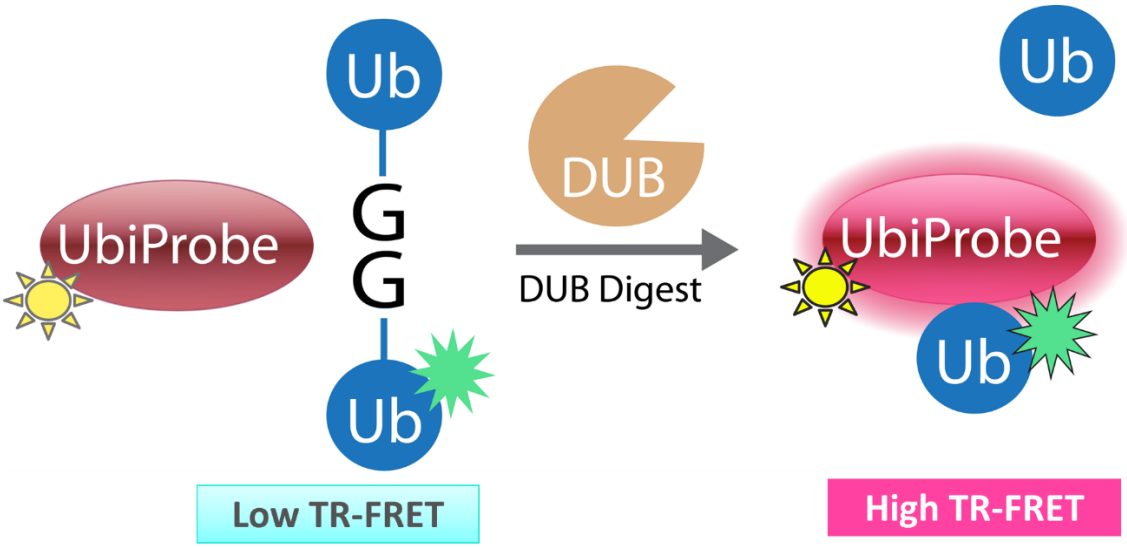
MANUAL

Ubiprobe: K48 Di-Ub DUB Assay Kit

Catalog Number: DU500



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Ubiprobe: K48 Di-Ub DUB Assay Kit

Cat. # DU500

BACKGROUND

The Ubiprobe assay consists of a designed Di-Ubiquitin (Di-Ub) fluorophore labeled as a reporter substrate, along with a ubiquitin binding reagent called "UbiProbe" serving as a detection probe. This arrangement generates a TR-FRET signal indicative of Deubiquitinase (DUB) enzyme activity. In the absence of DUB or in presence of DUB inhibitors, the uncleaved Di-Ub reporter substrate is considered inactive, leading to low TR-FRET signal. Upon cleavage of the Di-Ub-reporter substrate by the DUB, the cleaved ubiquitin interacts with UbiProbe, producing a TR-FRET signal. This coupled assay utilizes the signal generated via the cleavage of the Di-Ub substrate as a quantitative measure of isopeptidase activity in a homogeneous high-throughput format. Different Di-Ub reporter substrates, available with M1, K6, K11, K33, K48, and K63 linkages, allow for demonstration of selectivity and evaluation chain-specific DUBs.

SUGGESTED USES:

1. Real-time monitoring of DUB activity against DiUbiquitin substrates.
2. Profiling and validation of DUB inhibitors or activators.
3. High throughput screening (HTS) to identify DUB inhibitors.
4. Selectivity studies and DUBTACs screening.
5. Mechanism of action studies of DUB inhibitors or activators

