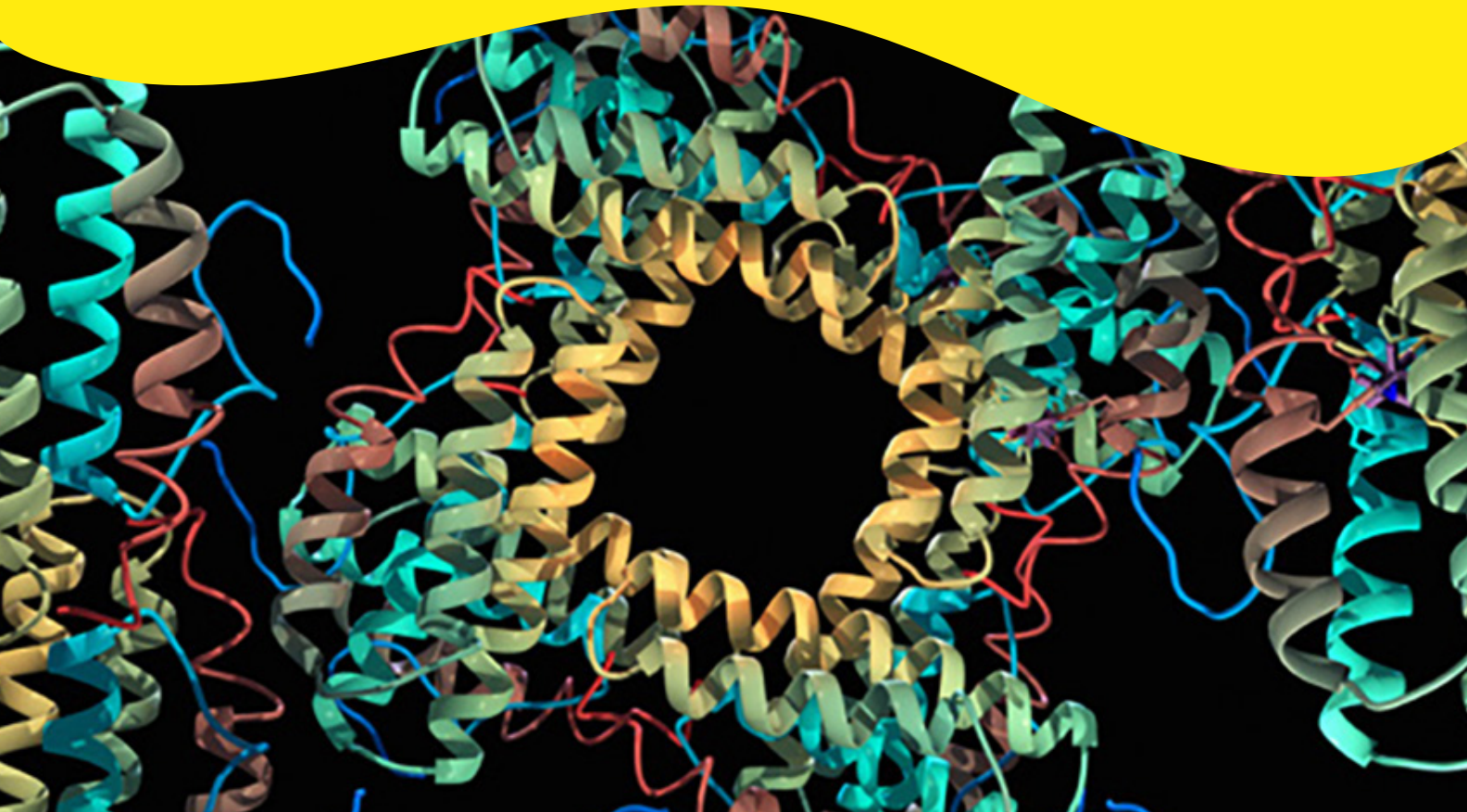
LANCE TR-FRET protein:protein interaction guide



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LANCE TR-FRET protein:protein interaction

Introduction

The interactions and binding of proteins (Protein-Protein Interactions, PPIs) are implicated in a large number of biological processes; thus the need for efficient, highly sensitive assays to study large protein interactions has become an exceedingly important part of the Drug Discovery process.

LANCE® (Lanthanide Chelate Excitation) is a TR-FRET technology that combines the ease of use and sensitivity of fluorescence along with the reduced background afforded by its time-resolved nature. It offers the ability to assay a wide variety of biological targets, including enzymes, receptor-ligand interactions, second-messenger levels, DNA, RNA, proteins, peptides, sugars, and small molecules.

