TR-FRET Assay Kits Simplify and Accelerate Drug Discovery

BPS Bioscience

Introduction

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) is a powerful technique commonly used to analyze the binding of two interacting molecules. Since most biological responses involve an interaction between at least two partners, TR-FRET is well suited to study a wide range of events, including many that characterize cellular signaling pathways. Possible applications are limitless: interaction between two proteins, between a receptor and its ligand or between an enzyme and its substrate; binding of a drug to its target or binding of a nucleic acid to a protein; measure of post-transcriptional modifications; and more.

The technology is a combination of Time-Resolved Fluorescence and Förster's Resonance Energy Transfer, a phenomenon in which a light-excited fluorophore can transfer its absorbed energy to a nearby acceptor fluorophore.

This eBook provides the technical bases of TR-FRET technology, with a few examples of assay design and validation data.

FRET

Fluorophores absorb high-energy light and emit light of lower energy than the absorbed light. Fluorescence Resonance Energy Transfer (FRET) is a phenomenon in which two fluorophores emitting at different wavelengths are coupled: the donor fluorophore excited by a high energy source transfers energy (not light) to an acceptor fluorophore. This results in excitation of the acceptor and fluorescence emission at the wavelengths inherent to the properties of the acceptor fluorophore.

TR-FRET technology takes advantage of the fact that the transfer of energy between the donor and the acceptor depends on physical proximity (<10 nm) and decreases rapidly with distance. Thus, partner molecules distributed in a solution are sufficiently far apart that FRET does not occur.

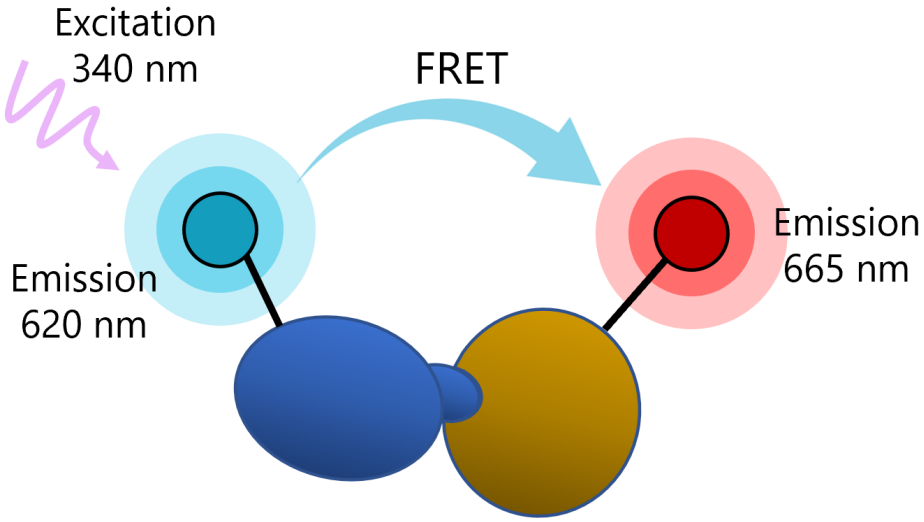


Figure 1: Illustration of FRET principle. In practice, one of the binding partners in the interaction of interest is labeled with a donor fluorophore such as a Europium chelate, whereas the other partner is labeled with an acceptor fluorophore. When direct labeling of the partners is not possible, the molecule of interest is tagged or biotinylated and the pairing is completed using streptavidin-coated donor or acceptor, or anti-tag antibodies.

Upon interaction, the partners complete the FRET pairing as they are now in proximity of each other.

Thus, FRET is applicable to any two interacting molecules, or to reactions in which a new molecular form appears providing that the new molecular form can be distinguished from the initial molecule. Examples include post-transcriptional modifications such as ubiquitination, methylation, or phosphorylation. The molecules under study can be directly labeled with a donor or acceptor fluorophore. Alternatively, a secondary reagent (for example, an antibody) that binds to the molecule of interest can be labeled with one of the fluorophores for indirect detection. This provides great flexibility regarding the design of an assay.