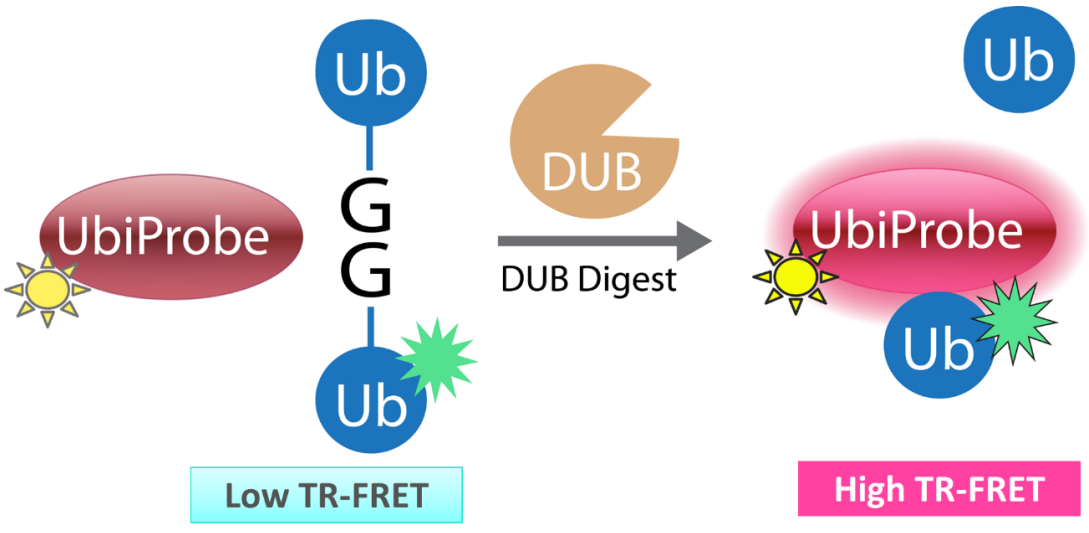


Figure 1. Schematic of UbiProbe DUB Assay. Cleavage of DiUb substrate by DUB results in high TR-FRET signal.

COMPONENTS

Store all materials at -80°C, avoid cycles of freezing and thawing. All components are stable for at least 2 months.

1. **10X Assay Buffer**
   Size: 1.0 mL (10X)
   Note: Add β-mercaptoethanol fresh to final concentration of 1mM in 1X assay buffer.

2. **UbiProbe**
   Size: 1x 18µl (125X, 5µM)

3. **K48-DiUb^{Alexa647}**
   Size: 1x 18µl (125X, 5µM)

4. **MonUb^{Alexa647}**
   Size: 1x 5µl (250X, 5µM)

5. **Positive Control DUB, USP2core**
   Size: 1x 5µl (400X, 20µM)

ADDITIONAL ITEMS REQUIRED BUT NOT INCLUDED IN THE KIT

1. Deubiquitinase of your choice. DUBs can also be purchased separately from Lifesensors Inc.
2. Centrifuge with microplate adaptor.
3. Multichannel pipettes suitable for dispensing 0.5µl and 5µl volumes.
4. 384 shallow-well black assay plate such as NUNC Catalog # 267461.
5. β-Mercaptoethanol (BME).
6. Streptavidin Europium Chelate W-1024 (Sa-Eu) purchased from Columbia Biosciences (Cat# D17-2212-50).
7. TR-FRET capable plate reader with UV (320nm +/- 20nM), 620nm, and 665nm optical filters.
8. 96 or 384 well polypropylene plate for preparing compound dilutions.
9. Plate seals (optional).

IMPORTANT NOTES

1. ***Do not add 10X assay buffer directly to DUB, UbiProbe, Di-Ub Substrate or Streptavidin Europium.***

2. ***After thawing the reagents, avoid making small volume (for example 2 µl) aliquots of UbiProbe and Di-Ubiquitins for freezing at -80°C. Components have been validated for activity after multiple freeze-thaw cycles.***

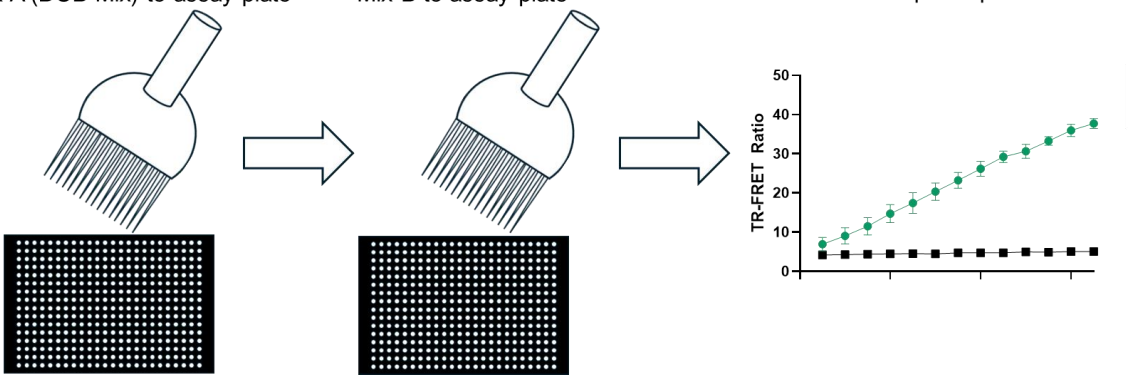
3. ***Streptavidin Europium should be stored according to manufacturer recommendations, typically at 2°C - 8°C.***

ASSAY SUMMARY

Step 1: Dispense 5 µl per well Mix A (DUB Mix) to assay plate

Step 2: Dispense 5 µl per well Mix B to assay plate

Step 3: Read Assay Plate with TR-FRET capable plate reader.



