Application Guide for Multimode Readers.

LEARN – SELECT – MEASURE

TECHNOLOGY ORIENTATION

TABLE OF CONTENT (ALPHABETICAL)

ABS – Absorbance.

Light is absorbed by the sample

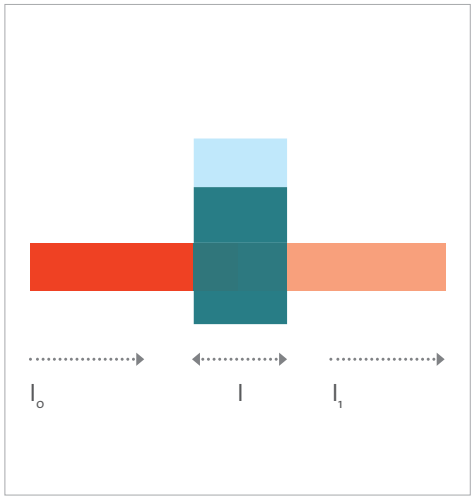

Figure 1: Schematic representation of an absorbance measurement performed in a cuvette.

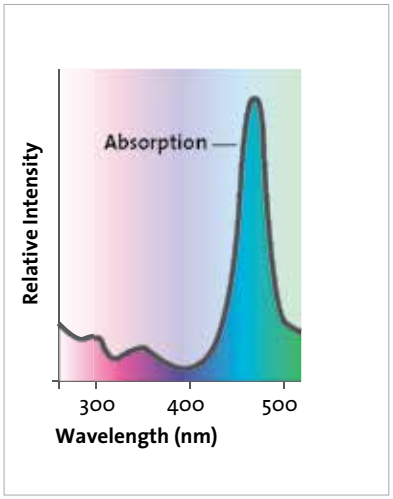

Figure 2: An absorbance spectrum shows the extent of light absorption at any specific wavelength.

Absorbance OD	Transmittance [%]
0	100
1	10
2	1
3	0.1
4	0.01

Table 1: The relationship between absorbance and transmittance values. An absorbance value of 3 means that only 0.1 % of the light is able to pass through the sample. Most multimode readers can only read samples up to an OD of 4.

TECHNOLOGY

When light shines through a turbid or colored liquid, some of its intensity is absorbed by the liquid's molecules or particles (Figure 1). The amount of light that penetrates the sample and reaches the detector is called the transmittance (T), and the light absorbed by the sample is called the absorbance (A, Abs), or optical density (OD)[1].

OD values commonly correspond to a 1 cm path length, which is the width of standard cuvettes (Figure 1). Note that absorbance is a logarithmic function (Table 1) of the transmittance, as defined by the lambert beer law[2]:

$$A = -\log_{10}(I_1/I_0)$$

The absorbance spectrum is a function of the molecule; light of different wavelengths can be absorbed differently depending on the sample, as shown in the absorbance spectrum in Figure 2.
For this reason, the absorbance is always stated together with the wavelength, for example OD_{600}.

Major applications

- DNA / RNA quantification (Abs)
- MTT / MTS assays
- BCA, Modified Lowry and Bradford assays – Protein quantification

[1] Bioanalytik. Von F. Lottspeich. Spektrum, Heidelberg, 1998
[2] Beer (1852) "Bestimmung der Absorption des rothen Lichts in farbigen Flüssigkeiten" (Determination of the absorption of red light in colored liquids), Annalen der Physik und Chemie, vol. 86, pp. 78–88.

DNA / RNA quantification (Abs)

DNA / RNA quantification based on absorbance

Figure 2: Tecan NanoQuant Plate™

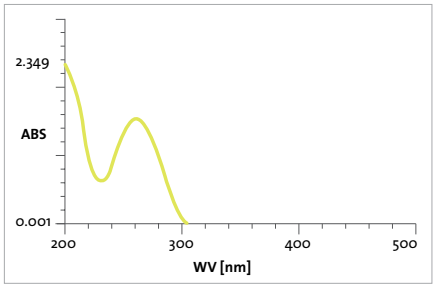

Figure 1: DNA absorbance spectum.

$1OD_{260}$	Concentration (µg/ml)
dsDNA	50
ssDNA	33
RNA	40
Oligonucleotides	20–30

Table 1: Nucleic acid concentrations at OD_{260} = 1 and neutral pH

ASSAY OVERVIEW

Technology

Absorbance

DNA and RNA can be quantified based on absorbance at 260 nm, which is in the UV range and not visible to the human eye (Figure 1). Tecan's multimode readers provide cuvette ports for DNA and RNA measurement (quartz or UV-transparent cuvettes must be used). Alternatively, Tecan's patented NanoQuant Plate™ (Figure 2) is ideal for smaller volumes (2 µl), higher throughput (16 samples at once), and more economical DNA / RNA quantification. 1 OD_{260} corresponds to different concentrations, depending on the type of nucleic acid being quantified (Table 1).

The ratio of absorption at 260 nm vs 280 nm is commonly used to assess DNA contamination of protein solutions, since proteins – in particular, the aromatic amino acids – absorb light at 280 nm[3,4]. It is generally acknowledged that pure DNA has a ratio of 2, and RNA, 1.8[5]. Recently researchers found that the A_{260}/A_{230} ratio also provides valuable information about the nucleic acid purity[6].