Time-Resolved Fluorescence

FRET has a lower background signal than classic fluorescence methods because the acceptor emission may not share much spectral overlap with the excitation pulse. The donor and the acceptor, on the other hand, must have good spectral overlap (the emission range of the first must overlap with the excitation range of the second), as well as good spectral resolution for a specific signal to be measured. However, it is the Time Resolved Fluorescence (TRF) technology element that allows for the ultra-low background advantage of TR-FRET.

Classic fluorescence intensity uses short-lived fluorophores such as fluorescein, with an emission speed in the order of the nanosecond. Excitation and emission occur at specific wavelengths that can be differentiated by a fluorescence reader. However, excitation and emission happen at the same time. If there is any amount of spectral overlap between excitation and emission, as there usually is, the reader will capture some of the excitation fluorescence, resulting in background signal and low signal-to-noise ratios.

TRF solves this by using long-lived inorganic fluorophores as donors and adding a time delay between excitation and measurement, which means that the excitation signal is gone by the time of the measurement, which considerably decreases background signals (Figure 2).

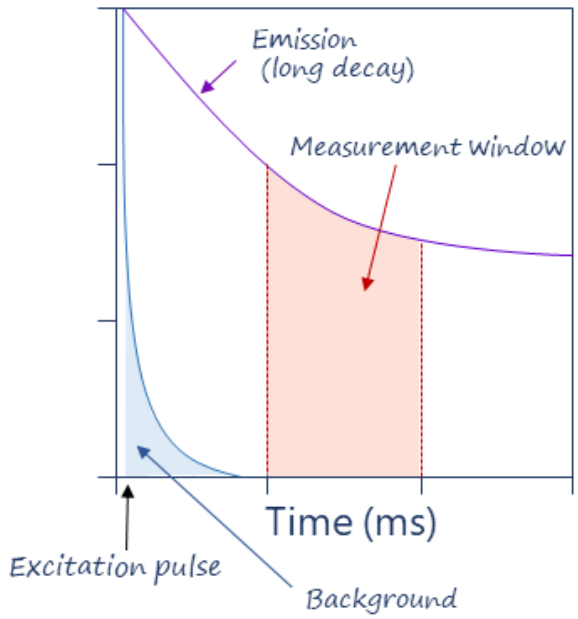


Figure 2: Illustration of TRF principle.

TRF also uses excitation pulses (not continuous excitation), so that a series of measurements are repeated over time. It eliminates the very transient background fluorescence generated from sample components such as buffers, proteins, and chemicals, which hinders classic FRET methods.

Ideal fluorophores have high signal intensity, are highly stable, and offer excellent signal-to-noise ratios. The most commonly used are "Lanthanide probes" which are metal ions referring to elements Cerium to Lutetium in the periodic table,

more specifically Europium and Terbium, which fluoresce over milliseconds instead of nanoseconds. Experimentally, a comparison measurement of the two emitted wavelengths over time needs to be calculated.

Monitoring BCL-2 binding to ligand

BCL-2 (B-cell lymphoma 2), a member of the BCL family, is an integral protein of the outer mitochondrial membrane. BCL-2 family members form hetero- or homodimers that regulate apoptosis. The main function of BCL-2 is to inhibit apoptosis and promote cell survival through control of mitochondrial membrane permeability, blocking the release of cytochrome c from mitochondria by pro-apoptotic BH3-containing proteins, and inhibition of caspase activity. However, BCL-2 may either promote or suppress apoptosis depending on context and partners. Constitutive expression of BCL-2 in B lymphocytes, caused by translocation of the *BCL2* gene to the immunoglobulin heavy chain locus, promotes follicular lymphoma. The protein contributes to cancer resistance to treatment in leukemia, melanoma, and breast and prostate cancer, owing to its pro-survival effects. Inhibitors used in the clinic are BH3-mimetic molecules that prevent BCL-2 interaction with its BH3-type partners. Navitoclax (ABT-263) inhibits BCL-2, BCL-xL, and BCL-w, while venetoclax (ABT-199) is highly selective of BCL-2.

