CAT.&Size: A1090005S (1,000 tests)
A1090005L (10,000 tests)
Storage at: -60 ℃ or Below

VKEYBIO-02-2025
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Not For Diagnostic Or Therapeutic Use

KeyTec® TR-FRET
Human VHL Binding Kit
Technical Manual

1. Introduction

KeyTec® TR-FRET human von Hippel – Lindau(VHL) binding kit is designed for the binding analysis of VHL ligands/inhibitors to wild-type human VHL. This kit provides a tag1- VHL/Elongin C/Elongin B protein complex (Tag1-VCB complex), a VHL ligand-LA*[1] (CAS#1948273-02-6), an anti-tag1 antibody labeled with KeyTec® TR-FRET Solar Eu*[2] (mAb anti-Tag1 - Solar Eu) and other detection reagents. These components enable the high-throughput screening of VHL ligands/inhibitors.

This assay is based on a competitive immunoassay method using KeyTec® TR-FRET technology, offering a simple, rapid, highly specific and sensitive, as well as reproducible detection process. The principle, outlined in Figure 1:

The binding of the mAb anti-Tag1 - Solar Eu, the Tag1-VCB complex and the VHL ligand-LA*[1] brings the TR-FRET donor and acceptor into close proximity, enabling resonance energy transfer (RET) upon excitation and generating a TR-FRET signal. VHL ligands/inhibitors compete with VHL ligand-LA*[1] for binding to the Tag1-VCB complex. The intensity of TR-FRET signal is inversely proportional to the apparent affinity of VHL ligands/inhibitors.

*[1] KeyTec® TR-FRET LA: TR-FRET Acceptor

*[2] KeyTec® TR-FRET Solar Eu: TR-FRET Donor

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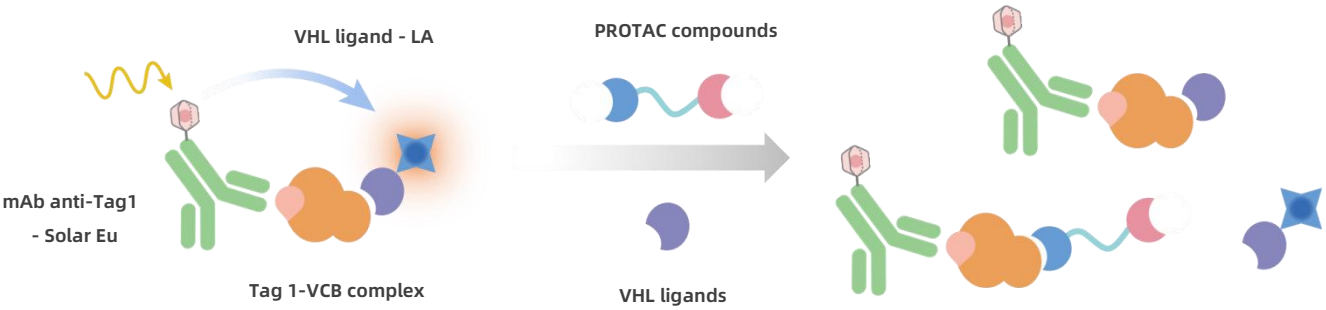

Figure1. The principle of KeyTec® TR-FRET human VHL binding detection

2. Components

Components	Storage	A1090005S (1,000 tests*3)	A1090005L (10,000 tests*3)
VHL binding standard 1 MZ 1 (20 mM in DMSO)	≤ -60 ℃	1 vial of 10 μL A1010037S	1 vial of 100 μL A1010037L
Tag1-VCB complex（50X）	≤ -60 ℃	1 vial 100 μL/vial	1 vial 1 mL/vial
VHL ligand - LA（100X）	≤ -60 ℃	1 vial 50 μL/vial	1 vial 500 μL/vial
mAb anti-Tag1 - Solar Eu Lyophilized	2-8 ℃	1 vial 1,000 tests/vial	1 vial 10,000 tests/vial
DTT Lyophilized	2-8 ℃	1 vial 120 μmole/vial A1010022S	1 vial 600 μmole/vial A1010022L
PROTAC Diluent Buffer	2-8 ℃	1 bottle 20 mL/bottle A1010025S	1 bottle 200 mL/bottle A1010025L
PROTAC Detection Buffer	2-8 ℃	1 bottle 20 mL/bottle A1010026S	1 bottle 120 mL/bottle A1010026L

*3 Tests refer to the number of assay wells that can be performed in 96-well or384-well plates with 20 μL in total reaction volume. The reagents of the kit are suggested to use as recommended.

