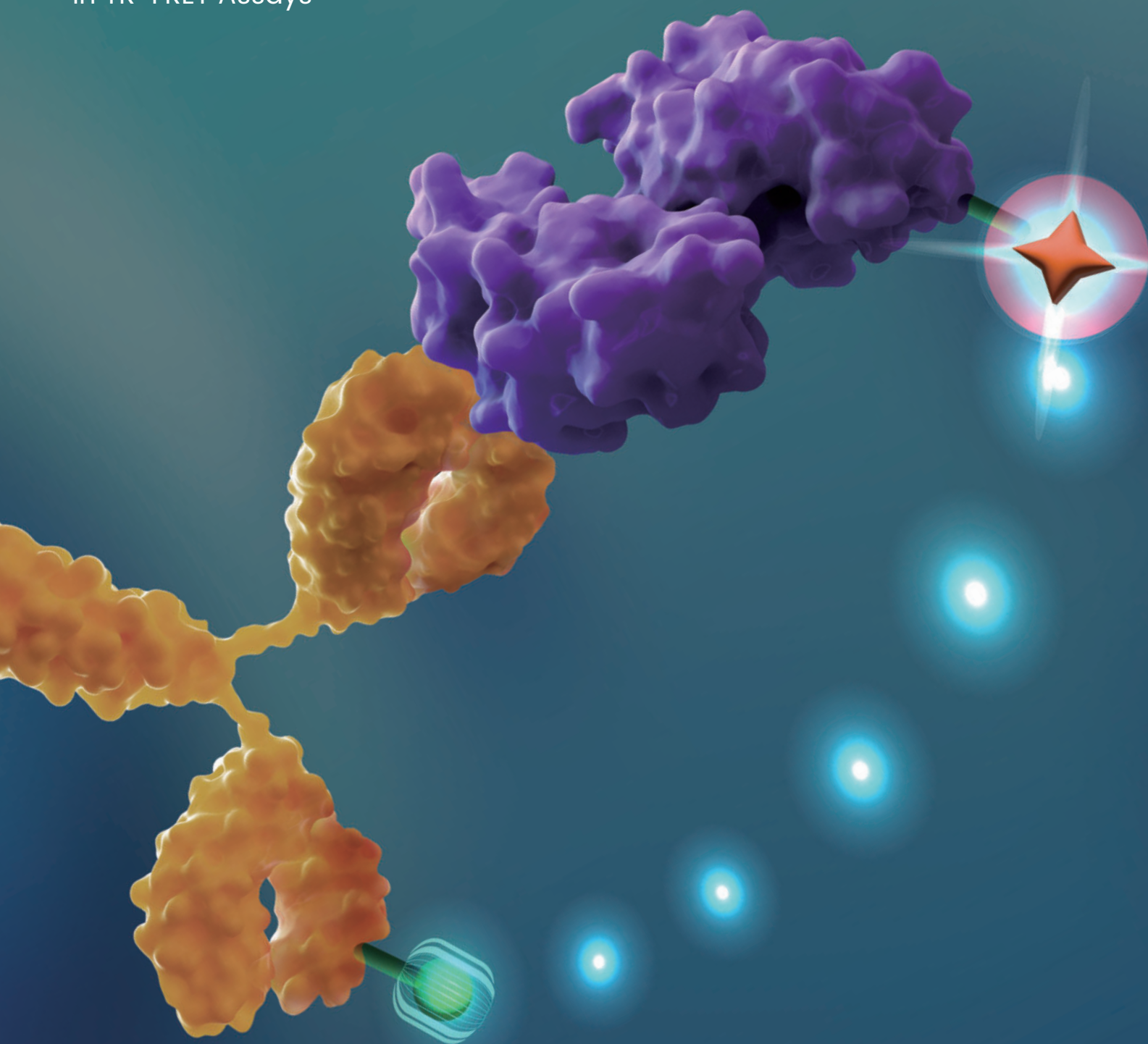




SwiftFluo[®] TR-FRET

Technical Q&A Handbook

Addressing Common Questions and Troubleshooting
in TR-FRET Assays





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I. Fundamentals

1. What is TR-FRET? How does TR-FRET differ from FRET assays?

TR-FRET uses long-lifetime lanthanide chelates as donor fluorophores. By applying time-delayed signal detection, background interference is substantially reduced, resulting in improved signal-to-background ratio and assay sensitivity. This makes TR-FRET particularly suitable for homogeneous assays. In contrast, conventional FRET assays rely on short-lifetime organic fluorophores and are more susceptible to interference from autofluorescence and light scattering.