LANCE and LANCE *Ultra* assays require two fluorophores, a donor and an acceptor, that energy may be transferred between. The donor (a chelated Lanthanide Europium) is excited by either a flash lamp or a laser at a wavelength of either 320 or 340 nm, which will cause an emission at 615 nm. This Europium emission can then go on to excite the acceptor dye (U*Light*™ or APC) if it's in close enough proximity (10 nm or less), which results in emission of light at 665 nm. Because the energy transfer requires close proximity, it is unnecessary to remove unbound binding partners or dyes, conferring the homogeneous, no-wash nature of the assay and making the technology highly amenable to automation (in contrast to ELISA and ECL, which require multiple wash steps).

In a LANCE protein-protein interaction assay, one protein is labeled, either directly or indirectly, by the donor, and the other by the acceptor. If the two proteins interact such that the donor and acceptor are brought into close proximity to each other, a signal can be generated. If the two proteins don't interact, or if their interaction is inhibited, this results in a corresponding reduction of signal. Both the donor and acceptor are small molecular entities that may be directly conjugated to a target of interest or indirectly targeted through the use of affinity reagents (e.g., labeling an anti-tag antibody or even antibody specific to the target of interest), thus enabling the detection of a myriad of unique biological events.

LANCE technology offers many features that make it a rapid, robust, reliable, and reproducible assay platform. It is a homogeneous assay format, thus requiring no wash steps, making interrogating low-affinity interactions much easier. The assay background is low due to its time-resolved nature, and the assay signal is stable for many days. Assay miniaturization is also quite simple – just keep all components at the same final concentrations in whatever volume you like for your plate type, whether your final assay volume is 100 µL in a 96-well plate or 6 µL in a 1536-well plate.

Toolbox reagents

LANCE europium donor reagents	U*Light* and SureLight® APC acceptor reagents	
Eu-anti-c-myc	U*Light*-anti-c-myc	APC-anti-FLAG
Eu-anti-FITC	U*Light*-anti-FITC	APC-anti-GST
Eu-anti-FLAG	U*Light*-anti-FLAG	APC-anti-6X His
Eu-anti-GST	U*Light*-anti-GST	APC-anti-mouse IgG
Eu-anti-HA	U*Light*-anti-6X His	APC-anti-rabbit IgG
Eu-anti-6X His	U*Light*-anti-Protein C tag	APC-streptavidin
Eu-anti-Protein C tag	U*Light*-anti-V5 tag	
Eu-anti-V5 tag	U*Light*-anti-human IgG	
Eu-anti-human IgG	U*Light*-anti-mouse IgG	
Eu-anti-mouse IgG	U*Light*-anti-rabbit IgG	
Eu-anti-rabbit IgG	U*Light*-Protein A	
Eu-Protein G	U*Light*-Streptavidin	
Eu-Streptavidin		

Assay design principles

The first goal of early assay development is to identify a configuration for your assay. Sometimes only one version of one or more of your binding partners is available, which helps narrow down the array of possible reagents that may be used. In this guide we use Glutathione-S-Transferase (GST) tagged MDM2 (Boston Biochem #E3-202) and Poly-Histidine (His6) tagged p53 (Boston Biochem #SP-450) as our binding partners, and some of our toolbox LANCE reagents that are directed against those affinity tags, as an alternative to directly labeling either molecule. Because the reagents targeting these affinity tags can be labeled with either the FRET donor or the FRET acceptor, there are multiple combinations that can be chosen and should be tested to determine which combination results in the best assay parameters.

In the case of the assay developed here, there were four possible combinations of FRET reagents that could have been tested. Figures 1 and 2 illustrate the two combinations that are possible in a LANCE *Ultra* assay configuration, where the U*Light* dye is used as the acceptor. Alternatively, the assay may be developed testing the same two configurations with APC as the acceptor. U*Light* is a small molecule dye that is brighter and smaller than APC and as such may cause less steric hindrance when developing a PPI assay. In some cases, however, the larger size of APC can be useful, as it may allow the acceptor fluorophore the freedom of movement to achieve the necessary proximity for signal generation.