Typical detection limits for absorbance based DNA quantification are within the single digit ng/µl range.

Alternative

LifeTechnologies' PicoGreen® and RiboGreen® quantification assays provide a broader dynamic range.

Sample protocol for DNA measurements

1. Blank the reader with the same buffer used to dilute the DNA
2. Take an appropriate volume of sample (NanoQuant Plate: 2µl, cuvettes: volume depends on the min. / max. filling volume)
3. Measure OD_{260} and OD_{280}
4. If the OD_{260} value is greater than two, dilute samples
5. To calculate the concentration, multiply the OD_{260} by the concentration factor shown in Table 2 and your dilution factor, if applicable
6. Determine the purity by dividing the value for OD_{260} by the value for OD_{280}

[3] a b c d e Sambrook and Russell (2001). Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor Laboratory Press. ISBN 978-0-87969-577-4.
[4] (Sambrook and Russell cites the original paper: Warburg, O. and Christian W. (1942). "Isolierung und Kristallisation des Gärungsferments Enolase". Biochem. Z. 310: 384–421.)
[5] Glasel, J.A. (1995) Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios, Biotechniques 18:62-63
[6] http://www.qiagen.com/literature/benchguide/default.aspx

Instrument parameters

		
Reader	**Infinite® 200 PRO**	**Spark®**
Assay	**DNA concentration**	**DNA concentration**
Measurement Mode	Absorbance	Absorbance
Wavelength	260 nm	260 nm
Bandwidth	5 nm	default
Flashes	25	25
Settle time	0 ms	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

MTT[7]/MTS[8] assay

Cell viability / cytotoxicity assays with absorbance readout

Figure 1: MTT assay in a 96-well format

ASSAY OVERVIEW

Technology

Absorbance

Principle, Major application

The MTT and MTS assays are colorimetric (Figure 1) – and hence absorbance – assays that measure cell viability. MTT / MTS is taken up by the cells and processed to varying extents depending on the cell viability. Viable cells reduce more MTS / MTT to formazan, yielding a more intense purple color.

Mechanism

While MTT assays need to use a reagent to make the formazan generated soluble, MTS assays yield water-soluble products, potentially making them homogeneous assays.

MTT assays use a solubilization reagent, such as dimethyl sulfoxide (DMSO) or isopropanol, to dissolve the non-water-soluble formazan product, yielding a colored solution that can be quantified by absorbance measurement at approximately 565 nm, dependent on the solvent employed[9].

MTS assays are an improved version of the MTT assay. Its reagents are reduced more efficiently within the cell than MTT, and the resulting product is water-soluble and less cytotoxic than the insoluble formazan used in the MTT assay. This makes it a one-step (homogeneous) assay, with the convenience of adding the reagent directly to the cell culture without the intermittent steps required in the MTT assay.

However, when MTS is used in a homogeneous way the assay becomes susceptible to colorimetric interference, as traces of colored compounds may remain in the microplate[10].

Provider

MTT, MTS and similar reagents are available from various chemistry distributors, or as the CellTiter Glo® 2.0 Assay (Promega)[11].

Alternatives

An alternative to absorbance-based cell viability assays, are fluorescence-based systems using the redox indicator Resazurin to detect the cell's metabolic activity. These assays are characterized by a more convenient assay handling and higher sensitivity levels.

[7] MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-phenylditetrazolium bromide, a yellow tetrazole)
[8] MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
[9] Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods 65 (1-2): 55–63.
[10] Cory AH, Owen TC, Barltrop JA, Cory JG (1991). Use of an aqueous soluble tetrazolium/formazan assay for cellgrowth assays in culture. Cancer communications 3 (7): 207–12.
[11] https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_glo-2_0-assay/?catNum=G9241

Instrument parameters

		
Reader	**Infinite 200 PRO**	**Spark**
Assay	**MTT assay/CellTiter Assay**	**MTT assay/CellTiter Assay**
Measurement Mode	Absorbance	Absorbance
Wavelength	565 nm	565 nm
Bandwidth	9 nm	3.5 nm
Flashes	25	10
Settle time	0 ms	50 ms

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- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
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BCA, Modified Lowry and Bradford assays – Protein quantification

Protein quantification assays with absorbance readout

Figure 1: Bradford Protein Assay measured in cuvettes, showing increasing protein concentrations.

ASSAY OVERVIEW

Technology

Absorbance

Principle, Major application

All three assays are designed to determine the protein concentration of a sample. For detection, a liquid reagent needs to be added to the samples. This reagent interacts with the proteins, leading to a visible color change (Figure 1) that is directly proportional to the concentration. Absolute concentrations are calculated using a standard curve.

Provider

Various companies have established their own assays for this purpose. The main differences between the various assays are the dynamic range and the measurement wavelength.

Mechanism

The **BCA**™ Protein Assay (Thermo Scientific Pierce) uses bicinchoninic acid (BCA) for colorimetric quantification of total protein in a sample[13]. The method is based on the reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium to form a colored watersoluble chelate that can be measured at its absorption maximum of 562 nm. The linear working range for BSA is 20 to 2000 µg/ml[14].

The **Bradford** Protein Assay (BioRad) is based on the Coomassie® Brilliant Blue G-250 dye which binds to basic and aromatic amino acid residues, particularly arginine. This induces a shift of the dye's absorbance maximum from 465 nm to 595 nm. The Bradford assay can be performed as a microassay procedure, with a linearity range of 125 to 1,000 µg/ml BSA[15].

