LanthaScreen™ DUB Substrate User Guide

Shipping: Dry Ice | **Storage: -80°C**

Part no. PV444X.pps | Rev. date: 11 April 2006

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1. REAGENTS AVAILABLE

Reagents	Size	Cat. No.
LanthaScreen™ DUB Substrate	10 µg	PV4443
	100 µg	PV4444

2. INTRODUCTION

When screening libraries of compounds, time-resolved FRET (TR-FRET) is a recognized method for overcoming interference from compound autofluorescence or light scatter from precipitated compounds. The premise of a TR-FRET assay is the same as that of a standard FRET assay: when a suitable pair of fluorophores are brought within proximity of one another, excitation of the first fluorophore (the donor) results in energy transfer to the second fluorophore (the acceptor). This energy transfer is detected by an increase in the fluorescence emission of the acceptor, and a decrease in the fluorescence emission of the donor. In high-throughput screening (HTS) assays, FRET is often expressed as a ratio of the intensities of the acceptor and donor fluorophores. The ratiometric nature of such a value corrects for differences in assay volumes between wells as well as quenching effects due to colored compounds.

TR-FRET assays use a long-lifetime lanthanide chelate as the donor species. Lanthanide chelates are unique in that their excited state lifetime (the average time that the molecule spends in the excited state after accepting a photon) can be on the order of a millisecond or longer. This is in sharp contrast to the lifetime of common fluorophores used in standard FRET assays, which are typically in the nanosecond range. Because interference from autofluorescent compounds or scattered light is also on the nanosecond timescale, these factors can negatively impact standard FRET assays. To overcome these interferences, TR-FRET assays are performed by measuring FRET after a suitable delay, typically 50 to 100 microseconds after excitation by a flashlamp excitation source in a microtiter plate reader. This delay not only overcomes interference from background fluorescence or light scatter, but also avoids interference from direct excitation due to the non-instantaneous nature of the flashlamp excitation source.

The most common lanthanides used in TR-FRET assays for HTS are terbium and europium. Terbium offers unique advantages over europium when used as the donor species in a TR-FRET assay. In contrast to europium-based systems that employ APC as the acceptor, terbium-based TR-FRET assays can use common fluorophores such as fluorescein as the acceptor. Because it is straightforward (and inexpensive) to label a molecule such as a peptide with fluorescein, directly labeled biomolecules may be used in terbium-based TR-FRET assays, rather than biotinylated molecules that must then be indirectly labeled via streptavidin-mediated recruitment of APC. The use of directly labeled molecules in a terbium-based TR-FRET assay reduces costs, improves kinetics, avoids problems due to steric interactions involving large APC conjugates, and simplifies assay development, since there are fewer independent variables requiring optimization in a directly labeled system.

Furthermore, unlike europium-based assays, green fluorescent protein (GFP) and its variants can be used as an acceptor in terbium-based assays, enabling use of genetically encoded substrates. Thus, production of GFP-fusions provides a convenient,

controlled means of producing an acceptor-labeled protein substrate. One advantage of GFP-fusions is that the acceptor is located in a defined position and is present at a 1:1 ratio with respect to the substrate.

A complete guide to commonly asked questions and answers regarding the LanthaScreen™ technology can be found at www.invitrogen.com/lanthascreen.

3. INSTRUMENT SETTINGS

The excitation and emission spectra of terbium and the GFP variant commonly referred to as yellow fluorescent protein (YFP) are shown below in Figure 1. YFP is used in the LanthaScreen™ DUB Substrate. As with other TR-FRET systems, the terbium donor is excited using a 340-nm excitation filter with a 30-nm bandwidth. However, the exact specifications of the excitation filter are not critical, and filters with similar specifications will work well. In general, excitation filters that work with europium-based TR-FRET systems will perform well with the LanthaScreen™ terbium chelates.

As is shown in the figure, the terbium emission spectrum is characterized by four sharp emission peaks, with silent regions between each peak. The first terbium emission peak (centered between approximately 485 and 505 nm) overlaps with the excitation peak of YFP. Energy transfer to YFP is measured in the silent region between the first two terbium emission peaks.