As shown in Figure 3, a TR-FRET assay was designed to monitor BCL-2 binding to its peptide ligand in a homogeneous 384-well format. In this assay, a terbium-labeled anti-His-tag antibody is used as the donor fluorophore. The antibody binds to His-tagged BCL-2, while the peptide ligand is labeled with biotin which allows binding to the dye-labeled streptavidin acceptor. The TR-FRET signal is generated by proximity induced upon interaction of the protein with the peptide ligand. The assay kit can be used to screen for small molecules that inhibit the interaction of BCL-2 with its partner peptide or to determine the IC_{50} of candidate inhibitors.

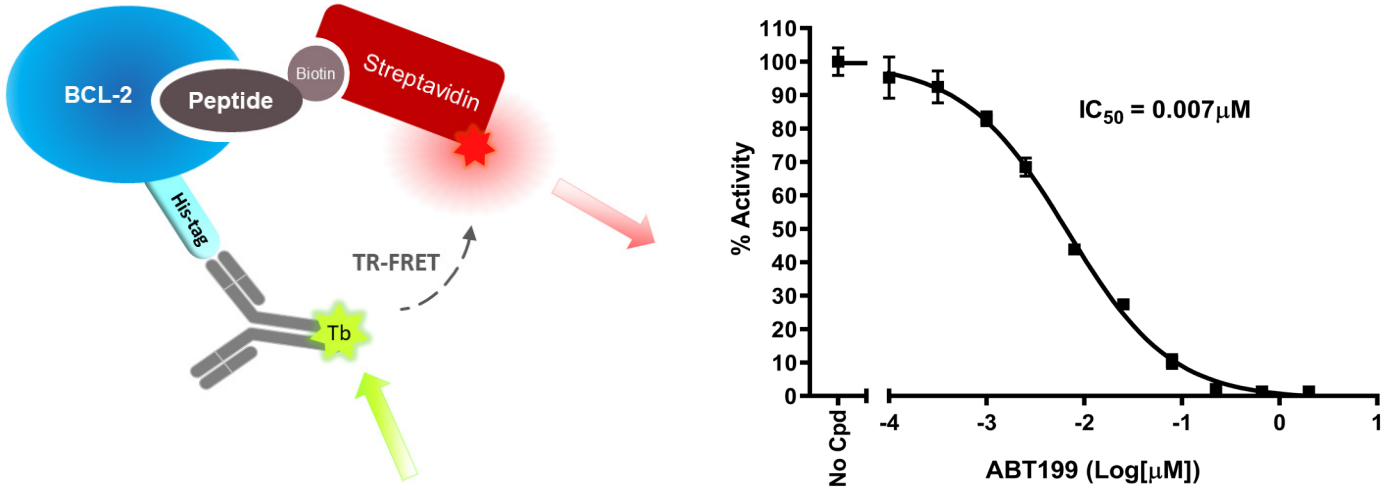
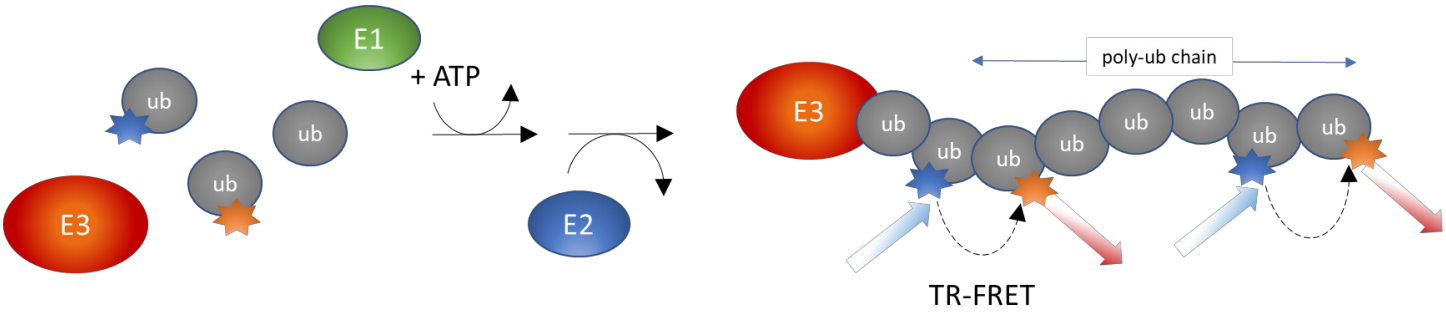