In the **Modified Lowry** Protein Assay (Thermo Scientific Pierce), the protein reacts with cupric sulfate and tartrate in an alkaline solution, which results in formation of a tetradentate copper-protein complex, reducing the Folin-Ciocalteu Reagent. The absorbance of the blue, water-soluble product can be measured at 750 nm. The assay – tested with BSA protein[16] – exhibits good linearity in the range of 1 to 1500 µg/ml.

Alternatives

Potential alternatives for protein quantification reach from absorbance-based methods using the protein extinction coefficient[17] to fluorescence based assays like NanoOrange® to even dedicated[18] devices.

[13] Smith, P.K., et al.: Measurement of protein using bicinchoic acid. Anal Biochem., 150, 76-85, 1985
[14] https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/protein-assays.html
[15] http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4110065A.pdf
[16] https://www.thermofisher.com/us/en/home/life-science/protein-biology/protein-assays-analysis/protein-assays.html
[17] http://web.expasy.org/protparam/protparam-doc.html
[18] http://www.millipore.com/techpublications/tech1/an2222en

Instrument parameters

Reader	**Infinite 200 PRO**	**Spark**
Assay	**BCA Assay**	**BCA Assay**
Measurement Mode	Absorbance	Absorbance
Wavelength	565 nm	562 nm
Bandwidth	9 nm	default
Flashes	25	25
Settle time	0 ms	0 ms

Assay	**Modified Lowry assay**	**Modified Lowry assay**
Measurement Mode	Absorbance	Absorbance
Wavelength	750 nm	750 nm
Bandwidth	9 nm	default
Flashes	25	25
Settle time	0 ms	0 ms

Assay	**Bradford assay**	**Bradford assay**
Measurement Mode	Absorbance	Absorbance
Wavelength	595 nm	595 nm
Bandwidth	9 nm	default
Flashes	25	25
Settle time	0 ms	0 ms

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FI – Fluorescence Intensity.

Light is absorbed and released (emitted)

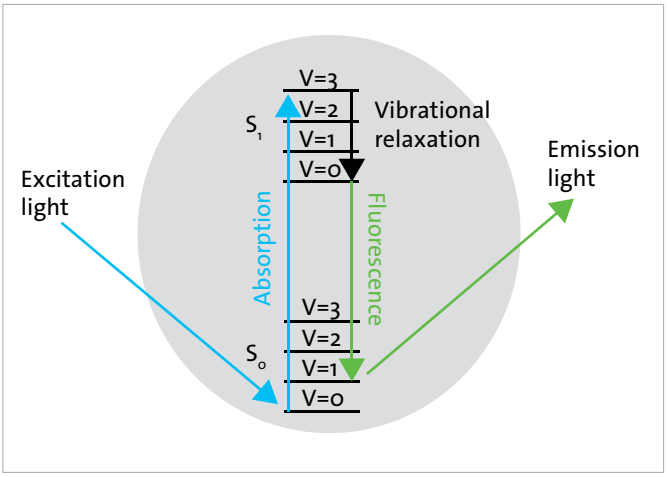

Figure 1: Jablonski diagram. S = electronic state, V = vibrational level. After photon absorption (= excitation), the molecule adopts a state of higher energy S_1 (= excited state) including several vibrationally excited substates. By vibrational relaxation, the molecule relaxes to the lowest excited S_1 state (black arrow). From this state the molecule relaxes into the vibrational states of S_0 by emitting light.

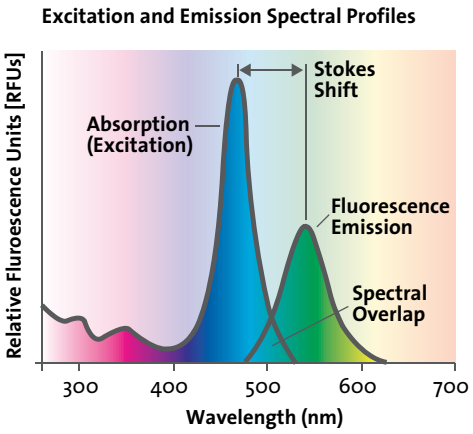

Figure 2: While the excitation spectrum describes how efficient it is to excite the fluorophore at a specific wavelength, the emission spectrum describes how efficient it is to detect the emitted light at any given wavelength. The Stokes shift describes the distance between the excitation and emission maximum, and is given in nanometers (nm).

TECHNOLOGY

Fluorescence describes a molecule's ability to emit (release) previously absorbed light (Figure 1). The emission occurs almost instantly (within 1 ns = nano second) and, according to the laws of physics, the emitted light will always have a higher wavelength and hence a lower energy. A fluorescence spectrum consists out of an absorption (excitation) and emission spectrum (Figure 2).

Fluorescence labels (fluorophores) can be attached to any available biomolecule and used to answer quantitative, as well as qualitative, questions. For example, 'does the sample contain the fluorophore?' (qualitative), and 'how much of the fluorophore is in the sample?' (quantitative). Signals are quantified as Relative Fluorescence Units [RFU].