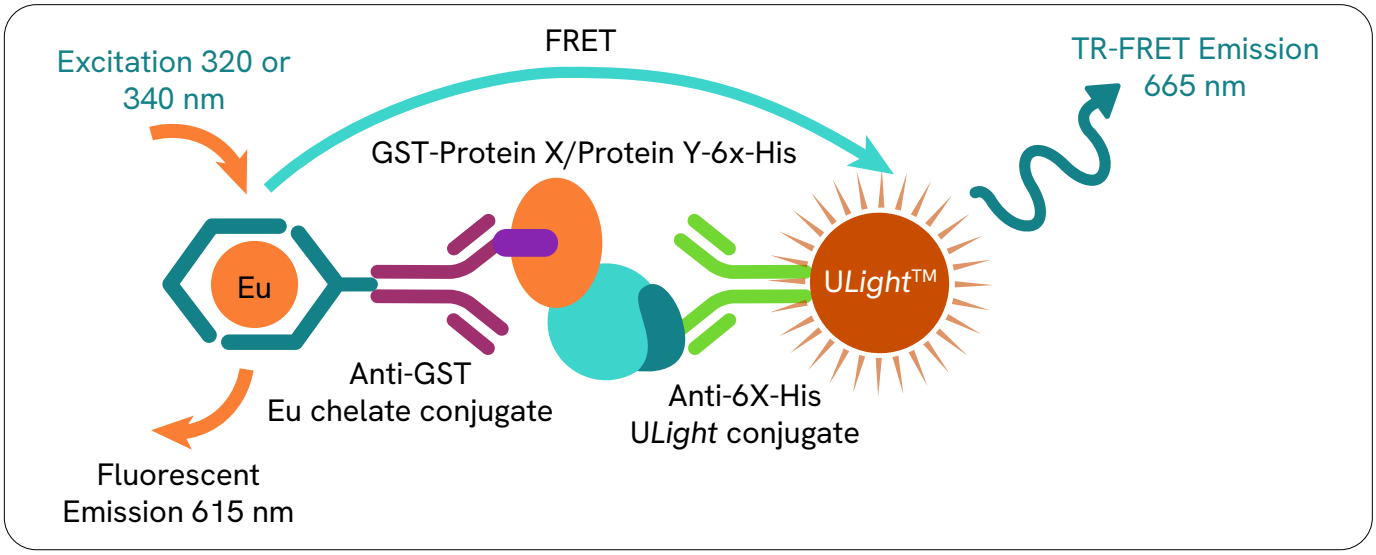


Figure 1: Illustration of a LANCE protein: protein interaction assay, using anti-6X-His U*Light* conjugate, Anti-GST W1024 Europium Chelate, 6X-His-p53 and GST-tagged MDM2.

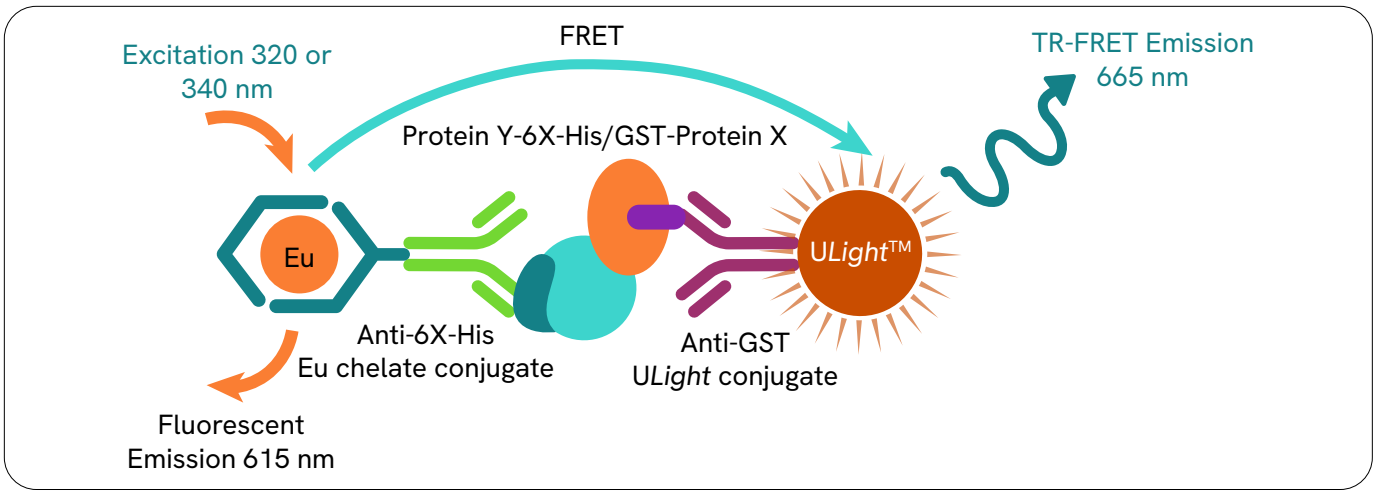


Figure 2: Illustration of a LANCE protein: Protein interaction assay, using anti-GST U*Light* conjugate, Anti-6X-His W1024 Europium Chelate, 6X-His-p53 and GST-tagged MDM2. Opposite configuration from that depicted in Figure 1.