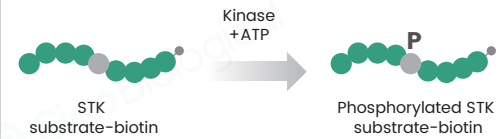
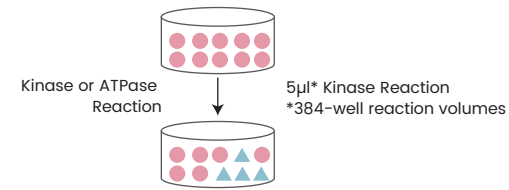
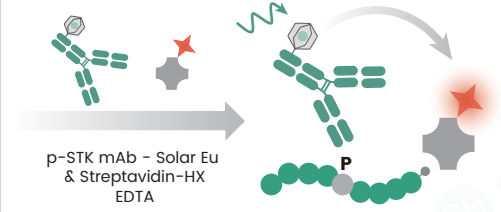
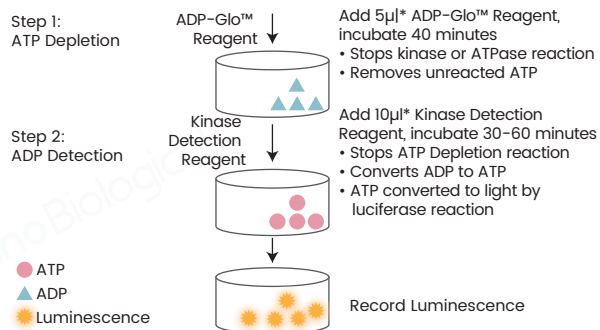
Comparison between TR-FRET and FRET

Method	TR-FRET	FRET
Fluorophores	Lanthanide donors (e.g., Eu, Tb, Sm, Dy)	Organic fluorophores (e.g., FITC)
Fluorescence lifetime	Long (1–2 ms)	Short (1–10 ns)
Detection mode	Time-delayed detection, minimizing background fluorescence	Simultaneous detection, more prone to background interference
Photostability	No photobleaching; allows repeated excitation	Photobleaching occurs
Applications	In vitro assays, high-throughput screening, protein–protein interactions, kinase activity assays; not suitable for real-time intracellular imaging	Live-cell imaging and spatial localization studies

2. For kinase activity assays, what are the key differences between TR-FRET and ADP-Glo™?

TR-FRET: Directly detects phosphorylated substrates, which helps avoid interference from kinase autophosphorylation. The assay signal is highly substrate-specific.

ADP-Glo™: Measures ADP generated during the reaction as an indirect readout of kinase activity and is not dependent on substrate specificity.

Assay	TR-FRET	ADP-Glo™
Enzymatic Reaction Step		
Detection Step		Step 1: ATP Depletion Step 2: ADP Detection 

3. What are the common applications of TR-FRET technology?

TR-FRET is commonly used in high-throughput drug screening, protein-protein interaction studies, enzyme activity assays, antibody screening, and cellular signaling pathway analysis. Owing to its high sensitivity, low background, and homogeneous assay format, TR-FRET is well suited for rapid quantitative measurements and multiplexed analysis.

4. How does TR-FRET compare with other detection methods (such as ELISA, MSD, and AlphaLISA) in terms of sensitivity, background, throughput, samples, instrumentation, and applications?

The specific differences between TR-FRET and other detection methods are summarized in the table below.

Comparison of TR-FRET with ELISA, MSD, and AlphaLISA

Method	TR-FRET	ELISA	MSD	AlphaLISA
Detection Format	Homogeneous	Heterogeneous (washing required)	Heterogeneous (washing required)	Homogeneous
Sensitivity	Wide dynamic range (3–5 orders of magnitude)	Based on colorimetric detection; typically, a narrower dynamic range (2–3 orders of magnitude) and more susceptible to matrix effects	High sensitivity with a wide dynamic range (4–5 orders of magnitude)	Wide dynamic range (3–5 orders of magnitude)
Background	Low	High	Moderate	Low
Throughput	Well suited for high-throughput screening; compatible with 96-, 384-, and 1536-well plates	Typically performed in 96-well plates; higher sample consumption and multiple steps limit suitability for ultra-high throughput	384-well formats are possible, but washing steps still limit throughput improvement	Suitable for high-throughput screening, but bead costs are generally high
Sample Types	Cell lysates, serum, culture supernatants, etc.	Cell lysates, serum, culture supernatants, etc.	Cell lysates, serum, culture supernatants, etc.	Cell lysates, serum, culture supernatants, etc.
Instrumentation	Microplate reader with TR-FRET module	Microplate reader	MSD imager	Microplate reader with Alpha detection module

II. Products

5. Which TR-FRET kinase assay kits are provided by Sino Biological?

The TR-FRET-based kinase assay kits offered by Sino Biological are listed in the table below.

Type	Cat#	Product Name	Coverage
TKs	SFKA001	SwiftFluo® TR-FRET TKs Kinase Kit	60+ TKs
STKs	SFKA002	SwiftFluo® TR-FRET Serine/Threonine Discovery Kinase Kit	150+ STKs
	SFKA003	SwiftFluo® TR-FRET Serine/Threonine Kinase Kit – S1 Substrate	50+ STKs
	SFKA004	SwiftFluo® TR-FRET Serine/Threonine Kinase Kit – S2 Substrate	30+ STKs
	SFKA005	SwiftFluo® TR-FRET Serine/Threonine Kinase Kit – S3 Substrate	50+ STKs
CDKs	SFKA006	SwiftFluo® TR-FRET CDKs Kinase Kit-Rb(S780) substrate	CDK1, 3, 4, 6, 7, 9, 10, 12, 13, 15–18, etc.
	SFKA007	SwiftFluo® TR-FRET CDKs Kinase Kit-ATF2(T71) substrate	CDK2, 5, 20, etc.