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3. Storage

- Store all reagents according to the recommended conditions. The products are stable for one year from the date of receipt.
- After thawing, aliquot the stock into single-use volumes (recommended minimum: 10 μL) to avoid repeated freeze-thaw cycles. Store these aliquots at -60 °C or below.

4. Required Components (Not Supplied)

Material	Brand	Catalog
Microplate	VKEY-BIO	M2000102N
Top sealing film	VKEY-BIO	M1000102N
Microplate Reader with TR-FRET module	TECAN	Infinite® 200 PRO

5. Procedure

5.1 Reaction system

Components	Volume[*4]	Stock Conc.	Working Conc.	Final Conc.
Test samples or standards	5 μL	\	\	\
Tag1-VCB complex	5 μL	50X	1X	\
VHL ligand - LA[*5]	5 μL	100X	1X	\
mAb anti-Tag1 - Solar Eu[*5]	5 μL	100X	1X	\

*[4] Recommended Format: Shallow-well 384-well microplate; For 96-well or 1536-well microplates, proportionally scale the reaction system.

*[5] It is recommended to premix VHL ligand-LA and mAb anti-Tag1 - Solar Eu , add 10 μL pre-mix solution in TR-FRET detection.

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5.2 Reagent preparation

- After thawing on ice, Aliquot the stock into single-use volumes (recommended minimum: 10μL) to avoid repeated freeze-thaw cycles. Store these aliquots at -60 ℃ or below. The buffers (PROTAC diluent buffer and PROTAC detection buffer) can be stored at 2-8 ℃.
- Before use, equilibrate all reagents to RT.
- Use the provided/recommended buffers to prepare sample and detection reagents.
- Prepare sample and detection reagent according to the kit technical manual.
- Prepare all reagents immediately before use, unless otherwise specified in the "Working Solution Preparation" section.
- Avoid vigorous mixing of all reagents.

6. Working Solution Preparation

6.1 PROTAC Diluent buffer

- **DTT stock solution**: Before reconstitution, centrifuge DTT to pellet the powder to the bottom (850×g,1-2 minutes). Add the recommended volume of Ultra-pure water to the powder and mix gently as below. Aliquot DTT stock solution into single-use tubes to avoid repeated freeze-thaw cycles. These aliquots can be stored at -60℃.

DTT Powder	Buffer	Volume	Stock Concentration
120 μmole/vial	Ultra-pure water	120 μL/vial	1 M
600 μmole/vial	Ultra-pure water	600 μL/vial	1 M

- **PROTAC Diluent buffer (1x, 1mM DTT)**: Dilute 1 volume of 1M DTT with 1000 volume of PROTAC diluent buffer and mix gently .

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6.2 Test sample or positive control preparation (1X)

- Dilute the test samples/standards with PROTAC Diluent buffer (1x, 1mM DTT). The percentage of DMSO must not exceed 2% for serial dilution.

Standard	Working Conc. (1X,nM)	Final Conc. (0.25X, nM)	Dilution
STD-11	360 000	90 000	2.7 μL standard stock solution + 147.3 μL Diluent Buffer
STD-10	120 000	30 000	50 μL STD-11 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-9	40 000	10 000	50 μL STD-10 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-8	13 333	3333.3	50 μL STD-9 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-7	4 444	1111.1	50 μL STD-8 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-6	1 481	370.4	50 μL STD-7 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-5	493.8	123.5	50 μL STD-6 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-4	164.6	41.15	50 μL STD-5 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-3	54.87	13.72	50 μL STD-4 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-2	18.29	4.57	50 μL STD-3 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-1	6.10	1.52	50 μL STD-2 + 100 μL Diluent Buffer (contain1.8% DMSO)
STD-0 (PC)	0.00	0	100 μL Diluent Buffer (contain1.8% DMSO)

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6.3 mAb anti-Tag1 - Solar Eu and VHL ligand - LA pre-mix solution

- **VHL ligand - LA working solution(1X)**: Dilute 1 volume of 100X VHL ligand - LA stock solution with 99 volume of PROTAC detection buffer and mix gently .

- **mAb anti-Tag1 - Solar Eu stock solution**: Before reconstitution, centrifuge mAb anti-Tag1 - Solar Eu to pellet the powder to the bottom (850×g,1-2 minutes). Add the recommended volume of Ultra-pure water to the powder and mix gently as below. Aliquot mAb anti-Tag1 - Solar Eu stock solution into single-use tubes to avoid repeated freeze-thaw cycles. These aliquots can be stored at -60℃.

Size	Buffer	Volume	Stock Concentration
1,000 tests/vial	Ultra-pure water	50 µL/vial	100X
10,000 tests/vial	Ultra-pure water	500 µL/vial	100X

- **mAb anti-Tag1 - Solar Eu working solution (1X)**: Dilute 1 volume of 100X mAb anti-Tag1 - Solar Eu stock solution with 99 volume of PROTAC detection buffer and mix gently .