ASSAY SETUP

– See Table 1 for example concentrations and volumes.

1. ***Prepare 1x Assay buffer***. Dilute 10x assay buffer in water and add β-Mercaptoethanol (BME) to a final concentration of 1.0mM. For example, 1mL 10x assay buffer in 9mL water with 0.7 µl BME (14.4M). If testing compounds, dispense 0.5 µl dimethylsulfoxide (DMSO) or compounds at a 21x concentration to assay plate.
   Note. A reducing agent must be added to the assay buffer for getting optimum DUB activity. DTT (5 to 10mM) can be used as an alternate reducing agent.
2. ***Prepare Mix A***: Prepare Mix A by diluting your deubiquitinase (DUB) at a 2x screening concentration in 1x assay buffer. For example, to set up USP2Core assay at a final concentration of 5nM, prepare 2x concentration at 10nM of USP2Core in required volume using 1x assay buffer.
3. Dispense 5µl of Mix A per well to the assay plate. Include blank control wells and positive control wells to ensure that the assay is working properly.
   a. For a blank control (-DUB), dispense 5µl of assay buffer into corresponding wells.
   b. For a positive control, prepare a 40nM solution of MonoUb^{Alexa647}. Dispense 5µl/well. The MonoUb^{Alexa647} will bind UbiProbe and generate a high TR-FRET signal.
4. Briefly centrifuge assay plate for ~30 seconds at ~2000 rpm. If testing compounds, the DUB may be preincubated with compound for 30min at room temperature. Seal or cover the assay plate until the next step.
5. ***Prepare Mix B*****:** Prepare Mix B by diluting UbiProbe, K48-DiUb^{Alexa647}, and Streptavidin Europium in assay buffer at 2x concentrations. For example, prepare a 2x mix containing 40 nM UbiProbe, 40 nM K48-DiUb^{Alexa647} substrate and 2 nM Streptavidin Europium in 1x assay buffer. Dispense 5 µl per well to the assay plate.
6. Briefly centrifuge assay plate for ~30 seconds at ~2000 rpm.

7. Read the assay plate in a plate reader capable of measuring TR-FRET signal. See table 2 for suggested plate reader parameters. For DUBs that are highly active or DUBs at high concentration (for example, >100nM), assay kinetics may be very rapid. It is recommended to titrate the DUB concentration to find an appropriate DUB concentration that shows linear assay kinetics.

EXAMPLE CONCENTRATIONS AND VOLUMES

Table 1. Example Volume calculations for a single full 384-well plate

Mix A (2.25ml)	2x Concentration	Volume	Final concentration
Assay Buffer	1x	2.25ml	1x
USP2c (20µM)	10nM	1.13 µl	5nM
Positive TR-FRET Control (125µl)			
Assay Buffer	1x	125µl	1x
Mono-Ub^{Alexa647} (5µM)	40nM	1 µl	20nM

Mix B (2.25ml)	2x Concentration	Volume	Final concentration
Assay Buffer	1x	2.25ml	1x
UbiProbe (5µM)	40nM	18 µl	20nM
K48-DiUbiquitin-Ub^{Alexa647} (5µM)	40nM	18 µl	20nM
Streptavidin Europium (1.85µM)	2nM	2.43µl	1nM

PLATE READER SETTINGS

Table 2. Suggested plate reader parameters for Time Resolved-FRET detection

Excitation	Wavelength	Integration Time Start	Integration Time Stop
Excitation	320nm, ± 10nm		
Channel 1 Emission	620nm, ± 10nm	60µs	400µs
Channel 2 Emission	665nm, ± 10nm	60µs	400µs