6. Which representative kinases are compatible with the SwiftFluo® TR-FRET TKs Kinase Kit?

SwiftFluo® TR-FRET TKs kinase kit (Cat#: SFKA001) has been specifically designed and optimized for activity measurements of more than 60 tyrosine kinases. These kinases are mainly classified into two categories: receptor tyrosine kinases (RTKs) and cytoplasmic tyrosine kinases (CTKs), as listed in the table below.

Receptor Tyrosine Kinases (RTKs)	Cytoplasmic Tyrosine Kinases (CTKs)
ALK, AXL, c-MET/HGFR, DDR2, EGFR, EphA2, EphA3, EphA4, EphA7, EphA8, EphB1, EphB2, EphB3, EphB4, ERBB4/Her4, FGFR1, FGFR2, FGFR3, FLT3, FRK, IGF1R, INSRR, Insulin Receptor, M-CSFR/CSF1R, Mer, MUSK, PDGF alpha receptor/PDGFRα, PDGF beta receptor/PDGFRβ, RET, RON/MSP receptor, Ros1, TIE-2, TrkA, TrkB, TYRO3, VEGFR1/FLT1, VEGFR2/KDR, VEGFR3/FLT4	ABL2, BLK, BMX, Brk, c-Abl/ABL1, CSK, FES, FGR, FYN, HCK, ITK, JAK2, JAK3, Lck, Lyn, PTK2/FAK, Ptk2b, Src, Syk, YES1, ZAP70

7. What are the differences between the SwiftFluo® TR-FRET STK Discovery Kinase Kit and the SwiftFluo® TR-FRET STK S1/S2/S3 Kinase Kits? Which target kinases have been validated?

The main difference among the STK series kits lies in the universal substrates provided. Each SwiftFluo® TR-FRET STK S1/S2/S3 Kinase Kit contains a single substrate (S1, S2, or S3, respectively). In contrast, the SwiftFluo® TR-FRET STK Discovery Kinase Kit includes all three substrates (S1, S2, and S3) and is intended for target exploration and assay development.

Substrate	Applicable Kinase
S1 Substrate	AMPK (G1/B1/A2) Heterotrimer, BRSK2, CAMK2D/CaMKII Delta, CAMKI, CaMKII alpha/CAMK2A, CaMKII beta/CAMK2B, CaMKII gamma/CAMK2G, CAMKIV, CHK1, CHEK2, DAPK1, DAPK2/DAP Kinase 2, DCAMKL2/DCLK2, DRAK1/STK17A, GRK4, GRK6, Haspin/GSG2, IRAK4, LATS1, LKB1/STK11, MAPKAPK2/MK2, MAPKAPK3, MARK1, MELK, MKNK1, MNK2/MKNK2, MYLK, Pask, PHKG1, PHKG2, PKC alpha/PRKCA, PKC delta/PRKCD, PKC epsilon/PRKCE, PKC iota, PKC theta, PKN2/PRK2, PLK1, PRAK/MAPKAPK5, PrKA, PRKA A2/B1/G1, PRKCB, PRKCG/PKC gamma, PRKCH/PKC eta, PRKCO, PRKCZ/PKC zeta, PRKDI/PKC mu, PRKG2, Protein Kinase D2/PRKD2, RSK1/RPS6KA1, RSK2, RSK3, SIK1, SIK2, SIK3, TBK1, TSSK1B, TSSK2, ZIP Kinase/DAPK3 AMPK (G1/B1/A2) Heterotrimer, BRSK2, CAMK2D/CaMKII Delta, CAMKI, CaMKII alpha/CAMK2A, CaMKII beta/CAMK2B, CaMKII gamma/CAMK2G, CAMKIV, CHK1, CHEK2, DAPK1, DAPK2/DAP Kinase 2, DCAMKL2/DCLK2, DRAK1/STK17A, GRK4, GRK6, Haspin/GSG2, IRAK4, LATS1, LKB1/STK11, MAPKAPK2/MK2, MAPKAPK3, MARK1, MELK, MKNK1, MNK2/MKNK2, MYLK, Pask, PHKG1, PHKG2, PKC alpha/PRKCA, PKC delta/PRKCD, PKC epsilon/PRKCE, PKC iota, PKC theta, PKN2/PRK2, PLK1, PRAK/MAPKAPK5, PrKA, PRKA A2/B1/G1, PRKCB, PRKCG/PKC gamma, PRKCH/PKC eta, PRKCO, PRKCZ/PKC zeta, PRKDI/PKC mu, PRKG2, Protein Kinase D2/PRKD2, RSK1/RPS6KA1, RSK2, RSK3, SIK1, SIK2, SIK3, TBK1, TSSK1B, TSSK2, ZIP Kinase/DAPK3
S2 Substrate	Aurora A, Aurora B, MARK2, NEK11, PAK2, PAK3, PAK4, PAK5, PAK6, PAK7, PLK4, PRKACA/PKAc alpha, PRKG1, PRKG2, PRKX, ROCK1, ROCK2, RPS6KA4/MSK2, RSK4
S3 Substrate	AKT1, AKT2, AKT3, AURKC/Aurora C, BRSK1, CDC42BPB, CHUK/IKK alpha, CLK3, Dmpk, DYRK2, EEF2K, GRK5, GSK3 alpha, HIPK2, IKK beta, LOK, MAP3K5/ASK1, MAP3K8, MINK1, MRCK alpha/CDC42BPA, MSK1, MST1, MST2/STK3, NEK2, NEK3, NEK6, NEK7, NLK/Nemo Like Kinase, NUAK1, PIM1, PIM2, PLK2, PLK3, PRKACG/PKAc gamma, RPS6KA4/MSK2, S6K1/RPS6KB1, SGK1/SGK, SGK2, SGK3, SRPK3, STK4, WNK2, WNK3
S1/S2/S3 Substrate	All kinase types listed above