Buffers

LANCE technology works with a wide range of buffers and additives, and is highly tolerant of many commonly used chemicals, including the solvent DMSO. If you are not sure what buffer will work best for your system, you can start with a Tris or HEPES-based buffer with salt and detergent as needed and then further optimize as necessary. The overriding factor in buffer selection should be choosing one that your binding partners are known to work well in, and then adding or removing any additives as necessary. For the work shown here, a HEPES-based buffer was used consisting of 50 mM HEPES, 100 mM Sodium Chloride (NaCl), 0.1 % Triton X-100 and 0.1% Bovine Serum Albumin (BSA). For more information please refer to www.revvity.com

Plates

There are many microplates on the market that can be used to run a LANCE assay. We recommend Revvity OptiPlates®, available in 96-well, 384-well, and 1536-well formats, which are used by our R&D teams. Appendix 1 lists all the part numbers for our recommended plate types.

Plate reader

LANCE assays require a microplate reader that is capable of time-resolved fluorescence. We recommend any of the Revvity plate readers, i.e. the EnVision®, the EnSight®, the EnSpire® or the Victor®. Many other plate readers are also capable of reading TR-FRET assays, though the results may differ depending on the instrument used. The chosen plate reader needs to be able to excite the Europium lanthanide chelate at 320 or 340 nm, and detect emission at both 615 nm and 665 nm. Regardless of what instrumentation you use to run your assay, your local Revvity representative can help you set up and optimize your reader.

Reagent storage

LANCE reagents are very stable when stored as labeled, and are tolerant of a wide range of temperatures at which to perform assays. They are not sensitive to light exposure, so experiments can be performed in ambient light. However, acceptor dyes may bleach over extended periods, so we recommend storing all stocks in the opaque tubes provided. Because the LANCE signal isn't sensitive to temperature, you can run your assay at higher or lower temperatures without having to equilibrate the plate before reading (though it is always a good idea

to check for condensation, especially when screening stacked plates). LANCE assay signals are quite robust and are stable for extended periods, assuming that the PPI complex that you are testing is stable for that long (e.g., a week, if the plate is sealed to prevent evaporation loss). This feature allows for read-time flexibility, for example dispensing your assay to 100 plates or more in a short period of time, and then reading all of those plates over the course of a day or night.

Data analysis

The data generated include three readouts per well: the 615 nm signal (Europium donor emission), the 665 nm signal (the FRET from the acceptor, U*Light* or APC) and the ratio of these two signals. Such ratiometric data can help to reduce well-to-well variability that can arise from pipetting error or fluorescent artifacts from compounds and buffer/media additives.

Assay development

Here we demonstrate in three simple experiments how to develop a LANCE PPI assay, complete with inhibitor testing. Using His6-p53 and GST-MDM2, we show the necessary experiments to develop a LANCE biochemical binding assay, including sample dilution schemes and plate layouts, assay protocols, and raw data from the development of this PPI assay, including inhibition with the commercially available small molecule Nutlin-3.

The two proteins chosen, MDM2 and p53, are lower affinity binding partners, so they are ideal candidates for choosing LANCE over a non-homogeneous assay technology. To reduce the number of samples tested here we used only the U*Light*-acceptor labeled antibodies (LANCE *Ultra)* and not APC-acceptor labeled reagents (LANCE). Thus there were four different detection reagents utilized in the first experiment, instead of six, and the number of 2D matrices tested, in duplicate, was reduced to two from four. It is up to the end user how many different configurations you want to test at once.

