ITCH E3 Ligase TR-FRET Kit

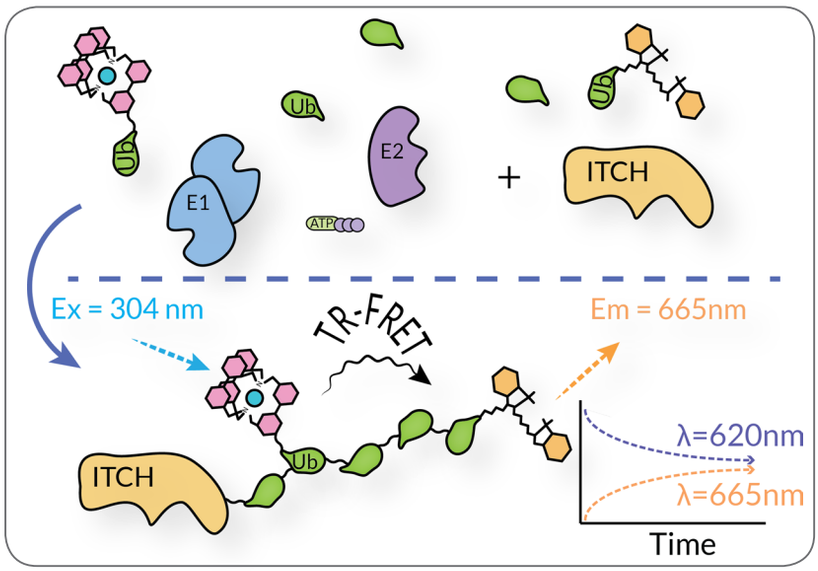
Cat. No. SBB-KF0035
Lot. No. 172440035

South Bay Bío

Introduction

South Bay Bio's ITCH E3 Ligase TR-FRET Kit provides a fast and sensitive method monitoring ubiquitin conjugation onto ITCH in solution, resulting from an enzymatic ubiquitin cascade without the need of running and staining an SDS gel. The kit enables continuous TR-FRET detection of ubiquitin chain formation onto ITCH in a real-time detection setup, or in an end-point configuration if desired. TR-FRET uses the extended fluorescence emission decay lifetimes typical of rare-earth lanthanides to impart a short time-delay between FRET donor excitation and emission. This delay provides a means to separate "true" signal from short-lived background fluorescence, and reduce interference from compound fluorescence and other assay artifacts.

The kit uses ubiquitin labeled with either Europium-Cryptate or Cyanine5 as FRET pair donor and acceptor fluorophores respectively, completely eliminating the need for antibody based detection setups. Enzymatic incorporation of the labeled ubiquitins into chains conjugated onto ITCH leads to an increase in fluorescence emission at 665 nm ($Em_{acceptor}$) and decrease at emission wavelength 620 nm (Em_{donor}).



Product Information

Quantity: 400 x 20 µL reactions
160 x 50µL reactions

Kit Components:

- 100x UBA1.....100µL
- 100x UBE2L3 .....100µL
- 100x ITCH.....100µL
- 100x TRF-Ubiquitin Mix 100µL
- 10x Reaction Buffer.....1 mL
- 10x Mg-ATP.....1 mL
- 384-well white low-volume microplate.....x 1

Storage: -80C, Avoid multiple freeze / thaw cycles. It is recommended to make aliquots of each reaction component upon first time use.

Setup Protocol

1) Mix components in this order: H_2O, 10x Reaction Buffer, 100x UBA1, 100x UBE2L3, 100x ITCH, and 100x trf-Ubiquitin Mix to a final volume concentration of 1x. Wait to initiate the reaction(s) with 10x Mg-ATP until the plate is ready to read in plate reader.

Example setup for <u>1 mL</u> final volume mixture:

To 760µL H_2O
Add... 100µL 10x Reaction Buffer
10µL 100x UBA1 (E1)
10µL 100x UBE2L3 (E2)
10µL 100x ITCH (E3)
10µL 100x trf-Ubiquitin Mix

Initiate reaction(s) with final addition of 10% final volume, 10x Mg-ATP <u>to each well</u>. For negative control(s) wells substitute Mg-ATP with 1x Reaction Buffer.