8. What validations have been performed for SwiftFluo® TR-FRET assay kits?

To ensure assay performance, comprehensive analytical validation has been conducted by Sino Biological. Key performance parameters include specificity, precision, sensitivity, linear range, and stability.

9. Do the kinase assay kits provide recommended ranges for kinase activity units (U/mL)?

No. The assay kits do not include kinases, and kinase activity can vary depending on the enzyme source. Users are advised to optimize kinase concentrations based on the specific enzyme used in their assays.

10. Do the TR-FRET assay kits include positive control inhibitors? Are IC₅₀ values available?

The kinase assay kits do not include positive control inhibitors. However, IC₅₀ data for the positive control compound staurosporine are provided.

11. How should donors (Solar Tb and Solar Eu) and acceptors (LA, HX, and Green) be selected when using the SwiftFluo® TR-FRET Toolbox reagents?

For protein-protein interaction assays, it is recommended to evaluate multiple donor-acceptor combinations to identify the most suitable pair.

If no specific preference is indicated, Eu is generally recommended as the donor and a red fluorophore as the acceptor.

Donor \ Acceptor	SwiftFluo® LA  Small-molecule red dye (MW: 800 D)	SwiftFluo® HX  6X APC (MW: 104 KD)	SwiftFluo® Green  Green dye (MW: /)
SwiftFluo® Solar Eu (Ex: 337 nm, Em: 620 nm)	✓	✓	
SwiftFluo® Solar Tb (Ex: 337 nm, Em: 490 nm/620 nm)	✓	✓	✓
SwiftFluo® Bright Eu (Ex: 337 nm, Em: 620 nm)	✓	✓	
SwiftFluo® Bright Tb (Ex: 337 nm, Em: 490 nm/620 nm)	✓	✓	✓

Excellent stability: Highly stable, generally unaffected by temperature, light, pH, ionic strength, or most biological and chemical substances.

12. What is the DMSO tolerance of the assay reagents? Can they be used for compound screening?

In typical compound screening assays, the DMSO concentration is generally kept at ≤2%. Under these conditions, the reagents are suitable for compound screening.

13. What is the typical signal-to-back ground (S/B) ratio of the TR-FRET assay kits? Are they suitable for HTS?

The S/B ratio is typically around 10, with only a small number of kits showing lower single-digit assay windows. The assay kits are suitable for high-throughput screening (HTS).

14. What is the upper limit of the assay detection window for the TR-FRET kits?

The upper limit of the detection window varies among different assay kits. Please refer to the individual kit datasheets for specific information.

15. Is TR-FRET kinase detection sensitive to ATP concentration? Is it prone to ATP competition or enzyme kinetics interference?

In TR-FRET kinase activity assays, ATP concentration primarily affects the enzymatic reaction rather than the TR-FRET fluorescence detection itself. During the reaction, with other conditions unchanged, the TR-FRET ratio increases as ATP concentration rises (up to ~300 μM). Above 300 μM, the TR-FRET ratio generally plateaus, and at excessively high ATP concentrations, the signal may decrease.

16. Are TR-FRET assays affected by pH, salt concentration, or detergents?

TR-FRET dyes (Eu/Tb) are generally insensitive to temperature, light, pH, ionic strength, and most biological and chemical reagents. However, conditions during the enzymatic reaction or protein-protein interaction phase may still be influenced by buffer components.

17. What range of binding affinities (dissociation constant, K_d) is suitable for TR-FRET detection?

There is no absolute fixed K_d range for TR-FRET detection. The suitable K_d depends on the assay model and experimental requirements. Note that K_d values measured by other methods may not directly translate to TR-FRET assays.

18. How stable are the TR-FRET assay kits, and how should they be stored?

Store reagents at the recommended temperature. From the date of customer receipt, the shelf life is one year.

Freeze-thaw stability: It is recommended to aliquot reagents and avoid repeated freeze-thaw cycles (no more than three times).