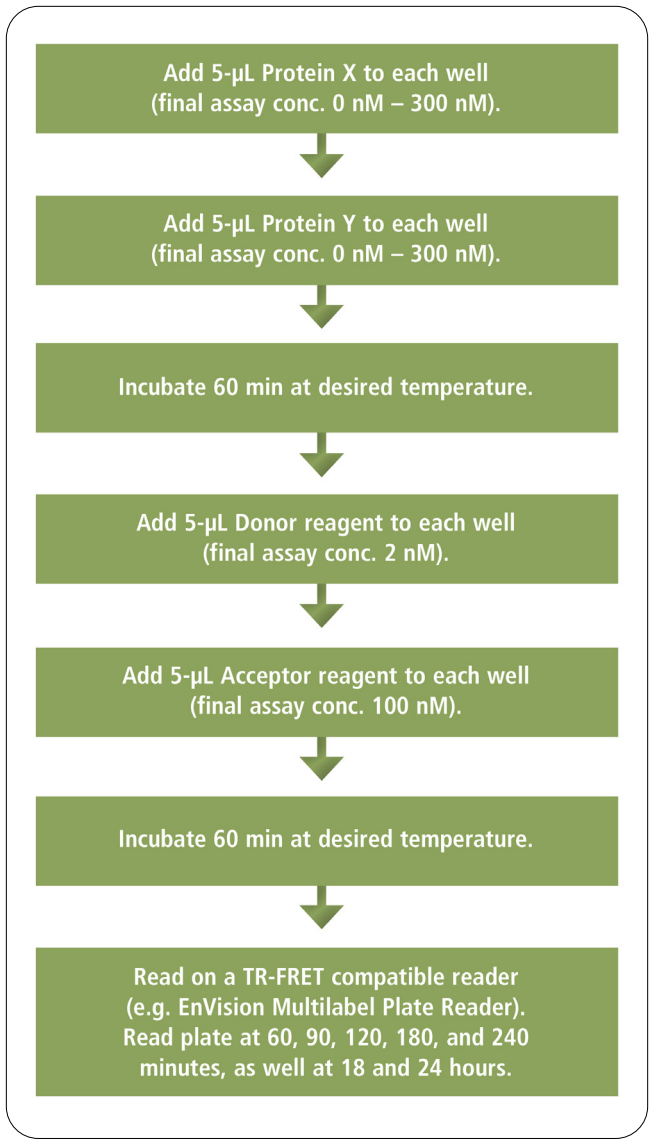


Figure 3: General assay protocol utilized.

Experiment one. Protein cross-titration with a fixed amount of donor: acceptor reagents at multiple assay incubation times

This multi-faceted experiment will allow you to not only determine the best donor: acceptor pairing for your particular binding partners, but also to determine the optimal concentration of binding partners and the optimal incubation time after your final reagent addition. Please note that other proteins may complex faster or slower than the one-hour time allotted for this pair, so you may have to examine different incubation times for the protein binding

portion of the assay. Two combinations of donor: acceptor pairs were tested - Anti-6X-His Eu W1024 (#AD0110) with Anti-GST U*Light* (#TRF0104-D), and Anti-GST Eu W1024 (#AD0252) with Anti-6X-His U*Light* (#TRF0105-D) but only the data from the better performing pair are shown (Anti-6X-His Eu and Anti-GST U*Light*).

For each combination of LANCE reagents a 2D titration of protein pairs is prepared (the 'matrix'), as shown in Figure 4, with each configuration run in singlet (i.e., if using a 96-well plate), duplicate, or triplicate as desired (e.g., in rows A-H and I-P, columns 1-8 for duplicate, and rows A-H, columns 1-8, 9-16 and 17-24 for triplicate).

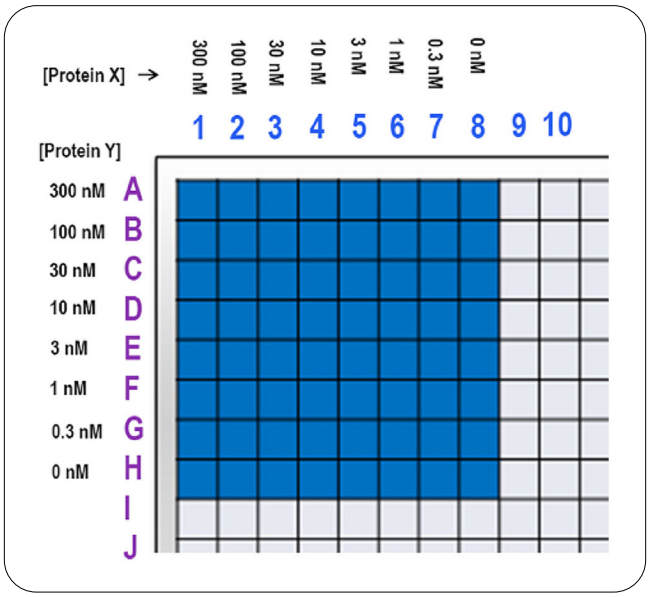


Figure 4: Representative plate layout for one replicate, 2D titration of each protein, at one concentration of donor: acceptor pair.

For each replicate matrix of proteins a single concentration of donor: acceptor reagents is added; in this example at 2 nM of the Europium-labeled reagent and 100 nM of the U*Light*-labeled reagent (these concentrations will be optimized in a later experiment). When first developing a PPI assay, it's generally a good idea to allow the protein pair to incubate before adding the detection reagents. This is especially true when one of the binding partners is tagged with biotin, and streptavidin is used as the tag-capture molecule, as the streptavidin-biotin interaction is extremely tight and very rapid, and this may alter the kinetics at which your assay achieves equilibrium. Upon confirmation that a specific assay configuration works, further optimization may be done to test the order-of-addition for all reagents.

For ease of pipetting, all four additions here were performed at the same volumes (5 µL per addition), though these volumes may be changed as long as the final concentration of each reagent is maintained in the assay. In this case (four 5-µL additions), each reagent would be made up at 4X of its final assay concentration.