It is recommended to pipette all the components (minus Mg-ATP), i.e. 90% of the final volume into each well first, then initiate the reactions with addition of the last 10% final volume of Mg-ATP or a solution of 1x Reaction Buffer for the negative control wells.

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2) Read 20-50µL reactions in a 384-well white low volume plate (supplied). You must use a plate-reader capable of taking top-read duel-channel TR-FRET measurements. Plate-readers tested with this kit include the Molecular Devices M5e Plate Reader and the PerkinElmer EnVision™ Multilabel Plate Reader. Plates can be read in either endpoint or continuous kinetic read mode.

Recommended Setting for Molecular Devices M5e

Channel 1: Excitation λ = 304 nm; Emission λ = 620 nm

Channel 2: Excitation λ = 304 nm; Emission λ = 665 nm

Delay Time = 50 µs; Integration Time = 400 µs

Cutoff λ_1 = 570 nm; Cutoff λ_2 = 630 nm

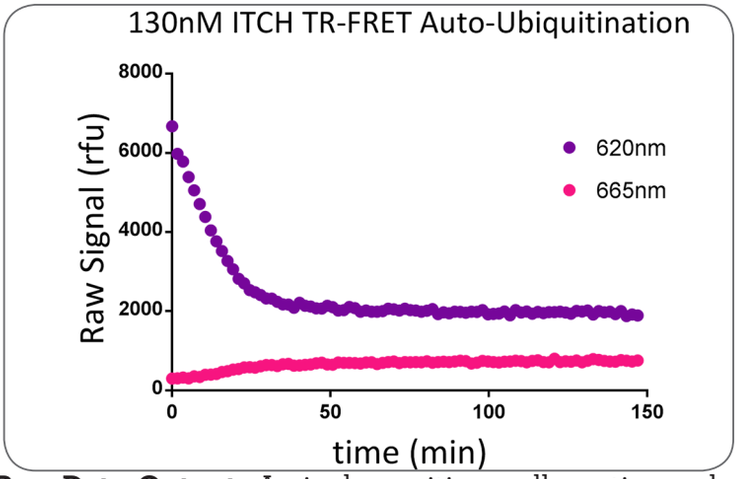
Recommended Setting for PerkinElmer EnVision

Top Mirror: LANCE/DELFIA Dual Enhanced (#662)

Excitation Filter = UV2 (TRF) 320 nm (#111); Emission Filter$_1$ = APC 665 nm (#205); Emission Filter$_2$ = Europium 615 nm (#203); Delay Time = 50 µs; Window Time = 400µs

Raw Data Output: Endpoint & Kinetic

Regardless of which type of readout that has been selected, the data must be collected on two channels; **Channel 1** measuring the emission signal of the donor cryptate at λ_1=620 nm, and **Channel 2** measuring the emission of the acceptor cyanine5 at λ_2= 665nm. Both emission channels are required during data reduction to calculate percent signal : background and Z-primes.



Raw Data Output: A single positive well continuously read in kinetic mode.

Data Reduction

Raw Signal: Aggregate TR-FRET signal detection is most typically quantified as the product of the ratio of acceptor to donor emission signal (λ = 665 nm/ λ = 620 nm), and a "convenience constant" (η=10^4):

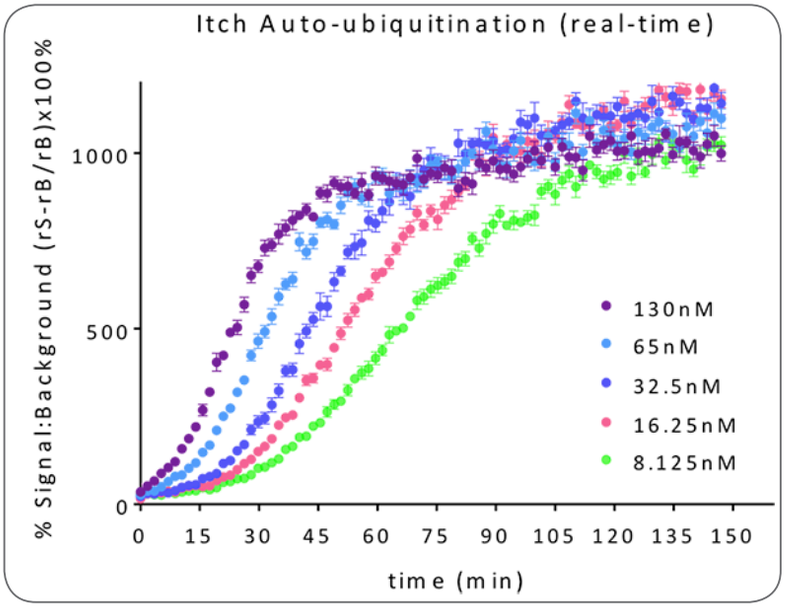
$$\frac{\text{Emission}_2}{\text{Emission}_1}\left(\frac{\lambda=665}{\lambda=620}\right) \times 10^4 = \text{Raw Signal or r(Signal)}$$