Major applications

- PicoGreen® and RiboGreen® DNA / RNA quantification
- Resazurin-based cell viability assays
- Fluorescent proteins (GFP, RFP, YFP, etc.)
- ORAC (Oxygen Radical Absorbance Capacity) assay


Rinse
Prime
Rinse
Speed
Temperature
Off
Off

PicoGreen® and RiboGreen® DNA / RNA quantification

High sensitivity, fluorescence-based DNA / RNA quantification

Figure 1: Green DNA

ASSAY OVERVIEW

Technology

Fluorescence Intensity

Assay design and provider

LifeTechnologies' PicoGreen[19] and RiboGreen[20] (Figure 1) quantification assays use a fluorescence approach to determine DNA and RNA concentrations. Using the Quant-iT PicoGreen dsDNA Assay Kit, you can selectively detect as little as 25 pg/ml of dsDNA in the presence of ssDNA, RNA, and free nucleotides.
The assay is linear over three orders of magnitude, and has little sequence dependence, allowing you to accurately measure DNA from many sources.

RiboGreen RNA reagent is one of the most sensitive detection dyes for the quantification of RNA in solution, offering linear fluorescence detection in the range of 1 to 200 ng/ml of RNA.

Mechanism

Both assays are easy to use; simply add the dye to the sample, wait five minutes, and detect the fluorescent signal.

Alternatives

If sensitivity is not a major issue, it may be possible to perform DNA quantification using absorbance at 260 nm.

[19] http://products.invitrogen.com/ivgn/product/P7589
[20] http://products.invitrogen.com/ivgn/product/R11490

Instrument parameters

		
Reader	**Infinite 200 PRO**	**Spark**
Assay	**PicoGreen/RiboGreen**	**PicoGreen/RiboGreen**
Measurement Mode	FI Top	FI Top
Excitation wavelength Donor	485 (9) nm	485 (20) nm
Emission wavelength Donor	535 (20) nm	535 (25) nm
Lag time	0	0
Integration time	20 µs	default
Flashes	25	10
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	0 ms	0 ms

SUPPORT

Links

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Resazurin assay

A Fluorescence Intensity-based cell proliferation assay

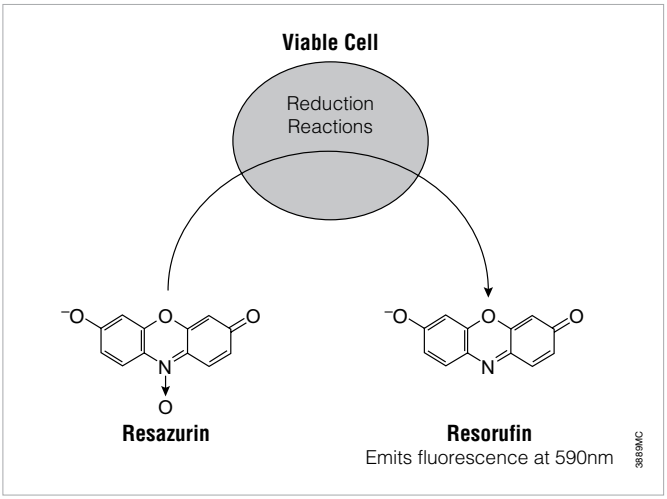

Figure 1: Viability dependet conversion of Resazurin to Resorufin (Promega)

ASSAY OVERVIEW

Technology

Fluorescence Intensity

Major application

Resazurin is a chemical compound that is frequently used for cell viability assays.

Provider

Resazurin was initially used for bacterial studies, but is now also available for eukaryotic cell-based applications under brand names such as the alamarBlue® assay[21] (Life Technologies) and CellTiter-Blue® Cell Viability Assay[22] (Promega).

Mechanism

Resazurin is a redox indicator that can be added directly to cells. Viable cells convert the dark blue, oxidized form of the dye (resazurin) into a red, fluorescent reduced form called resorufin (Ex: 570 nm; Em: 590 nm). The amount of fluorescence or absorbance is proportional to the number of living cells, and corresponds to the cell's metabolic activity. Damaged and non-viable cells have lower innate metabolic activity, and therefore generate a proportionally lower signal than healthy cells. The system is specific for cell viability as non-viable cells rapidly lose metabolic capacity and do not reduce resazurin. Consequently, a fluorescent signal[23] is not generated.

Alternatives

PrestoBlue Cell Viability Reagent[24], a new development from Invitrogen that offers much shorter incubation times.
The absorbance based MTT/MTS assay.

[21] http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/alamarBlue-Rapid-and-Accurate-Cell-Health-Indicator.html
[22] https://www.promega.com/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter_blue-cell-viability-assay/?catNum=G8080
[23] O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 2000, 267,5421–5426.
[24] http://www.invitrogen.com/site/us/en/home/brands/Molecular-Probes/Key-Molecular-Probes-Products/PrestoBlue-Cell-Viability-Reagent.html

Instrument parameters

		
Reader	**Infinite 200 PRO**	**Spark**
Assay	**alamarBlue, CellTiter-Blue**	**alamarBlue, CellTiter-Blue**
Measurement Mode	FI Bottom	FI Bottom
Excitation wavelength Donor	560 (9) nm	560 (20) nm
Emission wavelength Donor	600 (20) nm	600 (25) nm
Lag time	0	0
Integration time	20 µs	default
Flashes	25	10
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	0 ms	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

GFP (Green Fluorescent Protein)