REPRESENTATIVE DATA

1. Validation of USP2core as a pan-linkage specific DUB using UbiProbe kits

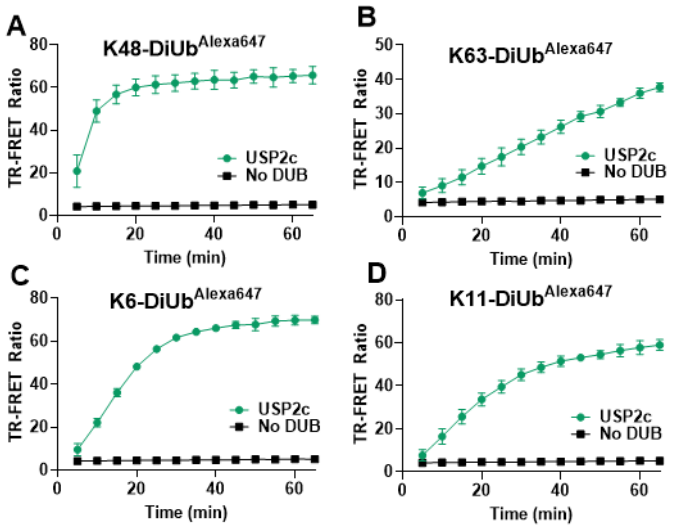


Figure 2. Robust USP2 activity obtained using UbiProbe assay with various DiUb substrates. USP2Core (5nM) was incubated with K48-DiUb (**A**), K63-DiUb (**B**), K6-DiUb (**C**) and K11-DiUb (**D**) and TR-FRET was measured for 65 minutes at 5 min intervals. DUB activity with each of the Di-ubiquitin probe represented as TR-FRET ratio and compared to negative control (-DUB). The TR-FRET signal was acquired using BMG Labtech ClarioStar. The error bars represent standard deviation, n=4.

2. Validation of OTUB1 as a K48-linkage specific DUB using UbiProbe kits

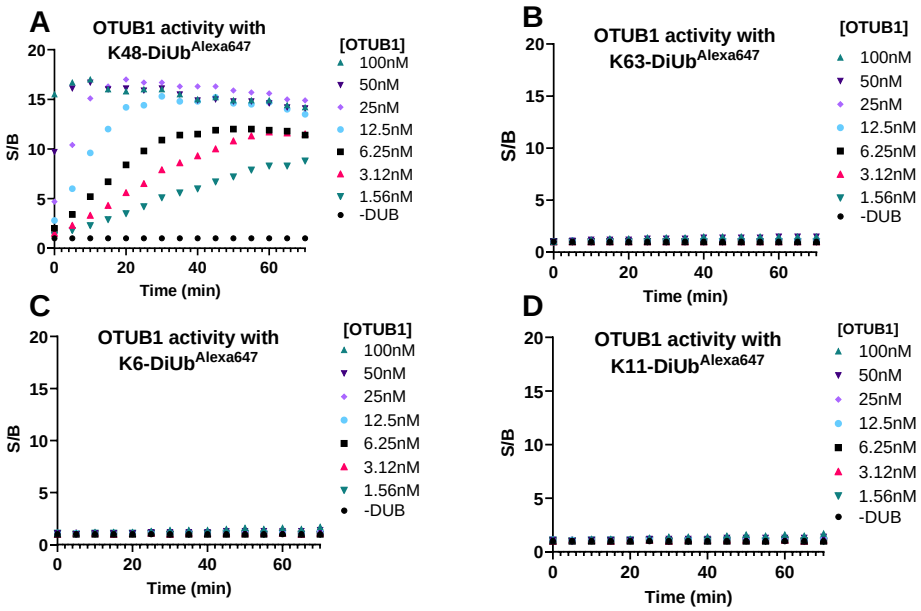


Figure 3. **Validation of ubiquitin chain linkage specificity of OTUB1 using UbiProbe assay.** All tests were carried out as suggested in the manual with OTUB1 and UbiProbe Kit (Lot-DB-48456.001, DA-48505.001). OTUB1 was tested in 7 point two-fold dose response starting at 100nM. The signal acquired using BMG Labtech ClarioStar in a kinetic measurement for 65 minutes with 5 min intervals. Activity is shown as signal-to-background relative to –DUB control. OTUB1 shows activity only with K48- **(A)** with >10-fold S/B with 1.56nM OTUB1. and do not show activity with a 10-fold s/b is observed even with 1.56nM OTUB1. No activity was observed when using K63-DiUb **(B),** K6-DiUb **(C)** and K11-DiUb **(D).**

REFERENCES

1. Hershko, A. and A. Ciechanover, The ubiquitin-proteasome pathway. *Annu Rev Biochem*, 1998. **67**: p. 425-479.
2. Schauer NJ, Magin RS, Liu X, Doherty LM, Buhrlage SJ. Advances in Discovering Deubiquitinating Enzyme (DUB) Inhibitors. J Med Chem. 2020 Mar 26;63(6):2731-2750. doi: 10.1021/acs.jmedchem.9b01138. Epub 2019 Nov 20. PMID: 31682427.
3. Mevissen TET, Komander D. Mechanisms of Deubiquitinase Specificity and Regulation. Annu Rev Biochem. 2017 Jun 20;86:159-192. doi: 10.1146/annurev-biochem-061516-044916. Epub 2017 May 12. PMID: 28498721.

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