19. How should the donors and acceptors of SwiftFluo® TR-FRET be stored, and how stable are they under recommended conditions?

Lyophilized reagents should be stored at 2–8°C, and liquid reagents at –80°C. Under recommended storage conditions, reagents remain stable for one year from the date of receipt.

20. Are SwiftFluo® TR-FRET assay kits available as stock? What is the lead time, and is supply stable?

SwiftFluo® TR-FRET assay kits are available in multiple packaging formats. Small packages are usually in stock, while larger packages have a lead time of approximately 2–3 weeks. The products are supplied consistently over the long term, suitable for ongoing projects.

III. Practical Considerations

21. What types of microplates can be used in TR-FRET assays, and how should microplates be selected?

Depending on experimental requirements, 96-, 384-, or 1536-well microplates can be used. A 384-well low-volume white microplate (Cat#: SFP01) is commonly recommended.

22. In protein–ligand binding assays, can donor and acceptor reagents be pre-mixed before adding to the plate?

Yes, pre-mixing of donor and acceptor reagents is allowed.

23. What incubation conditions (time and temperature) are recommended for protein–protein interaction (PPI) assays with SwiftFluo® TR-FRET reagent pairs?

Incubation time and temperature should be selected based on the properties of the proteins. Typical conditions are 1–3 h at room temperature.

24. How should controls be set up when using TR-FRET for kinase drug high-throughput screening (HTS)?

In TR-FRET kinase drug HTS assays, negative and positive controls are typically included to calculate the assay window and Z' factor, which help evaluate assay stability.

Example Setup

Control type	Setup
Positive control	Test compound + kinase + biotinylated substrate + ATP
Negative control	Test compound + reaction buffer (no kinase) + biotinylated substrate + ATP

25. Do common protein buffer components or additives (e.g., NaCl, imidazole, glutathione, glycerol) affect the performance of SwiftFluo® TR-FRET assay kits? Does Sino Biological have compatibility data?

Common protein buffer components or additives generally do not affect the performance of the assay kits.

26. How should the optimal concentration ranges of kinase, substrate, and compounds be determined? How do concentration settings affect assay results?

● Kinase Panel Screening

Recommended kinase reaction conditions can be used as a reference.

• Recommended Reaction Conditions for TK Panel

Kinase	UniProt	Enzyme (ng/well)	Incubation time (min)	ATP (μM)	Recommended substrate	Sub. fCo (nM)	Sta. IC ₅₀ (nM, ON)	S/B (ON)	Buffer additives
EGFR	P00533	0.05	60	10	TKs substrate - biotin	400	56.63	38.33	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
EGFR [d746-750/T790M]	P00533	0.05	60	300	TKs substrate - biotin	400	1.33	24.21	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
EGFR [L858R/T790M]	P00533	0.02	60	100	TKs substrate - biotin	400	3.44	44.36	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
EGFR [T790M/C797S/L858R]	P00533	0.02	60	100	TKs substrate - biotin	400	3.8	64.91	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
FGFR1	PII362	0.02	60	50	FGFRs substrate - biotin	400	12.87	44.34	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
FGFR2	P21802	0.2	60	50	FGFRs substrate - biotin	400	7.11	26.56	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
FGFR3	P22607	0.1	60	50	FGFRs substrate - biotin	400	49.87	58.29	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
FGFR4	P22455	0.5	60	100	FGFRs substrate - biotin	400	73.83	39.47	10 mM MgCl ₂ 1 mM MnCl ₂ 2 mM DTT
VEGFR1/FLT1	PI7948	0.2	60	300	TKs substrate - biotin	400	5.89	51.22	20 mM MgCl ₂ 2 mM DTT
VEGFR2/KDR	P35968	0.02	60	300	TKs substrate - biotin	400	3.56	40.37	20 mM MgCl ₂ 2 mM DTT
FLT3	P36888	10	60	100	JAKs substrate - biotin	200	0.55	56.21	10 mM MgCl ₂ 2 mM DTT
FLT3-ITD-NPOS	P36888	5	60	100	JAKs substrate - biotin	200	0.41	58.62	10 mM MgCl ₂ 2 mM DTT
FLT3-ITD-W51	P36888	5	60	100	JAKs substrate - biotin	200	1.27	67.79	10 mM MgCl ₂ 2 mM DTT
VEGFR3/FLT4	P35916	0.1	60	300	TKs substrate - biotin	400	1.2	31.76	20 mM MgCl ₂ 2 mM DTT
JAK1	P23458	10	60	100	JAKs substrate - biotin	200	28.58	39.29	0.5 mM MnCl ₂ 2 mM DTT

