**CAT.&Size:** A1080004S (1,000 tests)
A1080004L (20,000 tests)
A1080004B (100,000tests)
**Storage at:** 2-8 ℃

**VKEYBIO-01-2025**

**For Research Use Only**
**Not For Diagnostic Or Therapeutic Use**

# KeyTec® TR-FRET
# Serine/Threonine Kinase Kit - S2 Substrate
## Technical Manual

## 1. Introduction

**KeyTec® TR-FRET STK S2 kinase kit** is designed for measuring the Serine/threonine kinase (STKs) activity. Serine/threonine kinase family comprises multiple members. This assay kit has been validated for activity measurement of over 30 Serine/threonine kinases (For more details, please visit our official website or contact our technical support team). The assay provides a biotinylated substrate, Streptavidin-HX*[1] , a phospho STK-specific antibody labeled with KeyTec® TR-FRET Solar Eu*[2] and detection reagent. These components enable the quantification of kinase activity by detecting the phosphorylated substrate.

This assay is based on a sandwich 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, involves two main phases:

**Kinase Reaction:** The specific substrate is phosphorylated by the target kinase in the optimized reaction buffer with ATP.

**TR-FRET Detection:** Adding an antibody and Streptavidin-HX containing EDTA (to terminate the kinase reaction) that specifically binds to the phosphorylated substrate. This binding brings the TR-FRET donor and acceptor into close proximity, enabling resonance energy transfer (RET) upon excitation and generating a TR-FRET signal. The intensity of TR-FRET signal is directly proportional to the level of phosphorylated substrate.



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*1 KeyTec® TR-FRET HX: TR-FRET Acceptor

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

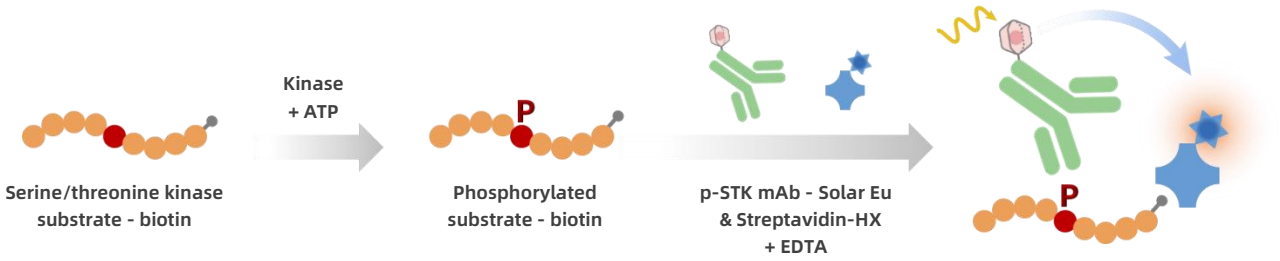


**Figure1.** The principle of KeyTec® TR-FRET kinase activity detection

## 2. Components

| Components | Storage | A1080004S (1,000 tests[*3]) | A1080004L (20,000 tests[*3]) | A1080004B (100,000 tests[*3]) |
|---|---|---|---|---|
| **p-STK mAb - Solar Eu** Lyophilized | 2-8 ℃ | 1 vial<br>1,000 tests/vial | 1 vial<br>20,000 tests/vial | 5 vials<br>20,000 tests/vial |
| **Streptavidin - HX** Lyophilized | 2-8 ℃ | 2 vials<br>840 pmoles/vial<br>A1020024S | 3 vials<br>8.4 nmoles/vial<br>A1020024L | 4 vials<br>40 nmoles/vial<br>A1020024B |
| **STK Substrate 2 - biotin** Lyophilized | 2-8 ℃ | 1 vial<br>25 nmoles/vial<br>A1080009S | 1 vial<br>250 nmoles/vial<br>A1080009L | 3 vials<br>250 nmoles/vial<br>A1080009L |
| **DTT** Lyophilized | 2-8 ℃ | 1 vial<br>120 μmoles/vial<br>A1010022S | 1 vial<br>600 μmoles/vial<br>A1010022L | 1 vial<br>3.0 mmoles/vial<br>A1010022B |
| **$MgCl_2$** (1 M) | 2-8 ℃ | 1 vial<br>1.5 mL/vial<br>A1010024S | 1 bottle<br>6.0 mL/bottle<br>A1010024L | 1 bottle<br>30 mL/bottle<br>A1010024B |
| **$CaCl_2$** (1 M) | 2-8 ℃ | 1 vial<br>1.5 mL/vial<br>A1010043S | 1 bottle<br>6.0 mL/bottle<br>A1010043L | 1 bottle<br>30 mL/bottle<br>A1010043B |
| **Kinase Enzymatic buffer** (5X) | 2-8 ℃ | 1 bottle<br>10 mL/bottle<br>A1010018S | 1 bottle<br>50 mL/bottle<br>A1010018L | 1 bottle<br>250 mL/bottle<br>A1010018B |
| **Kinase Detection buffer** (with EDTA) | 2-8 ℃ | 1 bottle<br>20 mL/bottle<br>A1010019S | 1 bottle<br>250 mL/bottle<br>A1010019L | 2 bottles<br>550 mL/bottle<br>A1010019B |