Figure 3, left panel: Illustration of the assay principle (BPS Bioscience #50222). Right panel: Example data. Increasing concentrations of ABT199 were added to a terbium-labeled donor, a dye-labeled acceptor, purified His-tagged BCL-2 protein, peptide ligand, and incubated for 3 hours before TR-FRET reading. Two sequential measurements were performed: Tb-donor emission was measured at 620 nm followed by dye-acceptor emission at 665 nm.

Measuring ubiquitination

Covalent conjugation of ubiquitin (Ub) to a protein is a common post-translational modification that regulates protein stability, function, and localization. Ubiquitination is the concerted action of three enzymes: a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase (E3). The specificity and efficiency of ubiquitination are determined by the E3 enzyme, which directs the last step of the conjugation cascade by binding to both an E2-Ub conjugate and a substrate protein, leading to its mono- or poly-ubiquitination.

The Intrachain TR-FRET Assay kits were designed to measure the auto-ubiquitination of a specific E3 enzyme in a homogeneous 384-well format. The E3 ligase, such as NEDD4, MDM2, VHL (Von Hippel Lindau), is affinity purified. The assays use a Europium-labeled Ub donor and a Cy5-labeled Ub acceptor to complete the TR-FRET pairing. Since both the donor and acceptor are incorporated into poly-ubiquitin chains forming on the E3 enzyme, the assays measure poly-ubiquitination and not mono-ubiquitination. They are used for high-throughput screening of E3 ligase inhibitors, to perform real-time kinetic analyses, or to accurately determine the IC_{50} of a compound.