- **(Optional) mAb anti-Tag1 - Solar Eu and VHL ligand - LA pre-mix solution (0.5X)**: The pre-mix solution should be combined in a 1:1 volume ratio of mAb anti-Tag1 - Solar Eu working solution and VHL ligand - LA working solution.

Component	Pre-mix Solution Conc.	Working Solution Conc.	Stock Solution Conc.	Dilution	Stock Vol. µL	1X PROTAC Detection Buffer µL	Total Vol. µL
VHL ligand - LA	0.5X	1X	100X	200	5	990	1,000
mAb anti-Tag1 - Solar Eu	0.5X	1X	100X	200	5		

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6.4 Protein solution (1X)

- Dilute 1 volume of 50X Tag1-VCB complex stock solution with 49 volume of PROTAC Diluent buffer (1x, 1mM DTT) and mix gently. After thawing on ice, Aliquot the stock into single-use volumes (recommended minimum: 10μL) to avoid repeated freeze-thaw cycles.

7. Procedure

- Follow the steps in the table below.

	Negative Control	Standard curve	Test samples
Step 1	5 μL Diluent Buffer	5 μL Standards (1X)	5 μL Test sample (1X)
Step 2	5 μL Diluent Buffer	5 μL Tag1-VCB complex (1X)	
Step 3	10 μL VHL ligand - LA and mAb anti-Tag1 - Solar Eu pre-mix solution(0.5X)		
Step 4	Seal the microplate to prevent liquid evaporation, Incubation（RT，25 ℃）for 60 minutes or over-night		
Step 5	Record the data in microplate reader with top sealing film		

8. Data Analysis

- Calculate the 665 nm/620 nm Ratio(TR-FRET Ratio)and the percentage coefficient of variation (CV %) for each well.

$$\text{TR-FRET Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10{,}000$$

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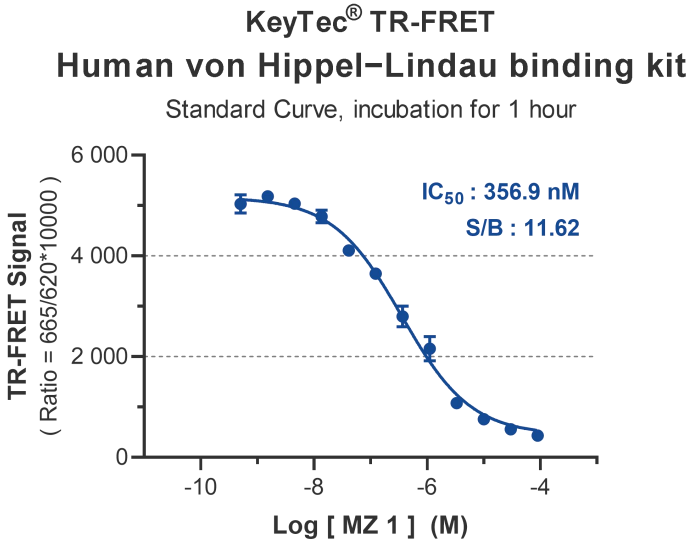
9. Summary

9.1 Standard Curve

Standard	MZ 1 (nM)	TR-FRET Ratio	CV%
STD-11	90 000	434	2.9
STD-10	30 000	556	9.5
STD-9	10 000	759	10.8
STD-8	3333.3	1,076	11.1
STD-7	1111.1	2,159	7.4
STD-6	370.4	2,800	3.1
STD-5	123.5	3,648	2.9
STD-4	41.15	4,109	2.7
STD-3	13.72	4,781	0.7
STD-2	4.57	5,036	1.4
STD-1	1.52	5,182	3.6
STD-0	0	5,034	1.7



Note: Exemplary data shown. Results are instrument-dependent.

9.2 Parameter

Parameter	
VHL ligand - LA K_d	44.40 nM
VHL ligand - LA concentration	50.00 nM
MZ 1 IC_{50}	356.9 nM
MZ 1 K_i	167.9 nM

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10. Instrument Model and Setting

Vendor	TECAN
Instrument model	Infinite® 200 PRO [Ref. 30050303]
Mode	Fluorescence Top Reading
Excitation filter	320 (25) nm [Ref. 30094454]
Emission filter 1	665 (8.5) nm [Ref. 30094518]
Emission filter 2	620 (10) nm [Ref. 30094505]
Mirror	Dichroic 510
Lag time	150 μs
Integration Time	500 μs
Number of reads	5 or user-defined
Gain	150 or optimal
Z -focus (mm)	Can be calculated on the well giving the highest signal

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