Specific Signal or Delta Signal: Subtracting the background raw-signal from the positive experimental-signal yields the "true" signal, also called the Delta Signal:

r(Experimental)-r(Background) = r(Delta)

% Signal to Background: Different plates or experiments from different days of the same assay can be compared using the Signal to Background:

$$\frac{\text{r(Experimental)-r(Background)}}{\text{r(Background)}} \times 100\% = \frac{\text{Signal}}{\text{Background}}\%$$



% Signal to Background of Continuous Real-Time TR-FRET ITCH titration (auto-ubiquitination): Serial dilutions of ITCH from 130 nM to 8.125 nM mixed with UBA1, UBE2L3, and trf-Ubiquitin mix. Reactions were initiated with addition of Mg-ATP.

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Z-Prime (Z'): Z-primes can be calculated by using the means and standard deviations of the positive and negative signal at each data-point over time.

$$\text{Estimated Z-prime} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

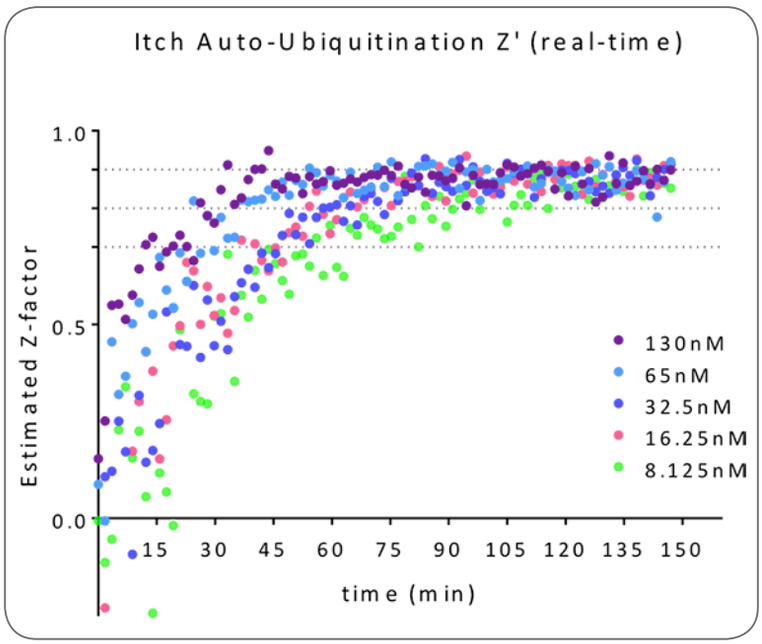
Where :

σ_p = standard deviation of the positive signal.

σ_n = standard deviation of the negative signal.

μ_p = mean of the positive signal.

μ_n = mean of the negative signal.



Estimated Z-primes of Continuous Real-Time TR-FRET MDM2 titration (auto-ubiquitination): Serial dilutions of ITCH from 130 nM to 8.125 nM mixed with UBA1, UBE2L3, and trf-Ubiquitin mix. Reactions were initiated with addition of Mg-ATP.

References

1) Magennis, S. W., Parsons, S., Pikramenou, Z., Corval, A., & Woollins, J. D. (1999). Imidodiphosphinate ligands as antenna units in luminescent lanthanide complexes. Chemical communications, (1), 61-62.

2) Zheng, N., & Shabek, N. (2017). Ubiquitin Ligases: Structure, Function, and Regulation.Annual Review of Biochemistry, (0).

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