*[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 and below.

## 4. Required Components (Not Supplied)

| Material | Brand | Catalog |
|---|---|---|
| **ATP** | Bidepharmatech | BD112724 |
| **AMP**（5 mM） | Sigma-Aldrich | A1752 |
| **cGMP**（1 mM） | Sigma-Aldrich | G6129 |
| **Lipid activator** | Sigma-Aldrich | 20-133 |
| **Microplate** | VKEY-BIO | M2000102N |
| **Top sealing film** | VKEY-BIO | M1000102N |
| **Microplate Reader with TR-FRET module** | TECAN | Infinite® 200 PRO |



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# 5. Procedure

## 5.1 Reaction system

| Phase | Components | Volume[*4] ( 20 μL) |
|---|---|---|
| Kinase reaction (10 μL) | Test samples or positive control | 2 μL[*5] |
| | Kinase | 4 μL[*5] |
| | Serine/threonine kinase substrate - biotin | 4 μL (Pre-mix)[*5] |
| | ATP | |
| TR-FRET detection (10 μL) | p-STK mAb - Solar Eu[*6] | 5 μL |
| | Streptavidin - HX[*6] | 5 μL |

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

*[5] The proportions of test sample, kinase, substrate and ATP may be adjusted, as long as the concentrations stay the same.

*[6] It is recommended to premix p-STK mAb - Solar Eu and Streptavidin - HX, add 10 μL pre-mix solution in TR-FRET detection.

## 5.2 Reagent preparation

- Before use, equilibrate all reagents to RT.
- **For lyophilized powder, centrifuge to collect the powder to the bottom (850×g, 1-2minutes).** It is normal for the fragmentation of lyophilized powder.
- 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.
- To avoid the degradation of active components, keep 1X Kinase Enzymatic buffer (containing supplemental components) on ice.
- To minimize the aggregation or non-specific binding, It is recommended to add surfactants in Kinase Enzymatic buffer (5X). Notes: Kinase Enzymatic buffer (5X)



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contained 0.05% BSA.

- Avoid vigorous mixing of all reagents.

## 6. Working Solution Preparation

### 6.1 Enzyme reaction buffer

- Prepare 1x enzyme reaction buffer: dilute 1 volume of 5X Kinase Enzymatic buffer and kinase supplements required (e.g., $MgCl_2$, $MnCl_2$, DTT, etc.) with 4 volume of ultrapure water.

- **DTT stock solution**: Before reconstitution, centrifuge DTT to pellet the powder to the bottom (850 × g,1-2 minutes). Add the recommended volume of ultrapure 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 |

### 6.2 Test sample or positive control preparation

- Dilute the test samples to working concentration (5X). For example, if the final test sample concentration is 10 nM in 10 μL of kinase reaction system, dilute test samples to working concentration (50 nM) using 1X enzyme reaction buffer and add 2 μL/well of test samples. The percentage of DMSO must not exceed 2% for serial dilution.

### 6.3 Substrate and ATP pre-mix solution

- **ATP stock solution**: Prepare 1 mM ATP stock solution with ultrapure water, aliquot it into single-use tubes to avoid repeated freeze-thaw cycles. These aliquots can be stored at -80℃ for up to 3 months.

