

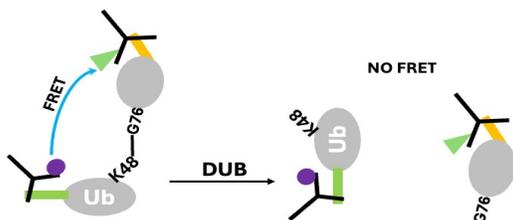
RELAY^{TR} K48 Linkage-specific Deubiquitinase Assay Kit I

(Catalog # T5210, 200 x 20 µl reactions)

Description

RELAY^{TR} K48 Linkage-specific Deubiquitinase Assay Kit I is based on the TR-FRET technology that offers a simple, sensitive, homogeneous, and stable assay to measure the activity of deubiquitinases (DUBs) that cleave K48-linked polyubiquitin chains. Each ubiquitin moiety in K48-linked diubiquitin (diUb) contains an HA tag or a DYKDDDDK tag, generating a robust FRET signal when binding with fluorescein-labeled anti-HA and terbium-labeled anti-DYKDDDDK antibodies. The mix of each individual ubiquitin is included as a negative control. The assay is high throughput compatible with a Z' value > 0.87.

Assay Principle - Detecting the decrease of FRET signal when diUb is cleaved



Implications

- 1) DUB activity or profiling assay
- 2) Hit/lead compound discovery and validation.

Components in the kit*

Components	Quantity
• 100X K48-linked Diubiquitin (DiUb, 1 µM)	40 µl
• 100X Monoubiquitin Mix (MonoUb Mix, 1 µM)	40 µl
• 100X RELAY ^{TR} Fluorescein-anti-HA/Terbium-anti-DYKDDDDK Antibody Mix (Ab Mix)	40 µl
• 10X Deubiquitination Buffer	1.25 ml
• 100X USP21 (1 µM, a positive DUB control)	20 µl
• Iodoacetamide (IAA, reaction stopper)	25 mg

* Dilution factor is referred to the final reaction concentration of each component.

* Aliquot components if needed. Use liquid nitrogen to snap freeze proteins.

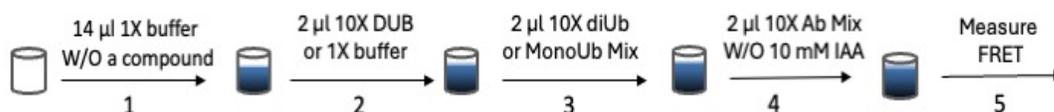
Materials not included

Plate reader capable of measuring terbium/fluorescein-based FRET; Low volume and non-binding 384-well black plate; Single and multi-channel pipets and tips; Sample dilution plate; Clear plate sealing film; Microfuge tubes; Plate shaker, MilliQ water

A general guide using RELAY^{TR} K48 Linkage-specific Deubiquitinase Assay Kit

USP21 is used to evaluate the kit by performing a concentration titration assay (see below). In general, user should test DUB concentration, reaction time, and instrument setting (such as gain, delay and integration times) to optimize an assay. For compound screening/validation assays, reaction should be at the steady state with ~40-80% of diUb being cleaved and the corresponding Z' value > 0.5.

[Reaction Schematic - 20 µl/well]



[Plate Layout]

No FRET background: MonoUb Mix + the highest USP21 concentration, A1-H1

100% of FRET signal: DiUb, A3-H3

USP21 concentration titration reactions: DiUb + USP21, 4 repeats for each USP21 concentration (wells 5-8), high to low USP21 concentration from row A to H.

	MonoUb Mix + USP21		DiUb		diUb + USP21 (four repeats for each USP21 concentration from Well 5 - Well 8)			
	1	2	3	4	5	6	7	8
A	↓		↓		→	→	→	→
B					→	→	→	→
C					→	→	→	→
D					→	→	→	→
E					→	→	→	→
F					→	→	→	→
G					→	→	→	→
H	↓		↓		→	→	→	→

[Plate Reader Setup]

- User should use the kit to adjust plate reader settings to achieve a desirable signal-to-background (S/B) ratio (see Data Processing below). The terbium and fluorescein pair can be excited at 320-340 nm with dual emission detections centered at 490 nm and 520 nm. A delay time of 50 - 100 µs with the integration time of 200 - 400 µs is often used. The number of flashes should follow manufacturer's recommendation. The 100% of FRET signal is the emission ratio of samples with diUb + Ab Mix. The no FRET background is the emission ratio of samples with monoUb Mix + Ab Mix. The S/B ratio is ~10 in our tests.