Figures 5 and 6 show the data from the protein 2D titration (Experiment #1), read on the EnVision both utilizing the FRET laser (#5) and the Flash lamp (#6). In both cases the signal-to-background ratio were similar (close to 10, at the peak), so for future experiments all data reported were read using the FRET laser. Figure 7 shows the signal to background ratio data generated on both the Laser and the flash lamp.

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	97521	126996	113913	77712	50232	34208	30901	22007
	100	89545	145112	177578	142836	93059	61009	49565	21958
	33	76105	140992	199731	202668	153257	102161	80199	21110
	11	53272	87186	132982	162271	143067	89644	53988	21979
	3.7	36277	50759	71296	90427	87288	67978	46241	22138
	1.2	27062	32559	40144	49435	48845	43899	35591	21844
	0.41	24288	27016	29108	31647	31663	29777	27695	23546
	0	23244	22592	23740	23065	22717	22200	22316	23705

Figure 5: Heat map of data from experiment #1, read using the FRET laser on the EnVision. Data are averages of two replicates, where red is high signal and green is low signal.

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	6240	8061	7257	4930	3159	2206	1945	1378
	100	5776	9421	11525	9306	6008	3900	3157	1401
	33	4908	9128	13163	13483	10080	6638	5227	1369
	11	3418	5622	8616	10672	9355	5887	3509	1410
	3.7	2312	3245	4618	5877	5757	4443	3009	1437
	1.2	1727	2095	2574	3176	3176	2848	2309	1423
	0.41	1557	1724	1870	2032	2039	1933	1789	1517
	0	1483	1448	1509	1476	1455	1431	1439	1525

Figure 6: Heat map of data from experiment #1, read using the Flash Lamp on the EnVision. Data are averages of two replicates, where red is high signal and green is low signal.

The peak signal generated was at 3:1 p53 to MDM2 (33 nM p53 to 11 nM MDM2), respectively. Concentrations over and above the peak for either or both proteins show a decrease in signal, which is due to a phenomenon termed the "hook effect." The hook effect is caused by having an excess of analyte, in this case either of the two proteins, resulting in saturation of one of the binding partners. Concentrations of each protein below those that gave the peak signal (i.e., below the hook point – 10 nM for each protein) were chosen.

To show the signal stability of the LANCE assay, and to discern whether the binding partners had reached equilibrium, the assay plate was read at multiple times over the course of a few days (1 hour, 2 hours, 3 hours, 4 hours, 6 hours, 18 hours, 24 hours, 34 hours, 44 hours, and 62 hours). The data in Figure 8 show the signal and signal-to-background from the 62-hour plate read. These data show that the assay is quite stable, even when reading the same well ten times.

A

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	4.4	5.8	5.2	3.5	2.3	1.6	1.4	1.0
	100	4.1	6.6	8.1	6.5	4.2	2.8	2.3	1.0
	33	3.6	6.7	9.5	9.6	7.3	4.8	3.8	1.0
	11	2.4	4.0	6.1	7.4	6.5	4.1	2.5	1.0
	3.7	1.6	2.3	3.2	4.1	3.9	3.1	2.1	1.0
	1.2	1.2	1.5	1.8	2.3	2.2	2.0	1.6	1.0
	0.41	1.0	1.1	1.2	1.3	1.3	1.3	1.2	1.0
	0	1.0	1.0	1.0	1.0	1.0	0.9	0.9	1.0

B

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	4.5	5.8	5.3	3.6	2.3	1.6	1.4	1.0
	100	4.1	6.7	8.2	6.6	4.3	2.8	2.3	1.0
	33	3.6	6.7	9.6	9.8	7.4	4.8	3.8	1.0
	11	2.4	4.0	6.1	7.6	6.6	4.2	2.5	1.0
	3.7	1.6	2.3	3.2	4.1	4.0	3.1	2.1	1.0
	1.2	1.2	1.5	1.8	2.2	2.2	2.0	1.6	1.0
	0.41	1.0	1.1	1.2	1.3	1.3	1.3	1.2	1.0
	0	1.0	0.9	1.0	1.0	1.0	0.9	0.9	1.0

Figure 7: Signal-to-background ratio data from Experiment #1. A shows data utilizing the FRET Laser while B shows data utilizing the Flash Lamp.