Fluorescent protein frequently used as an expression / activation reporter

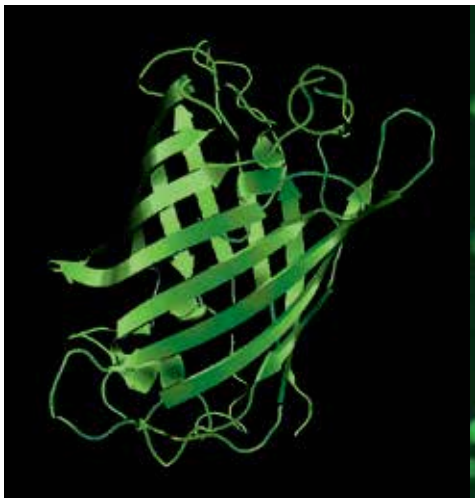

Figure 1: Protein structure of GFP

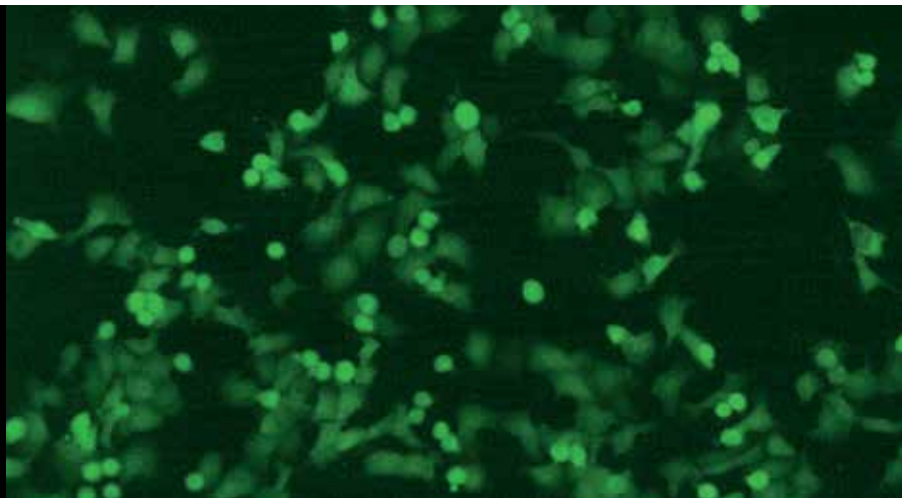

Figure 2: GFP-transfected eukaryotic cells

ASSAY OVERVIEW

Technology

Fluorescence Intensity

Principle

GFP (Figure 1) is a protein derived from a jellyfish which has the ability to fluoresce in the green wavelength range and can be detected using standard FI measurements.

Major applications

GFP can be used in an almost unlimited number of ways, for example as a BRET / FRET partner in binding studies, or for gene activation, where it is often fused / cloned to a gene of interest and co–expressed once the gene is activated (Figure 2). Commonly, it is used to differentiate between constitutive (permanent) and temporary expression. Constitutive expression is mostly used to monitor growth or proliferation of cells or bacteria, while temporary expression is used for gene activation studies.

Format, provider

Due to multiple engineering efforts, an almost unlimited number of mutants exist, resulting in a large bandwidth of excitation and emission values. Some of these variants are commercially available, while others are published and therefore not protected. Consequently, only a selection of measurement parameters can be given, since the wavelength depends on the mutant type of the protein.

Alternatives

Technology-wise, there are a lot of alternative fluorescent proteins available, such as CFP (cyan), YFP (yellow) and RFP (red). From an assay perspective, the alternative selected depends on the application. For gene expression studies, DLR® (Dual luciferase reporter assay) or GeneBLAzer® assays may be suitable. For FRET / BRET studies, fluorescent labels might be an alternative.

Instrument parameters

Reader	**Infinite 200 PRO**	**Spark**
Assay	**GFP**	**GFP**
Measurement Mode	FI Bottom	FI Bottom
Excitation wavelength Donor	485 (9) nm	485(20) nm
Emission wavelength Donor	535 (20) nm	535(25)
Lag time	0 µs	0 µs
Integration time	20 µs	default
Flashes	25	15 (5x3, optimal read)
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
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TRF – Time-Resolved Fluorescence.

Light is absorbed and emitted for a relatively long period of time

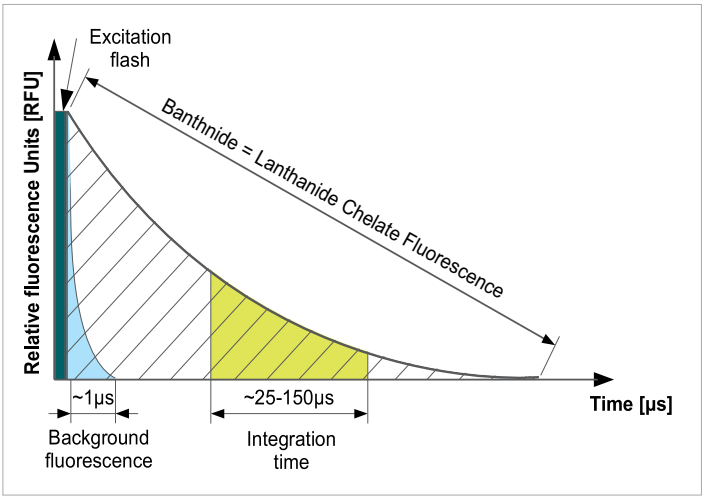

Figure 1: Schematic drawing of a time resolved emission spectrum

TECHNOLOGY

TRF is similar to standard fluorescence, except that the light is emitted for a much longer period of time (Figure 1). The advantage of this is that the signal can be measured once all the background fluorescence (noise) has subsided, increasing the signal to noise ratio, and hence the sensitivity. Only lanthanides – also called rare earth metals – are capable of this kind of fluorescence[25].