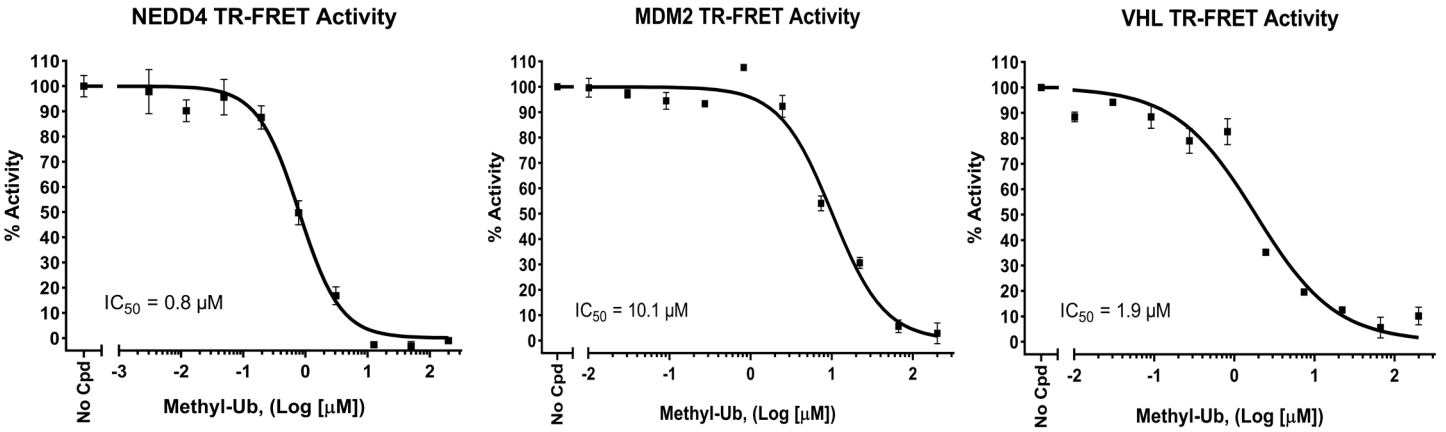


Figure 4, upper panel: Illustration of the assay principle. Lower panels: Increasing concentrations of methyl-ubiquitin were added to a Europium-labeled donor, a Cy5-labeled acceptor, purified E1 and E2 proteins and a purified E3 ligase (NEDD4, MDM2 or VHL) in the presence of ATP, and incubated for 2 hours prior to TR-FRET reading. Two sequential measurements were performed: donor emission was measured at 620 nm followed by dye-acceptor emission at 665 nm.

Measuring the activity of RdRp

The pandemic coronavirus disease 2019 (COVID-19) is caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). One of the most promising druggable SARS-CoV-2 targets is the RNA-dependent RNA Polymerase (RdRp), a crucial enzyme in the life cycle of coronaviruses which operates as a complex of NSP12, NSP7, and NSP8 viral proteins.

The RdRp TR-FRET Assay kit is designed to measure RdRp-mediated incorporation of biotinylated ATP into a double-stranded RNA substrate. The increase in TR-FRET signal is proportional to the amount of ATP incorporated in the RNA and therefore directly measures enzymatic activity. This assay is ideal for high-throughput screening of enzyme inhibitors, to perform kinetic studies, or to accurately measure a drug IC_{50}.