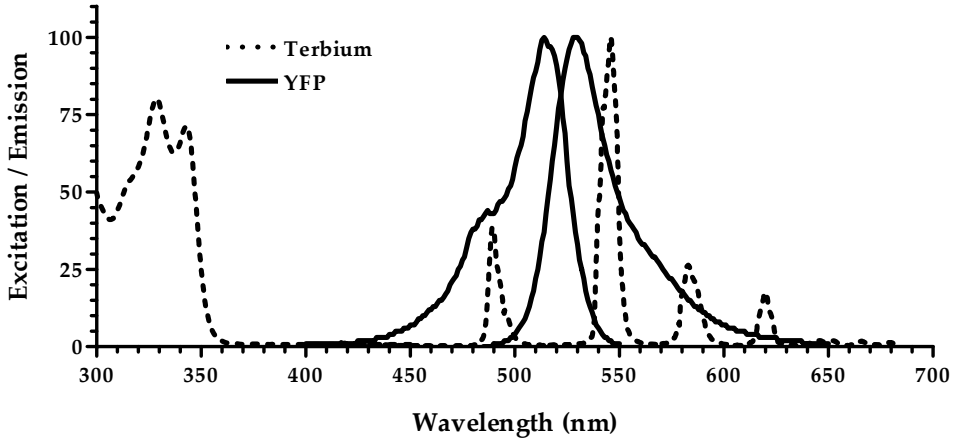

Figure 1. Excitation and Emission spectra of YFP and terbium.

Because it is important to measure energy transfer to YFP without interference from terbium, a filter centered at 520 nm with a 25 nm bandwidth is used for this purpose. The specifications of this filter are more critical than those of the excitation filter. In general, **standard "fluorescein" filters may not be used**, because such filters also pass light associated with the terbium spectra as well. The emission of YFP due to FRET is referenced (or "ratioed") to the emission of the first terbium peak, using a filter that isolates this peak. This is typically accomplished with a filter centered at 490 or 495 nm, with a 10 nm bandwidth. In general, a 490 nm filter will reduce the amount of YFP emission that "bleeds through" into this measurement, although instrument dichroic mirror choices (such as those on the Tecan Ultra instrument) may necessitate the use of a 495 nm filter. The effect on the quality of the resulting measurements is minimal in either case. The dichroic mirror should ideally have a cutoff of less than 490 nm. A dichroic mirror meeting this specification is available from Chroma as part of filter set PV001, or from the instrument manufacturer. In some instruments (Tecan instruments, for example) the fluorescein dichroic performs well but must be manually selected. In other instruments a 50:50 mirror may be selected, but in general will not give as high performance as a dedicated dichroic. The LanthaScreen™ filter module from BMG contains an optimal dichroic mirror. Filters suitable for LanthaScreen™ assays are available from Chroma (www.chroma.com) as filter set PV001, or from other vendors. A LanthaScreen™ filter module for the BMG LABTECH PHERAstar is available direct from BMG LABTECH.

Aside from filter choices, instrument settings are typical of the settings used with europium-based technologies. In general, guidelines provided by the instrument manufacturer can be used as a starting point for optimization. A delay time of 100 µs, followed by a 200 µs integration time, would be typical for a LanthaScreen™ assay. The number of flashes or measurements per well is highly instrument dependant and should be set as advised by your instrument manufacturer. In general, LanthaScreen™ assays can be run on any filter-based instrument capable of time-resolved FRET, such as the Tecan Ultra, BMG LABTECH PHERAStar, Molecular Devices Analyst, or PerkinElmer Envision. LanthaScreen™ assays have also been performed successfully on the Tecan Safire^2 monochromator-based instrument and the Molecular Devices M5 instrument. Contact Invitrogen Technical Services for instrument-specific setup guidelines.

4. DESCRIPTION OF LANTHASCREEN™ DUB SUBSTRATE

Deubiquitinating enzymes (DUBs), also referred to as isopeptidases or ubiquitin-specific proteases, are a large group of proteases that specifically cleave peptide or isopeptide bonds at the ubiquitin C-terminus. These proteases are believed to play both "housekeeping" functions by maintaining pools of active ubiquitin as well as regulatory functions by rescuing specific target proteins from degradation by the proteasome via removal of ubiquitin from the target protein. The LanthaScreen™ DUB Substrate enables a sensitive, robust, and general assay for DUBs. The substrate is an N-terminal YFP fusion of ubiquitin with a short C-terminal extension containing an engineered cysteine residue that has been labeled with a terbium chelate (Figure 2). There is a high degree of FRET in the intact substrate, and a very low FRET signal after cleavage at the ubiquitin C-terminus by a DUB (Figure 3). The substrate contains a relatively small peptide leaving group and thus is expected to be a general substrate for both USP and UCH family enzymes, some of which have been shown to prefer small, flexible leaving groups *in vitro*.