In most cases, it is possible to substitute fluorescence applications with TRF to achieve higher sensitivity and / or lower background noise.

Major applications

- DELFIA® – Dissociation-Enhanced Lanthanide Fluorescent Immunoassay

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[25] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers

FRET – Fluorescence Resonance Energy Transfer.

Light is absorbed, transferred to another fluorophore and then emitted

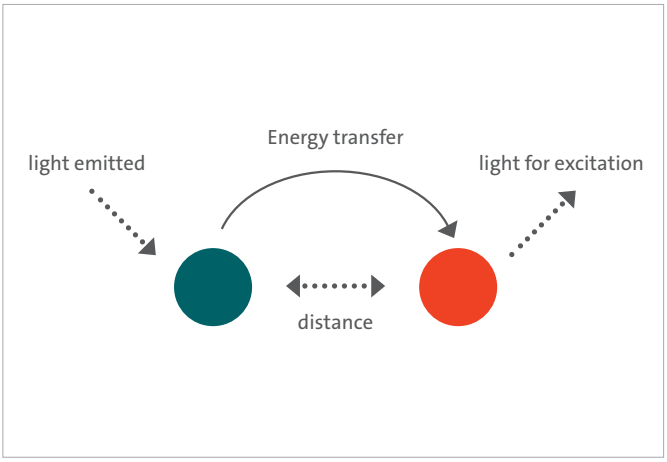

Figure 1: Schematic principle of FRET. Light emitted from the green molecule is used to excite the red molecule.

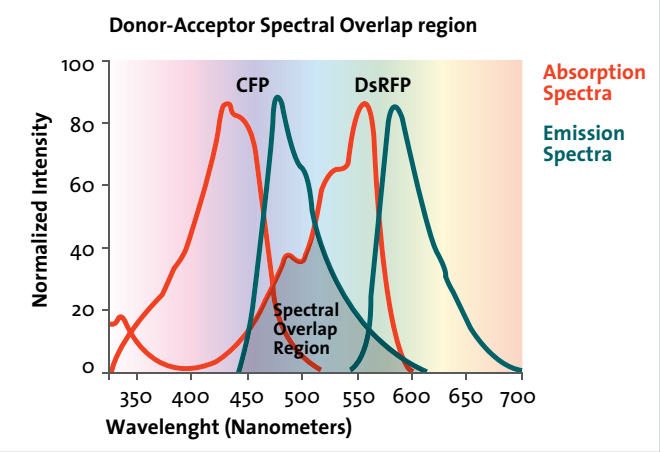

Figure 2: FRET is possible because the emission spectrum of CFP and the excitation spectrum of DsRFP overlap between 450 and 600 nm.

TECHNOLOGY

As the name implies, FRET involves energy transfer between two fluorescent molecules (Figure 1). However, there are some specific requirements for this transfer to take place. Firstly, the emission spectrum of the donor fluorophore and the excitation spectrum of the acceptor fluorophore need to overlap (Figure 2), as the emission light of the donor fluorophore is used to excite the acceptor fluorophore. Secondly, the distance between the two fluorophores – the Förster radius – should be less than 10 nm (Figure 3)[28].

One way in which FRET is used is to determine if two biomolecules are in close proximity. In this case, both biomolecules must be labeled with fluorophores and then combined. After an incubation period, the assay is performed. Samples are excited at the donor excitation wavelength, and measured at the donor and acceptor emission wavelength. To compensate for well-to-well variation, for example from pipetting errors, the ratio of both values is calculated (ratiometric assay). If donor and acceptor are in close proximity, FRET will take place, otherwise only the emission signal of the donor is measurable.

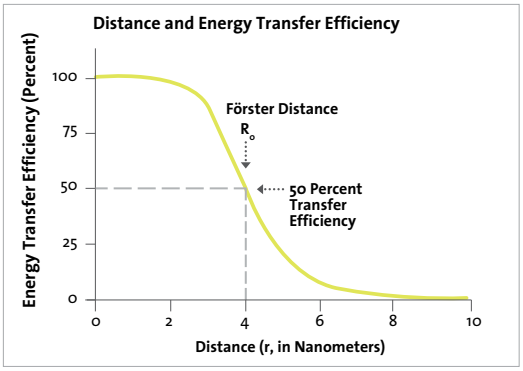

Figure 3: Förster radius – the distance where the FRET signal intensity is reduced to 50 %.

Major applications

- GeneBLAzer and Tango™ GPCR Assay System

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

[28] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers

TR-FRET – Time-Resolved Fluorescence Resonance Energy Transfer.