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	39197	79154	104498	83128	63675	48067	45752	23199
	100	48761	102433	148157	139321	109376	78077	67766	23436
	33	47816	110585	172424	197656	162850	123597	102537	22668
	11	44442	85008	139221	178137	168088	109508	73268	23484
	3.7	36596	50403	74215	96135	99104	77316	51800	22991
	1.2	31215	32941	40720	52627	52931	46783	36885	18177
	0.41	29183	27745	29448	32615	34665	31536	29199	22439
	0	20931	23251	23947	23817	23384	22149	22667	24193

		[MDM2] nM							
		300	100	33	11	3.7	1.2	0.41	0
[p53] nM	300	1.7	3.4	4.5	3.6	2.7	2.1	2.0	1.0
	100	2.1	4.4	6.3	5.9	4.7	3.3	2.9	1.0
	33	2.1	4.9	7.6	8.7	7.2	5.5	4.5	1.0
	11	1.9	3.6	5.9	7.6	7.2	4.7	3.1	1.0
	3.7	1.6	2.2	3.2	4.2	4.3	3.4	2.3	1.0
	1.2	1.7	1.8	2.2	2.9	2.9	2.6	2.0	1.0
	0.41	1.3	1.2	1.3	1.5	1.5	1.4	1.3	1.0
	0	0.9	1.0	1.0	1.0	1.0	0.9	0.9	1.0

Figure 8: Signal and Signal-to-background ratio data from Experiment #1, read at 62 hours after the final incubation.

Experiment two. concentration response of vehicle (dMSO) at different concentrations of detection Reagents

The first experiment allowed for determination of optimal reagent configuration, incubation time, and protein concentrations. Next, we will examine the effect of DMSO on our assay while simultaneously optimizing Europium-labeled and U*Light*-labeled antibody concentrations.

For our assay, a small molecule inhibitor shown to inhibit the interaction of MDM2 and p53, Nutlin-3, was commercially available from multiple sources. The solvent used to prepare this small molecule is DMSO, so the effects of DMSO concentration must first be examined. This next experiment tested both the effect of DMSO concentration and various concentrations of detection reagents using the conditions gleaned from Experiment #1.

Figure 9 shows the data from an eight-point DMSO titration curve, and the effects of DMSO upon the binding assay at different concentrations of detection reagent. The wells highlighted in green correspond to the conditions where the signal generated was within 5% above or below the no-DMSO signal. For the wells that aren't highlighted in green, the values generated were all quite close to the no-DMSO signal, and any deviation is attributed to hand-pipetting of duplicate samples. These data show that LANCE technology itself has no inherent sensitivity to solvents such as DMSO and that it has no effect on the MDM2/p53 binding interaction either.

As shown in Figure 10, for any given concentration of detection reagents, the effect of DMSO is negligible. In this case, 1% final concentration DMSO solvent vehicle, 1 nM of the Europium reagent, and 50 nM of the U*Light* reagent were chosen as the best balance of signal and background.

		DMSO %							
		8	4	2	1	0.5	0.25	0.125	0
1 nM Eu	100 nM U*Light*	102	106	101	100	100	99	99	100
	50 nM U*Light*	102	109	105	102	109	106	105	100
	25 nM U*Light*	92	98	98	95	100	97	95	100
	12.5 nM U*Light*	97	98	98	94	102	101	95	100
2 nM Eu	100 nM U*Light*	112	110	107	104	103	103	102	100
	50 nM U*Light*	104	107	106	105	109	105	104	100
	25 nM U*Light*	102	103	103	101	105	103	101	100
	12.5 nM U*Light*	99	103	101	103	104	101	98	100

Figure 9: Effect of DMSO at different detection reagent concentrations on the MDM2/p53 LANCE assay (data shown are average of duplicates).

		DMSO %								
		8	4	2	1	0.5	0.25	0.125	0	Blank
1 nM Eu	100 nM U*Light*	169531	175998	167273	166065	166130	164763	164281	165670	12203
	50 nM U*Light*	135037	144170	139171	135066	144456	139991	138699	132094	9851
	25 nM U*Light*	98770	105159	105559	101632	106838	103966	101963	107227	8666
	12.5 nM U*Light*	81915	82729	82589	79100	85491	84980	80001	84226	6427
2 nM Eu	100 nM U*Light*	237454	231346	225227	219361	218395	216610	215611	211243	23185
	50 nM U*Light*	170834	175090	173948	172039	178769	171967	170536	164128	19945
	25 nM U*Light*	111489	112178	112184	110923	114629	112831	110727	109420	18074
	12.5 nM U*Light*	86418	90274	88447	90235	91183	88564	85841	87615	16676

Figure 10: Signal generated in LANCE assay at different detection reagent concentrations upon addition of DMSO.