Kinase	UniProt	Enzyme (ng/well)	Incubation time (min)	ATP (μM)	Recommended substrate	Sub. fCo (nM)	Sta. IC ₅₀ (nM, ON)	S/B (ON)	Buffer additives
JAK2	O60674	2	60	100	JAKs substrate - biotin	200	5.34	45.33	0.5 mM MnCl ₂ , 2 mM DTT
JAK3	P52333	0.5	60	100	JAKs substrate - biotin	200	0.48	29.12	0.5 mM MnCl ₂ , 2 mM DTT
BTK	Q06187	2	60	50	BTKs substrate - biotin	200	18.61	21.95	10 mM MgCl ₂ , 1 mM MnCl ₂ , 2 mM DTT, 10 mM EAB
BTK [C481R]	Q06187	2.5	60	300	BTKs substrate - biotin	200	3,386	43	10 mM MgCl ₂ , 1 mM MnCl ₂ , 2 mM DTT, 10 mM EAB
BTK [C481S]	Q06187	5	60	50	BTKs substrate - biotin	200	30.33	40.59	10 mM MgCl ₂ , 1 mM MnCl ₂ , 2 mM DTT, 10 mM EAB

• Recommended Reaction Conditions for CDK Panel

Kinase	UniProt	Enzyme (ng/well)	Incubation time (min)	ATP (μM)	Rb(S780)-LA (nM)	Sta. IC ₅₀ (nM, ON)	S/B (ON)	Buffer additives
CDK1 & Cyclin A2	P06493/P20248	0.02	60	50	30	11.25	46.04	5 mM MgCl ₂ , 0.5 mM TCEP
CDK1 & Cyclin B1	P06493/P14635	0.5	60	50	30	24.7	73.33	5 mM MgCl ₂ , 0.5 mM TCEP
CDK1 & Cyclin E1	P06493/P24864	0.1	60	50	30	54.15	54.73	5 mM MgCl ₂ , 0.5 mM TCEP
CDK1 & Cyclin E2	P06493/O96020	1	60	50	30	24.45	52.06	5 mM MgCl ₂ , 0.5 mM TCEP
CDK3 & Cyclin E1	Q00526/P24864	1	60	50	30	5.02	27.26	5 mM MgCl ₂ , 0.5 mM TCEP
CDK3 & Cyclin E2	Q00526/O96020	1	60	50	30	5.59	30.73	5 mM MgCl ₂ , 0.5 mM TCEP
CDK4 & Cyclin D1	P11802/P24385	25	60	300	30	331.8	14.88	5 mM MgCl ₂ , 1.0 mM TCEP
CDK4 & Cyclin D2	P11802/P30279	1	60	100	30	25.4	18.95	5 mM MgCl ₂ , 1.0 mM TCEP
CDK4 & Cyclin D3	P11802/P30281	1	60	100	30	42.1	68.22	5 mM MgCl ₂ , 1.0 mM TCEP
CDK6 & Cyclin D1	Q00534/P24385	5	60	50	30	33.93	46.2	5 mM MgCl ₂ , 1.0 mM TCEP
CDK6 & Cyclin D2	Q00534/P30279	0.5	60	100	30	18.1	51.49	5 mM MgCl ₂ , 1.0 mM TCEP
CDK6 & Cyclin D3	Q00534/P30281	0.25	60	50	30	32.92	15.33	5 mM MgCl ₂ , 1.0 mM TCEP
CDK7 & CCNH & MNAT1	P50613/P51946/P51948	20	60	100	30	31.27	51.28	5 mM MgCl ₂ , 0.5 mM TCEP
CDK9 & Cyclin K	P50750/O75909	2	60	100	30	6.3	17.07	5 mM MgCl ₂ , 1.0 mM TCEP
CDK9 & Cyclin T1	P50750/O60563	1.5	60	100	30	15.37	21.55	5 mM MgCl ₂ , 1.0 mM TCEP
CDK9 & Cyclin T2	P50750/O60583	5	60	100	30	11.91	12.73	5 mM MgCl ₂ , 1.0 mM TCEP

Kinase	UniProt	Enzyme (ng/well)	Incubation time (min)	ATP (μ M)	Rb(S780)-LA (nM)	Sta. IC ₅₀ (nM, ON)	S/B (ON)	Buffer additives
CDK10 & Cyclin M	Q15131/O8NIB3	10	60	300	30	21.47	36.62	5 mM MgCl ₂ , 1.0 mM TCEP
CDK15 & Cyclin Y	Q96040/Q8ND76	10	60	25	30	44.24	36.24	5 mM MgCl ₂ , 0.5 mM TCEP
PCTK1 (CDK16) & Cyclin Y	Q00536/Q8ND76	0.5	60	50	30	48.34	54.75	5 mM MgCl ₂ , 0.5 mM TCEP
PCTK2 (CDK17) & Cyclin Y	Q00537/Q8ND76	0.5	60	25	30	33.96	48.62	5 mM MgCl ₂ , 0.5 mM TCEP
PCTK2 (CDK17) & p35	Q00537/Q15078	10	60	25	30	22.4	33.14	5 mM MgCl ₂ , 1.0 mM TCEP
PCTK3 (CDK18) & Cyclin Y	Q07002/Q8ND76	0.5	60	100	30	161.1	65.41	5 mM MgCl ₂ , 0.5 mM TCEP

Note

Sub. fCo: Recommended final substrate concentration in a 10 μ L enzyme reaction. Sta. IC₅₀: IC₅₀ data for staurosporine. ON: Over Night.