[Reagent Preparation]

2. Thaw kit components and chill 5 ml MilliQ water on ice.
Note: Diluting each component is necessary. Do not stock diluted components for future use.
3. *Preparation of 1 ml 1X Deubiquitination Buffer (referred to as 1X buffer).* In a 1.5 ml microfuge tube, add 100 μ l 10X Deubiquitination Buffer and 900 μ l cold MilliQ water. Invert the tube to mix well. Keep under room temperature.
4. *Preparation of 20 μ l 10X MonoUb Mix.* In a 1.5 microfuge tube, add 2 μ l 10X Deubiquitination Buffer, 16 μ l cold milliQ water, and then 2 μ l 100X MonoUb Mix, tab the tube to mix well. Keep under room temperature.
5. *Preparation of 100 μ l 10X K48-linked DiUb.* In a 1.5 microfuge tube, add 10 μ l 10X Deubiquitination Buffer, 80 μ l cold milliQ water, and then 10 μ l 100X K48-linked DiUb, tab the tube to mix well. Keep under room temperature.
6. *Preparation of 120 μ l 10X Ab Mix.* In a 1.5 microfuge tube, add 12 μ l 10X Deubiquitination Buffer, 96 μ l cold milliQ water, and then 12 μ l 100X Ab Mix, tab the tube to mix well. Keep on ice.
7. *(Optional) Preparation of 120 μ l Ab Mix with 10 mM IAA only when DUB inhibition is planned during antibody incubation.* Add 1.35 ml milliQ water into 25 mg IAA powder, vortex to dissolve. This is 100 mM IAA stock (10X IAA). In a 1.5 microfuge tube, add 12 μ l 10X Deubiquitination Buffer, 84 μ l cold milliQ water, 12 μ l 10X IAA, and then 12 μ l Ab Mix, tab the tube to mix well. Keep on ice.
8. Preparation of 10X USP21 serial dilutions at 6400/3200/1600/800/400/200/100/50 nM. Making the highest concentration at 6400 nM first, then make 2X serial dilution using a microtiter plate. Keep the stocks under room temperature.

Note: Provided USP21 in the kit serves as a positive DUB control for a 10 nM USP21 reaction.

	2X serial dilution	10X [C], nM		
A	50 μ l in 1X buffer	6400		
B	20 μ l A +20 μ l 1X buffer	3200		
C	20 μ l B +20 μ l 1X buffer	1600		
D	20 μ l C +20 μ l 1X buffer	800		
E	20 μ l D +20 μ l 1X buffer	400		
F	20 μ l E +20 μ l 1X buffer	200		
G	20 μ l F +20 μ l 1X buffer	100		
H	20 μ l G +20 μ l 1X buffer	50		

[Reaction Setup]

9. Add 14 μ l 1X buffer into A1-H1, A3-H3, A5-H5, A6-H6, A7-H7, and A8-H8. This is STEP 1 in Reaction Schematic.
Note: 1) Use a multichannel/repetitive pipette to reduce sample addition error. 2) In compound screening assays, add 0.5 μ l chemical at 40X in DMSO into each well, then add 13.5 μ l 1X buffer and mix well. For control reactions without a chemical, use 0.5 μ l DMSO. The final DMSO concentration should be no more than 2.5%.
10. In A1-H1, add 2 μ l 10X USP21 at 6400 nM.

In A3-H3, add 2 μ l 1X buffer.

In A5-A8, add 2 μ l 10X USP21 at 6400 nM. In B5-B8, add 2 μ l 10X USP21 at 3200 nM, and so on. Use a plate shaker to mix at 300 rpm for 2 min. This is STEP 2 in Reaction Schematic.

Note: 1) Add either 2 μ l the highest USP21 concentration or 1X buffer (no DUB) in negative control reactions in A1-H1. 2) In compound screening assays, once DUB being added and mixed well, incubate under room temperature for at least 15 min to allow DUB to interact with compounds.

11. In A1-H1, add 2 μ l 10X MonoUb Mix prepared in STEP 4.

In A3-H3, A5-8, B5-8, C5-8,, H5-8, add 2 μ l 10X DiUb prepared in STEP 5.

Use a plate shaker to mix at 300 rpm for 2 min. Incubate 40 min under room temperature. This is STEP 3 in Reaction Schematic.

Note: Incubation time and DUB concentration should be optimized for a specific assay. Seal the plate with a sealing film if necessary, especially when incubation time is more than 1 hour.

12. Add 2 μ l 10X Ab Mix prepared in STEP 6 into each well. Mix well by shaking the plate at 300 rpm for 2 min. Incubate 30 min under room temperature. This is STEP 4 in Reaction Schematic.

Note: DUB can still cleave diUb during antibody binding incubation. IAA can be added to inhibit most DUBs if needed (see STEP 7).

13. Transfer the plate to a plate reader to record FRET signals. We use a PHERAstar FS plate reader with the 337nm/520nm/490nm filter. Integration starts at 50 μ s and integration time is 400 μ s. This is STEP 5 in Reaction Schematic.

[Data Processing]

14. TR-FRET signal is calculated as the product of the ratio of acceptor emission intensity at 520 nm to donor emission intensity at 490 nm multiplies a "convenience constant" of 10^4 . FRET ratio (R) = $(\text{Fluorescence Intensity}_{520\text{nm}} / \text{Fluorescence Intensity}_{490\text{nm}}) \times 10^4$.

15. Signal-to-background (S/B) ratio is calculated using the formula of $(R_{\text{signal}} - R_{\text{background}}) / R_{\text{background}}$. The S/B ratio of reactions with diUb (no DUB) is referred to as 100% of FRET signal, in which the average R of reactions with diUb (no DUB) or with monoUb and the highest USP21 concentration are considered as R_{signal} and $R_{\text{background}}$, respectively. S/B ratios of reactions with diUb and USP21 are calculated and referenced to the 100% FRET signal (see results below). Error bars represent S.D. of four repeats.