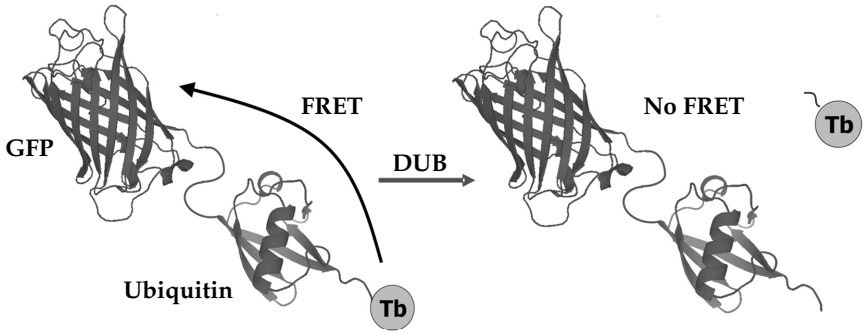

Figure 2. Graphical representation of the LanthaScreen™ DUB substrate, before and after cleavage by a DUB.

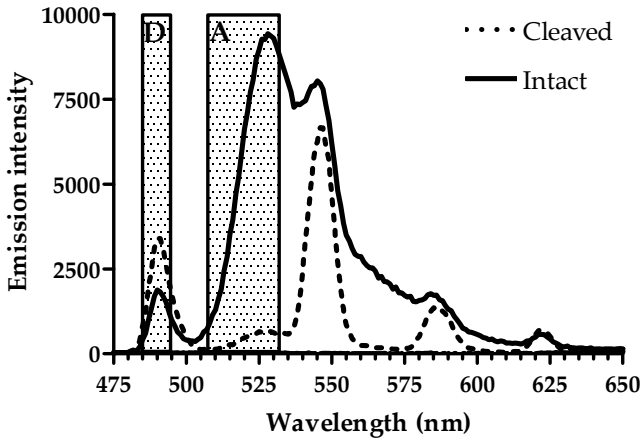

Figure 3. Emission spectra of 10 nM LanthaScreen™ DUB substrate, before and after cleavage by the model DUB, UCH-L3. Donor emission (D) is typically measured at 495 nm and acceptor emission intensity is measured at 520 nm (A). Results are typically expressed as a ratio of acceptor emission to donor emission (A:D).

5. APPLICATIONS OF LANTHASCREEN™ DUB SUBSTRATE

The LanthaScreen™ DUB Substrate is a general DUB substrate that is cleaved by both USP and UCH family enzymes (Figure 4) and can be used in either a kinetic or end-point assay (Figures 4 and 5). Due to the TR-FRET readout, the substrate is ideally suited for screening compound libraries and examining the potency and selectivity of inhibitors (Figures 6 and 7). Because the substrate exhibits a very strong change in FRET upon cleavage, high Z' values are obtained with as little as 20% of the substrate consumed. (Table 1).

For most applications, such as compound screening and profiling, the ratio of acceptor fluorescence to donor fluorescence is calculated, which increases precision and minimizes interference from color quenchers. For some applications, it is useful to

determine the exact percent of substrate converted, which can be calculated based solely on the acceptor emission (E_{520nm}) by equation 1.

Equation 1.

$$\text{\% conversion of substrate at ``}X\text{'' concentration of protease} = \frac{(E_{520nm} \text{ without protease} - E_{520nm} \text{ with ``}X\text{'' protease}) \times 100}{(E_{520nm} \text{ without protease} - E_{520nm} \text{ with excess protease})}$$

The recommended concentration of substrate for screening and inhibitor profiling is 10 nM, which provides a strong FRET signal comparable to other robust TR-FRET assays. Because K_m values for most DUBs are expected to be in the micromolar range, increasing the amount of substrate beyond 10 nM would increase initial velocities of the reaction, but would actually not increase the sensitivity assay with respect to the amount of protease required. This result is due to the fact that the read-out is based on the *percent* of substrate converted, which is independent of the substrate concentration in this case.

Some consideration should be given to specific assay parameters, including reducing agents, pH, stop reagents, and plate type. Some DUBs will require a reducing agent such DTT or TCEP to maximize activity. For the classical DUB enzymes of the cysteine protease UCH and USP families, a thiol reactive compound such as N-methylmaleimide (NMM) or iodoacetamide (IAA) can be added to stop the reaction, if necessary. The concentration NMM or IAA must be sufficient to react with any DTT in the buffer in addition inactivating the enzyme. LanthaScreen™ TR-FRET dilution buffer (PV3574) supplemented with DTT has been used successfully to perform DUB assays, though buffer selection will likely be enzyme-specific. Buffers with a pH of 7.0 or higher are recommended, as YFP fluorescence decreases below pH 7.0. Invitrogen recommends black Corning® 384-well, low-volume, round-bottom (non-binding surface) assay plates (#3676). Other black-walled, low-binding assay plates, while not tested, may be suitable.