Experiment three. concentration response of small molecule inhibitor

Next, we need to demonstrate the specificity of the binding assay. One way to test for this is using an untagged version of one or the other of the binding partners to perform a competition experiment, wherein the untagged molecule acts as an inhibitor by binding to the tagged partner and producing a non-viable binding complex. Alternatively, you could use a known inhibitor of the binding interaction. Inhibitor molecules are not always readily available, but when they are it's generally better to choose option two, as doing so allows for demonstration that the interaction between the two binding partners is druggable. Using the optimized conditions determined in Experiments 1 and 2, the specificity of the assay was then tested with a known inhibitor of the MDM2/p53 interaction: Nutlin-3. Nutlin-3 is a small molecule that can displace p53 from the binding pocket of MDM2. Nutlin-3a is the active enantiomer and Nutlin-3b is the inactive enantiomer, which has few, if any, of the downstream cellular effects of Nutlin-3a (Vassilev et al (2004) Science 303 844). Figure 11 shows the IC50s generated for both enantiomers of Nutlin-3 in the LANCE binding assay that was developed. The IC50s generated are in agreement with accepted values: 90 nM for 3a and 13.6 µM for 3b. The curve for Nutlin-3b has an approximate IC50 of 8.4 µM because we have chosen the bottom of that curve to be the same as the bottom of the Nutlin-3a curve, but because the top compound concentration tested was 10 µM it should be reported as > 5 µM.

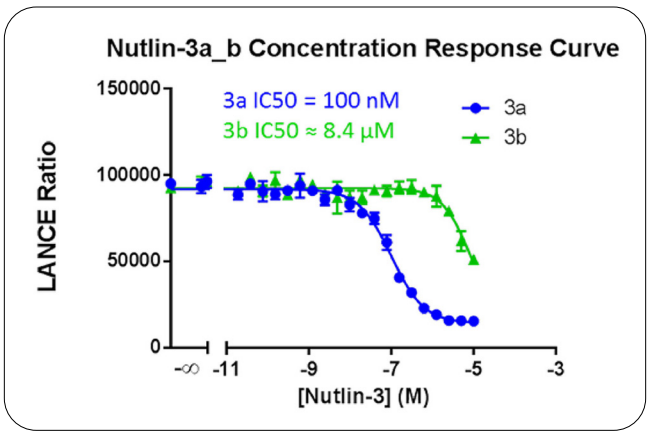


Figure 11: Concentration response curves for Nutlin-3 enantiomers, a small molecule inhibitor of the MDM2/p53 interaction.

Using the top and bottom points from the concentration curve, the Z-factor for the assay was determined to be a robust 0.88. These data together show that the LANCE PPI assay developed in these three experiments is suitable for screening compounds in both single-point and IC50 mode screening campaigns.

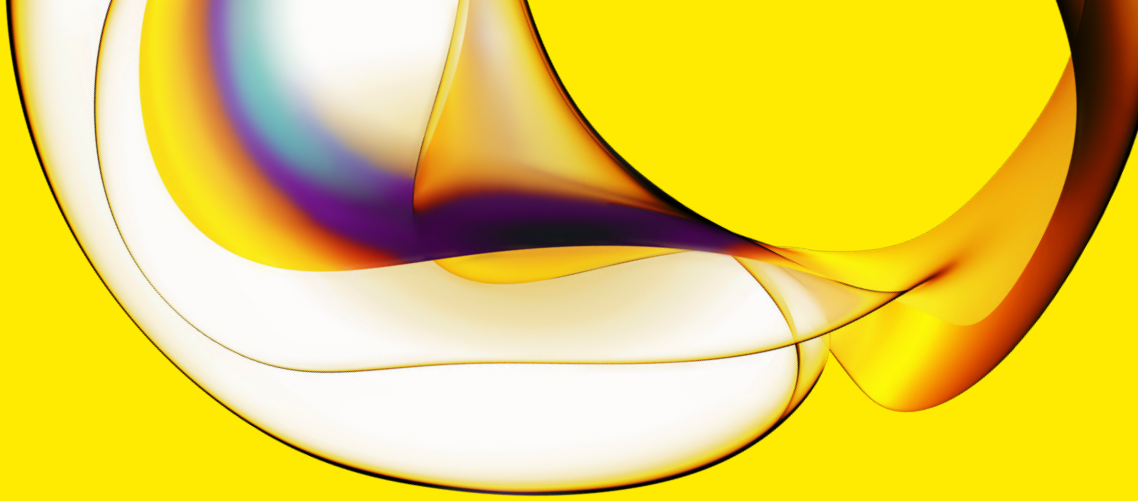
Appendix 1

Table 2: Microplates for LANCE TR-FRET Assays.

Well format	Plate type	Color	Number of plates	Catalog number
96	OptiPlate™	White	50	6005290
			200	6005299
		Black	50	6005270
			200	6005279
	½ AreaPlate	White	50	6005560
			200	6005569
384	OptiPlate	White	50	6007290
			200	6007299
		Black	50	6007270
			200	6007279
	Shallow ProxiPlate™	White	50	6008280
			200	6008289
		Black	50	6008260
			200	6008269
1536	OptiPlate	White	50	6004290
			200	6004299

References

1. Vassilev et al (2004) *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. Science 303 844. PMID: 14704432



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