● Single Kinase Screening

It is recommended to determine the kinase K_m for ATP first, then optimize enzyme concentration and reaction time. Substrate concentration can be fixed as recommended by the kit.

27. How do sample handling and storage conditions affect TR-FRET results? How can sample degradation or loss of activity be prevented?

In general, the effect is minimal. For labile samples, store at -80°C in aliquots and avoid repeated freeze-thaw cycles.

28. Do cell lysate or serum samples cause high background interference? How should it be handled?

Some serum samples may show high background due to high protein concentration. Diluting with buffer or using blank serum as a negative control is recommended. Cell culture media with high FBS content should also be diluted with buffer.

29. Should samples be centrifuged? Is filtration or removal of particulates necessary?

Centrifugation is recommended.

30. Are there any wash steps during the assay? Can it be performed by simply adding reagents?

TR-FRET is a homogeneous assay with no wash steps. The experiment can be completed by simply adding reagents, incubating, and reading the signal.

31. How long should the reaction be incubated? What is the minimum time before reading the signal?

Incubation time depends on the analyte. Signals can typically be read after 30 min to 1 h.

IV. Instrumentation

32. What are the instrument requirements for TR-FRET detection? What excitation/emission wavelengths are used? Can a standard microplate reader be used?

TR-FRET detection requires a microplate reader equipped with a TR-FRET module. For example, Eu/LA assays use 320/340 nm excitation and 620/665 nm emission.

33. How should delay and integration times be set for Solar Eu and Solar Tb to minimize background and autofluorescence?

Set parameters based on the instrument model, or perform a preliminary experiment using background and positive control wells to determine the optimal delay and integration times.

Example Settings for TECAN Instruments

Vendor	TECAN	TECAN
Instrument model	Infinite® 200 PRO [Ref. 30050303]	Infinite® 200 PRO [Ref. 30050303]
Mode	Fluorescence Top Reading	Fluorescence Top Reading
Excitation filter	320 (25) nm [Ref. 30094454]	320 (25) nm [Ref. 30094454]
Emission filter 1	520 (10) nm [Ref. \]	665 (8.5) nm [Ref. 30094518]
Emission filter 2	620 (10) nm [Ref. 30094505]	620 (10) nm [Ref. 30094505]
Mirror	Dichroic 510	Dichroic 510
Lag time	150 µs	150 µs
Integration Time	500 µs	500 µs
Number of reads	5 or user-defined	5 or user-defined
Gain	150 or optimal	150 or optimal
Z-focus (mm)	Can be calculated on the well giving the highest signal	Can be calculated on the well giving the highest signal

V. Data Analysis

34. How is the TR-FRET Ratio calculated?

EU/LA: TR-FRET Ratio = $665/620 \times 10000$

TB/GREEN: TR-FRET Ratio = $520/620 \times 10000$

35. Can the Z' factor be calculated? What values are typically achieved?

The Z' factor is generally ≥ 0.5

36. What should be done if the dose-response curve cannot be fitted or the EC_{50} is unstable?

First, identify the possible cause of the fitting issue, which may include compound, operational, or kit-related problems:

- Compound issue: Use a new batch or a different compound.
- Operational issue: Check for experimental errors and repeat the assay to verify.
- Kit issue: Confirm that the assay is performed within the linear range of the kit.

37. How can kinase activity or compound inhibition be accurately determined from TR-FRET signals (e.g., 665/620 ratio)?

In kinase assays, a higher 665/620 ratio indicates active kinase and increased substrate phosphorylation, while a lower ratio indicates kinase inhibition and reduced substrate phosphorylation.

VI. Experimental Troubleshooting

38. What are the possible causes of weak TR-FRET signals or low S/B, and how can it be fixed?

For commercial assay kits:

- Check experimental setup and raw data (including positive and background readings).
- Check the 620 nm reading to confirm correct reagent addition.
- Confirm instrument settings (it is recommended to confirm before starting the assay).

For in-house assay development:

- Optimize the protein and antibody pairing
 - Ensure donor and acceptor labels are at an appropriate distance for efficient TR-FRET
 - Check if antibody epitopes are sterically blocked or affected by protein conformation.
- Choose the right reaction buffer
 - Avoid buffers with high concentrations of chelators, reducing agents, or fluorescent-interfering components.
 - Compare different buffers (e.g., HEPES, PBS) if necessary to assess signal strength.
- Refer to published literature and established protocols
 - Use TR-FRET studies with similar protein types and assay formats (binding, kinase, PPI) as reference.
- Optimize key experimental parameters
 - Protein and antibody concentration ratios
 - Donor and acceptor reagent amounts
 - Incubation time and temperature
 - Total reaction volume

39. Why is the TR-FRET signal window less than 2, and how can it be increased to meet high-throughput screening requirements?

A signal window below 2 usually indicates weak positive signals, high background, or low overall signal-to-background ratio. It is generally not recommended to use a system with a signal window < 2 for subsequent screening. For HTS, the signal window should typically be stable above 2.5 (ideally ≥ 3) to ensure good reproducibility and robustness.