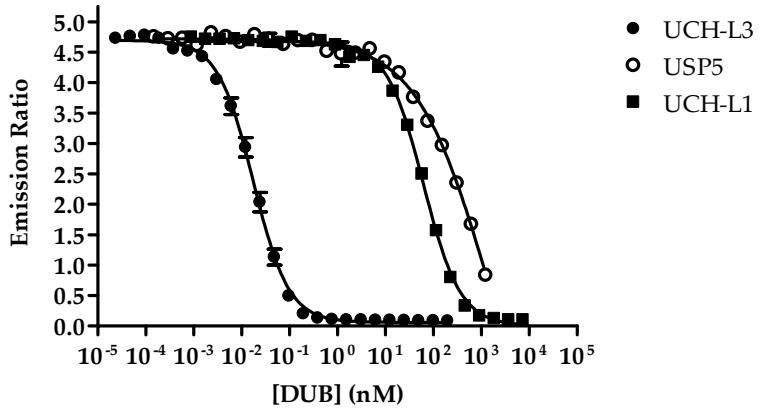

Figure 4. Protease titrations. Assays were performed for three DUBs at various concentrations of protease and with 10 nM LanthaScreen™ DUB Substrate, and 1 mM DTT in LanthaScreen™ TR-FRET dilution buffer. 20 µL reactions were performed in 384 well plates (Corning 3676) and read following a 60 minute incubation in a BMG LABTECH PHERAstar.

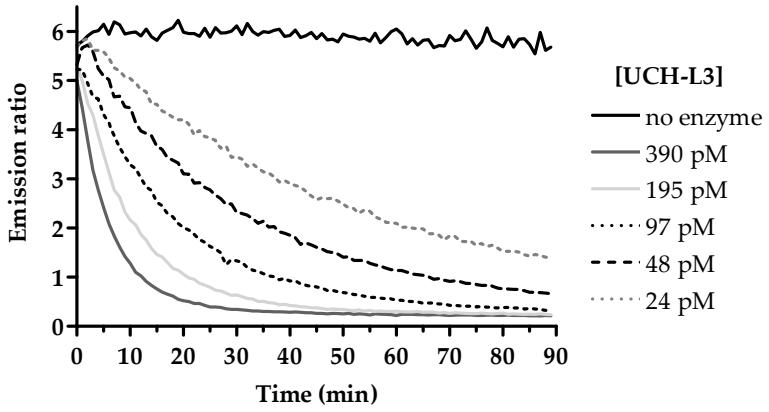

Figure 5. Real-time measurements of DUB activity. Assays were performed at various concentrations of UCH-L3 essentially as described in Figure 4 and measured continuously.

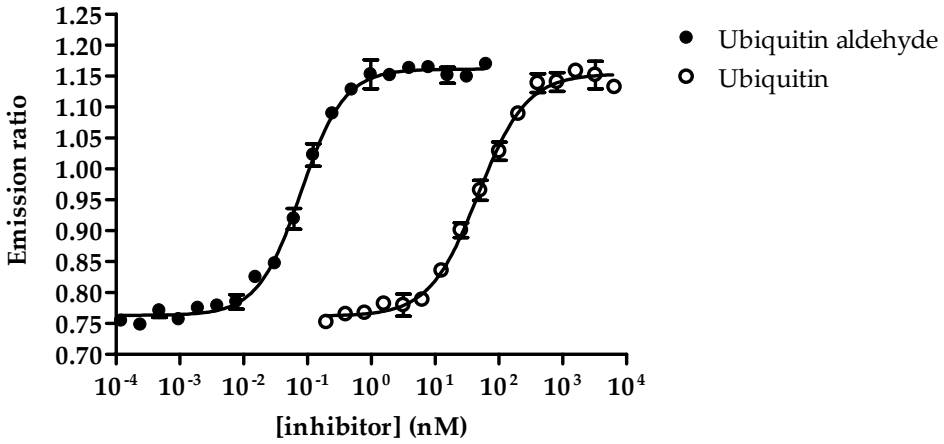

Figure 6. Inhibitor titrations. Protease assays of 20 µL each were performed as in Figure 4, except at a fixed concentration of UCH-L3 (15 pM) and with various concentrations of the competitive inhibitors ubiquitin and ubiquitin aldehyde. Based on emission at 520 nm, approximately 27% of the substrate was converted to product in absence of the inhibitors.