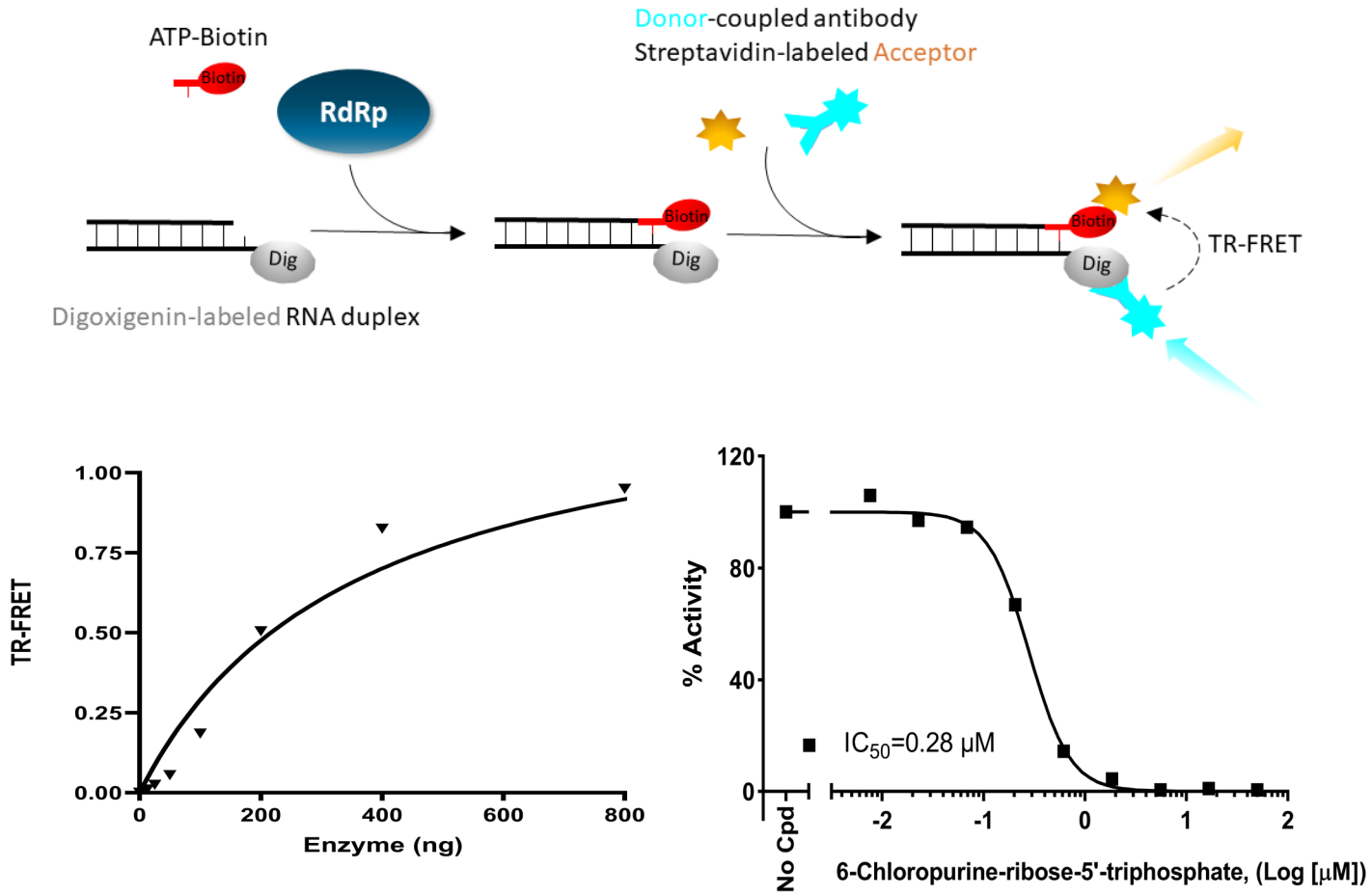
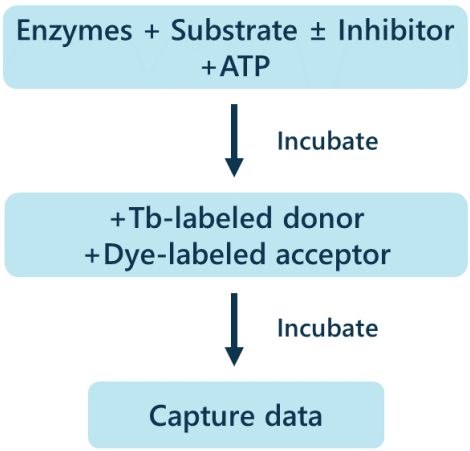


Figure 5, upper panel: Illustration of the assay principle (BPS Bioscience #78553). Lower panels: the assay was performed in two steps. First, a test compound was incubated with the purified RdRp enzyme in a reaction mixture consisting of optimized buffers, substrate, and biotinylated ATP. Next, a dye-labeled acceptor and a Europium-labeled anti-Digoxigenin antibody were added for one hour before TR-FRET reading. Two sequential measurements were performed: donor emission was measured at 620 nm followed by dye-acceptor emission at 665 nm. The experiment was performed using increasing amounts of enzyme (lower left panel) or in the presence of increasing concentrations of an inhibitor (lower right panel).

Conclusion

TR-FRET is an ultra-low background technique allowing the measurement of any reaction in which two labeled entities come in proximity. The main drawback of the technique is that it requires two optimized labeled entities, in addition to a low dynamic range. However, these drawbacks are offset by several advantages:

- Small volumes
- Homogeneous: no need for washing steps or for physical separation from the unbound entities
- Robust, sensitive signal
- Ultra-low background with high signal-to-noise ratio
- Stable signal: use of lanthanide donor fluorophores minimizes photobleaching



Simple operation

The growing commercial availability of ready-to-use TR-FRET immunoassay kits has opened the technique to mainstream use. BPS Bioscience offers over 80 assay kits for drug discovery in the TR-FRET format with new products developed regularly. Optimized, validated, high quality assay kits ensure reliable results, quickly.

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