-test comparison to sample without UBE3A in panels (b) and (c).

**Figure 3. p53 with multiple epitope tags provides assay versatility**

(a) SDS-PAGE gel indicating successful production of a p53 substrate with triple HIS-HA-CMYC epitope tags. (b) The assay performs similarly when using magnetic beads pulling down the p53 substrate using each of the three epitope tags. N=3 separate replicate reactions. Error bars represent 95% confidence intervals. \*\*p<0.01, Two-tailed t-test relative to sample without UBE3A.

**Figure 4. Optimization of assay conditions for different target UBE3A concentrations**

(a) Fluorescence signal from the assay performed with four incubation times and five concentrations of UBE3A. Fluorescence signal from the assay performed with (b) 5 minute, (c) 60 minute, and (d) 90 minute incubation times, with incubation of the magnetic beads at 4 °C for 30 minutes. (e) Fluorescence signal from the assay performed with a 120 minute incubation time as well as incubation of the magnetic beads at room temperature for 30 minutes. (f) The assay detects lower ubiquitination by the R482P UBE3A mutant, with assay conditions of 1 μM UBE3A and 5 minutes incubation. N=3 separate replicate reactions. Error bars represent 95% confidence intervals. Samples with the same letters are not statistically significant from each other. Otherwise, p<0.05 relative to samples without the same letter. ANOVA with Tukey-Kramer post-hoc.

**Table Captions**

**Table 1.** Concentrations of assay components in the order they were added to the reaction mixture. The concentration of UBE3A varies according to the experiment performed, but the concentrations of the other components remain the same. 1 μM HPV-E6 was also included in experiments in Figure 2-4 and was added directly after UBE3A was added.

**Table 2.** Technical differentiators of this assay compared to western blot assays.

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