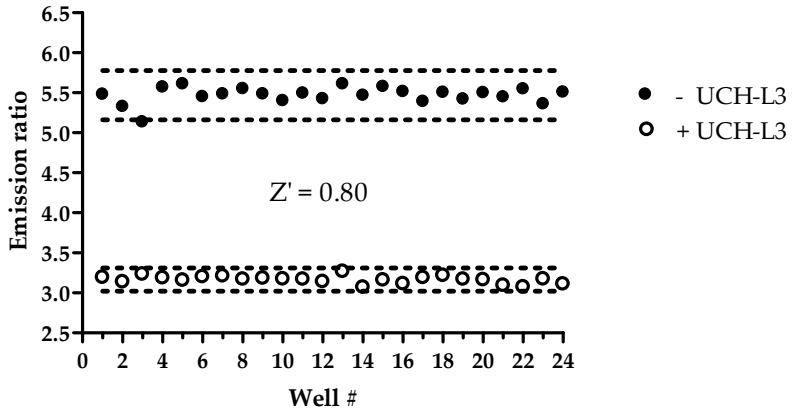

Figure 7. Example Z′ factor determination for UCH-L3. Protease assays of 20 µL each were performed as in Figure 4, except the concentration of UCH-L3 was fixed. Calculations were based on 24 replicate reactions with UCH-L3 and 24 control reactions without protease. Under these screening conditions, 29% of the substrate was converted to product.

% substrate converted	Z′ factor
14%	0.15
17%	0.53
29%	0.77
32%	0.80
42%	0.83
47%	0.83
60%	0.89
80%	0.92

Table 1. Z′ factors were calculated for UCH-L3 at various enzyme concentrations. Reactions were performed as described in Figure 4. Calculations were based on 24 replicate reactions performed at each concentration of UCH-L3 and 24 control reactions without protease.

6. ASSESSING DATA QUALITY IN RATIOMETRIC MEASUREMENTS

The TR-FRET value is a unitless ratio derived from the underlying donor and acceptor signals. Because the underlying donor and acceptor signals are dependant on instrument settings (such as instrument gain), the TR-FRET ratio and the resulting "top" and "bottom" of an assay window will depend on these settings as well, and will vary from instrument to instrument.

What is important in determining the robustness of an assay is not the size of the window as much as the size of the errors in the data relative to the difference in the maximum and minimum values. For this reason, the "Z prime" value (Z′) proposed by Zhang and colleagues (Zhang *et al.*, 1999), which takes these factors into account, is the correct way to assess data quality in a TR-FRET assay.

7. RELATED PRODUCTS

Reagents	Volume	Cat. No.
LanthaScreen™ TR-FRET Dilution Buffer	100 ml	PV3574
LanthaScreen™ Tb-Streptavidin, 1 mg/ml	50 µg	PV3576
	1 mg	PV3577
LanthaScreen™ Tb-Ubiquitin	5 g	PV4375
	25 g	PV4376
Fluorescein-Ubiquitin	50 g	PV4377
	500 g	PV4378
Biotin-Ubiquitin	10 g	PV4379
	100 g	PV4380
LanthaScreen™ Tb-anti-GST Antibody	25 µg	PV4216
	1 mg	PV4217
LanthaScreen™ Tb-anti-His-Tag Antibody	25 µg	PV3568
	1 mg	PV3569
LanthaScreen™ Tb-anti-Mouse Antibody	25 µg	PV3765
	1 mg	PV3767
LanthaScreen™ Tb-anti-Goat Antibody	25 µg	PV3769
	1 mg	PV3771
LanthaScreen™ Tb-anti-Rabbit Antibody	25 µg	PV3773
	1 mg	PV3775
LanthaScreen™ Tb-anti-Human Antibody	25 µg	PV3777
	1 mg	PV3779
LanthaScreen™ Amine Reactive Tb Chelate	10 µg	PV3583
	100 µg	PV3582
	1 mg	PV3581
LanthaScreen™ Thiol Reactive Tb Chelate	10 µg	PV3580
	100 µg	PV3579
	1 mg	PV3578

For a complete, up-to-date listing of products, visit www.invitrogen.com/lanthascreen.

8. REFERENCES

Zhang, J. H., Chung, T. D., and Oldenburg, K. R. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screen.*, *4*, 67-73

9. NOTICE TO PURCHASER

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