- **Substrate stock solution**: Before resuspension, centrifuge STK substrate 2 - biotin



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to pellet the powder to the bottom (850 × g,1-2 minutes). Add the recommended volume of ultrapure water to the powder and mix gently as below.

| Substrate Powder | Buffer | Volume | Stock Concentration |
|---|---|---|---|
| 25 nmoles/vial | Ultra-pure water | 500 µL/vial | 50 µM |
| 250 nmoles/vial | Ultra-pure water | 500 µL/vial | 500 µM |

- **Substrate and ATP pre-mix solution(2.5X)**: Prepare 1 mL substrate and ATP pre-mix Solution(2.5X), dilute 50 µL of STK substrate 2 - biotin stock solution and 12.5 µL of ATP stock solution with 937.5 µL of 1x enzyme reaction buffer.

| Component | Final Conc. µM | Working Conc. µM | Stock Conc. µM | Dilution | Stock Vol. µL | 1X Enzyme Reaction Buffer µL | Total Vol. µL |
|---|---|---|---|---|---|---|---|
| Substrate - biotin | 1.0 | 2.5 | 50 | 20 | 50 | 937.5 | 1,000 |
| ATP | 5 | 12.5 | 1,000 | 80 | 12.5 | | |

### 6.4 Kinase solution

- Dilute the kinase to working concentration (2.5X). For example, if the final kinase concentration is 1 ng/µL in 10 µL of kinase reaction system, dilute 100 ng/µL to working concentration ( 2.5 ng/µL) using 1X enzyme reaction buffer and add 4 µL/well of kinase solution. **Prepare it immediately prior to addition.**

### 6.5 Detection solution

- **p-STK mAb - Solar Eu stock solution**: Before reconstitution, centrifuge p-STK mAb - Solar Eu to pellet the powder to the bottom (850 × g,1-2 minutes). Add the recommended volume of Kinase detection buffer to the powder and mix gently as below.

| Kit Cat. | Size | Buffer | Volume | Stock Concentration |
|---|---|---|---|---|
| A1080004S | 1,000 tests/vial | Kinase Detection buffer | 0.5 mL/vial | 10X |
| A1080004L/B | 20,000 tests/vial | Kinase Detection buffer | 1.0 mL/vial | 100X |



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- **p-STK mAb - Solar Eu working solution(1X)**: Dilute 10X p-STK mAb - Solar Eu stock solution to working concentration(1X). For example, prepare 1 mL working solution(1X), dilute 100 µL of p-Tyr mAb - Solar Eu stock solution with 900 µL of kinase detection buffer. Working solution(1X) can be stored at 2-8℃ for 24 hours or -60℃ for 3 months .

- **Streptavidin - HX stock solution**: Before reconstitution, centrifuge Streptavidin - HX 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.

| Component Cat. | Size | Buffer | Volume | Stock Concentration |
|---|---|---|---|---|
| A1020024S | 840 pmoles/vial | Ultra-pure water | 42 µL/vial | 20 µM |
| A1020024L | 8.4 nmoles/vial | Ultra-pure water | 420 µL/vial | 20 µM |
| A1020024B | 40 nmoles/vial | Ultra-pure water | 2.0 mL/vial | 20 µM |

- **Streptavidin - HX working solution(1X, 250nM)**: The molar ratio of substrate-biotin: Streptavidin-HX has been optimized at 8:1. Dilute Streptavidin-HX stock solution to working concentration (1X). For example, prepare 0.8 mL working solution(1X), dilute 10 µL of Streptavidin - HX stock solution with 790 µL of kinase detection buffer. Working solution(1X) can be stored at 2-8℃ for 24 hours or -60℃ for 3 months .



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- **(Optional) p-STK mAb - solar Eu and streptavidin - HX pre-mix solution(0.5X)**: The pre-mix solution should be combined in a 1:1 volume ratio of p-STK mAb-Solar Eu working solution and streptavidin-HX working solution.

| Component | Volume | Kinase reaction Conc. (10 μL in total) | Final Conc. (20 μL in total) | Working Conc. |
|---|---|---|---|---|
| Test samples or positive control | 2 μL/well | \ | \ | \ |
| Kinase | 4 μL/well | 0.1 ng/μL | \ | 2.5 ng/μL |
| Substrate - biotin | 4 μL/well (Premix with ATP) | 1000 nM | 500 nM | 2500 nM |
| Streptavidin - HX (1/8*[7]) | 5 μL/well | \ | 62.5 nM[*7] | 250 nM[*7] |
| p-Tyr mAb - Solar Eu | 5 μL/well | \ | \ | 1X |

*[7] Streptavidin - HX / substrate - biotin = 1/8 (Final Conc.).