Before entering HTS, the TR-FRET signal window can be improved by optimizing the following key aspects:

- Optimize the protein and antibody pairing
 - Ensure donor and acceptor labels are at an appropriate distance for efficient TR-FRET
 - Check if antibody epitopes are sterically blocked or affected by protein conformation.

- Choose the right reaction buffer
 - Avoid buffers with high concentrations of chelators, reducing agents, or fluorescent-interfering components.
 - Compare different buffers (e.g., HEPES, PBS) if necessary to assess signal strength.
- Refer to published literature and established protocols
 - Use TR-FRET studies with similar protein types and assay formats (binding, kinase, PPI) as reference.
- Optimize key experimental parameters
 - Protein and antibody concentration ratios
 - Donor and acceptor reagent amounts
 - Incubation time and temperature
 - Total reaction volume

40. What causes high well-to-well variability (CV > 20%) in TR-FRET assays, and how can it be reduced?

High well-to-well CV is usually related to operational or consumable factors. Common causes and recommended measures include:

- Inaccurate pipetting: Calibrate the pipette.
- Uneven sample mixing: Gently mix multiple times with the pipette.
- Air bubbles: Centrifugation
- Microplate quality issues

41. How can non-specific binding or false positives be distinguished, and why do they occur?

Non-specific binding or false positives can be identified by testing with a known positive compound or by competing with unlabeled protein. If the signal does not decrease, the result is likely non-specific or a false positive.

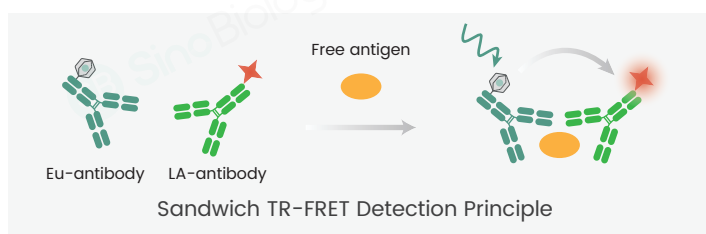
Common causes of false positives include:

- Buffer lacking surfactants (applicable to peptides or small molecules)
- Non-specific binding of compounds to proteins or antibodies (particularly His-tag antibodies)
- Compound structures affecting Eu/Tb luminescence

42. What is the Hook effect and how can it be addressed?

The Hook effect typically occurs in sandwich assays.

Sandwich assay model: The antigen binds both the capture antibody (donor) and the detection antibody (acceptor) to form a “sandwich” complex. The donor and acceptor are brought into proximity, allowing energy transfer upon donor excitation.



When antigen is in excess, some antigens bind only to the donor and some only to the acceptor, preventing formation of the complete sandwich complex and resulting in reduced FRET signal.

Mitigation strategies mainly include diluting the antigen or protein concentration and switching to a competitive assay format.

43. Do EDTA or EGTA affect kinase activity, and how can this be avoided?

EDTA and EGTA can inhibit kinase activity. During the kinase reaction, avoid using buffers containing EDTA or EGTA (it is recommended to use the kit-provided Kinase Enzymatic Buffer, which does not contain EDTA/EGTA).

Note: The kit-provided Kinase Detection Buffer contains EDTA to stop the kinase reaction.

44. Do DTT, TCEP, or other reducing agents affect donor/acceptor fluorescence?

No. DTT or TCEP are required during the kinase reaction.

VII. Support

45. Is technical support available, such as remote guidance for experiments?

Yes, technical support is available.

46. Is there a return or replacement policy?

Products with quality issues can be returned, exchanged, or refunded.

**Sino Biological US Inc. (U.S.A.)**

Address: 37 Industrial blvd., suite B, Paoli, PA 19301

Tel: +1-215-583-7898 Fax: +1-267-657-0217

Email: order_us@sinobiologicalus.com

Sino Biological Europe GmbH (Europe)

Düsseldorfer Str. 40, 65760 Eschborn, Germany

Tel: +49(0)6196 9678656

Fax: +49(0)6196 9678657

Email: order_eu@sinobiologicaleu.com

株式会社日本シノバイオロジカル (Japan)

〒2130001 神奈川県川崎市高津区溝口1-19-11 グランデール溝口706

ホームページ: jp.sinobiological.com

お問い合わせ: info@sinobiological.co.jp

ご注文: order@sinobiological.co.jp

受託サービス: cro-service@sinobiological.co.jp

電話番号: 044-400-1330

(주)시노바이오로지컬코리아 (Korea)

Address: Room 1932-1933 (Rosedale, Suseo-dong), 280

Gwangpyeong-ro, Gangnam-gu, Seoul, Republic of Korea

Tel: 02-6339-5150

Email: order@sinobiological.co.kr

Sino Biological, Inc. (Global)

Address: Building 9, No.18 Kechuang 10th St, BDA

Beijing, 100176, P.R.China

Tel: +86-400-890-9989

Fax: +86-10-5095-3282

Email: order@sinobiological.com

www.sinobiological.com

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