FRET with a longer lifetime and hence a lower background

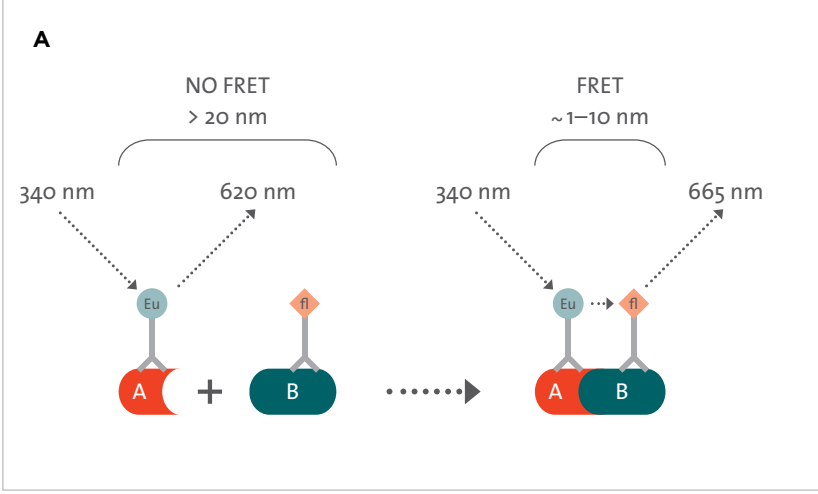

Figure 1: From a technology perspective TR-FRET is equivalent to FRET. In order to reduce the background of the assay, a lanthanide lable (e.g. Eu) and a standard fluorescence molecule (fl) as acceptor molecule.

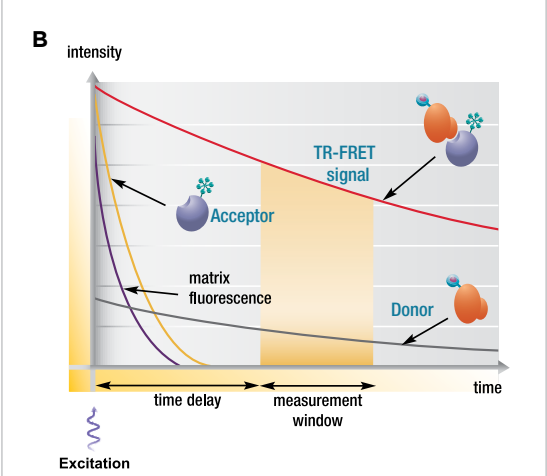

Figure 2: Signal vs. time plot showing the advantages of using lanthanide lables in a FRET process molecule.

TECHNOLOGY

TR-FRET combines the advantages of Time-Resolved Fluorescence (TRF) with the functionality of Fluorescence Resonance Energy Transfer (FRET) (Figure 1). While most standard FRET assays have difficulties with high background resulting from the excitation of matrix molecules. This issue can be resolved by using time-resolved donor molecules. As shown in Figure 2, by the time donor and acceptor emission signals are measured all the unspecific background noise has vanished, giving a high signal to background noise ratio and therefore greater sensitivity.

Assay design

TR-FRET assays are commonly designed to detect whether molecules are in close proximity. This can be exploited to determine if, for example, a protein, peptide substrate, small molecule, phosphorylation, or acetylation is present, or if binding has occurred, for example a receptor-ligand interaction. A major limitation of this technology is that the maximum distance between the donor and the acceptor molecule cannot exceed 10 nm[32].

Major applications

- HTRF® – Homogeneous Time-Resolved Fluorescence
- Adapta® Universal Kinase Assay and Substrates
- LanthaScreen™ Kinase Activity Assays
- Transcreener® TR-FRET Assays

[32] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers


te-inject.
LUMI.
TECAN.

DELFIA® – Dissociation-Enhanced Lanthanide Fluorescent Immunoassay[26]

TRF-based alternative to absorbance-based ELISA

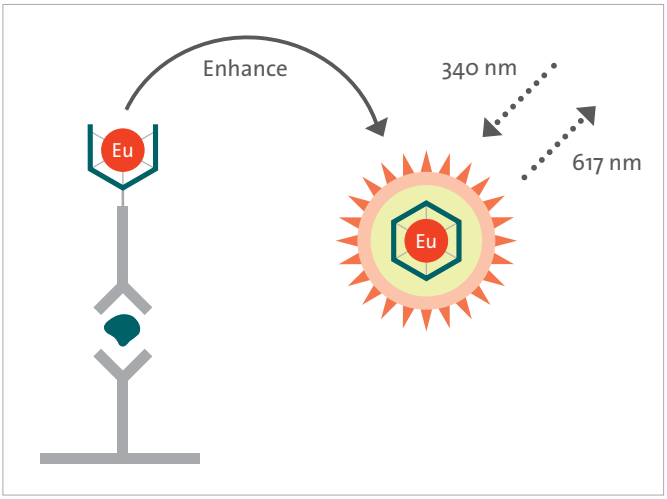

Figure 1: Schematic principle of DELFIA

ASSAY OVERVIEW

Technology

TRF – Time-Resolved Fluorescence

Principle, Provider

PerkinElmer offers the most common lanthanide chelates, including Europium (Eu), Samarium (Sm), Terbium (Tb) and Dysprosium (Dy), under the brand name DELFIA, making DELFIA a technology rather than a single assay.