## 7. Procedure

- Follow the steps in the table below.

| | | Kinase Group | Negative Control |
|---|---|---|---|
| Kinase reaction | Step 1 | 2 μL Test sample (5X)<br>4 μL Kinase (2.5X)<br>4 μL STK substrate 2 - biotin and ATP pre-mix solution (2.5X) | 2 μL Test sample (5X)<br>4 μL Enzymatic buffer<br>4 μL STK substrate 2 - biotin and ATP pre-mix solution (2.5X) |
| | Step 2 | Seal the microplate to prevent liquid evaporation, Incubation (RT, 25 ℃) for 60 minutes | |
| TR-FRET detection | Step 3 | Remove the top sealing film, add detection solution:<br>5 μL p- STK mAb - Solar Eu (1X)<br>5 μL Streptavidin - HX (1X)<br>or 10 μL p-STK mAb - solar Eu and streptavidin - HX 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 | |



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# 8. Data Analysis

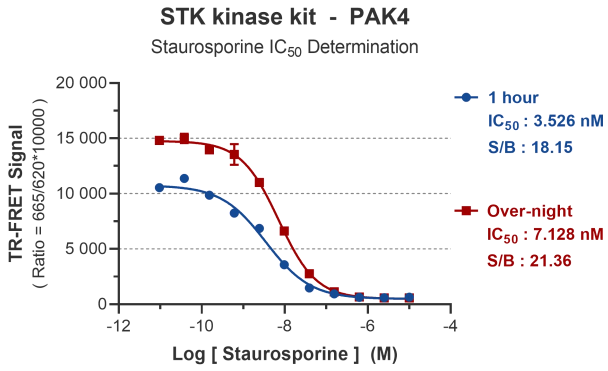
- Calculate the 665 nm/620 nm Ratio (TR-FRET Ratio) and the percentage coefficient of variation (CV %) for each well.

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

# 9. Summary

## 9.1 Results

| | |
|---|---|
| **PAK4** | UniProt ID: O96013 |
| **Enzyme concentration** | 0.1 ng/μL [ Final concentration in 10 μL buffer ; 1.0 ng/well ] |
| **Incubation time at RT** | 60 min |
| **Substrate** | STK Substrate 2 - biotin, 1 μM [ Final concentration in 10 μL buffer ] |
| **Streptavidin - HX** | 62.5 nM [ SA/Substrate = 1/8 for final concentration in 20 μL buffer ] |
| **Detection antibody** | p-STK mAb - Solar Eu, 1X [ Working concentration ] |
| **ATP concentration** | 100 μM |
| **Buffer additives** | 5 mM $MgCl_2$ , 1 mM DTT |



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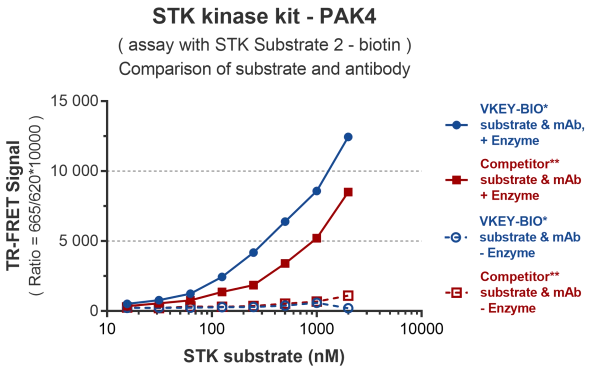
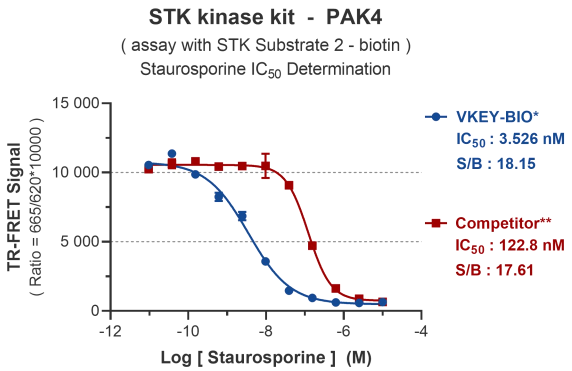
## 9.2 Performance

- The comparative data presented below show the performance of the VKEY-BIO KIT and Company C KIT under the same experimental conditions.




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

# 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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