Major applications

In addition to the self-labeling kits, which allow users to label almost any biomolecule with the lanthanide chelates, PerkinElmer offers pre-coupled antibodies and DNA probes. DELFIA is also available as a ready-to-go assay for cytotoxicity or cell proliferation studies. Other major applications include: receptor-ligand binding, enzyme assays, protein-protein and protein-DNA interaction studies.

Alternatives

As a common application, it is used as an alternative approach to the well-established, absorbance-based ELISA[27].

Mechanism

The biomolecule (antibody, DNA probe, etc.) used for detection is labeled with one of the lanthanide chelates. Assays are performed in an endpoint manner and only need to be read once, when all pipetting steps are complete. All steps are performed according to a standard ELISA protocol. Instead of a substrate an enhancement solution is added, that disconnects the chelate lanthanide-chelate complex from the antibody to increase the signal intensity.

[26] http://www.perkinelmer.com/Catalog/Category/ID/delfia%20trf%20assays%20and%20reagents
[27] Enzyme linked Immunosorbent assay

Instrument parameters

Reader	**Infinite 200 PRO**	**Spark**
Assay	**Delfia**	**Delfia**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength	340(35)	340(35)
Emission wavelength	612(10)	612(10)
Lag time	200 µs	100 µs
Integration time	400 µs	400 µs
Flashes	25	150
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	0 ms	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

GeneBLAzer and Tango™ GPCR[29] Assay System[30]

Gene activator assay with FRET readout

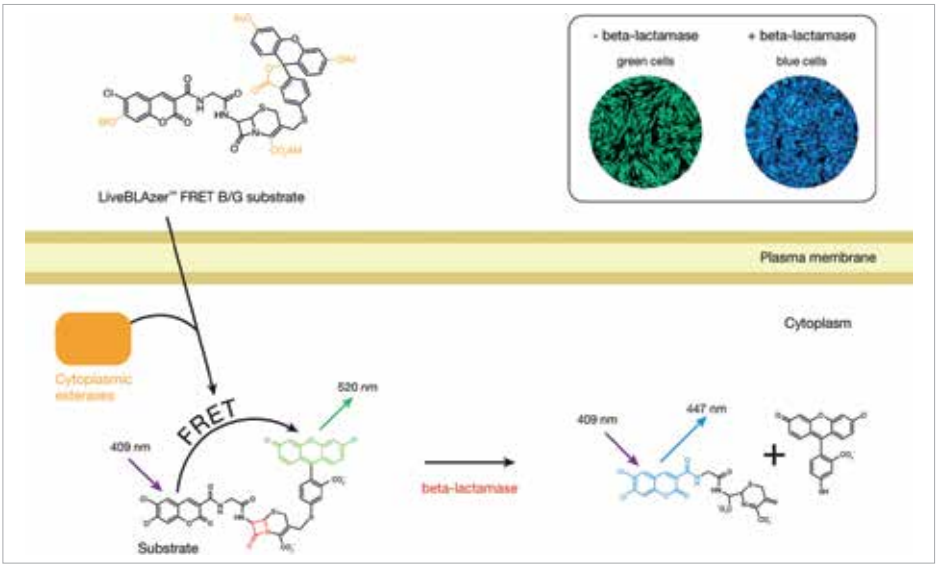

Figure 1: Principle of the GeneBLAzer assay. If the substrate is cleaved, the FRET signal is disrupted.

ASSAY OVERVIEW

Technology

Fluorescence Resonance Energy Transfer (FRET), ratiometric

Major application, Principle

Life Technologies' GeneBLAzer assays are designed to monitor the activation of genes, including surface and intracellular reporters, a wide range of signal transduction pathways, ion channels and other transporters. The basis for the GeneBLAzer assay are cell lines possessing a β-lactamase[31] (BLA) gene under the control of a promotor which is downstream of the monitored target protein.

Provider

The β-lactamase-transfected cell lines can either be purchased from Life Technologies or self-transfected. Tango cell lines are also based on the GeneBLAzer technology, but are designed exclusively for GPCR activation assays.

Mechanism

As shown in Figure 1, after the transfection, cells are loaded with an engineered fluorescent substrate which is an assembly of two fluorophores: coumarin and fluorescein. If the target protein is inactive, BLA is not expressed and the substrate molecule remains intact. In this state, excitation of the coumarin results in FRET to the fluorescein moiety and emission of green light.

However, in the presence of BLA expression, the substrate is cleaved, causing the separation of the fluorophores, and FRET cannot occur. This results in the emission of a blue fluorescence signal from coumarin.

Reporter assays are often measured over several hours, or even days. During this time period the plate can either be shuttled between the incubator and the reader, or a temperature and gas controlled multimode reader such as the Infinite 200 PRO or Spark may be used.

Alternatives

LiveBLAzer™, which is a combination of GeneBLAzer and resazurin.

[29] G-Protein Coupled Receptors: important group of cell surface receptors for cellural signalling
[30] http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Drug-Discovery/Target-and-Lead-Identification-and-Validation/g-protein_coupled_html/GPCR-Cell-Based-Assays/GeneBLAzer-Theory.html
[31] β-lactamase is an enzyme that can cleave specific substrates

Instrument parameters

Reader	**Infinite 200 PRO**
Assay	**GeneBLAzer**
Measurement Mode	FI Bottom (2 labels)
Excitation wavelength Donor	415(20)
Emission wavelength Donor	460(20)
Lag time	0 µs
Integration time	40 µs
Flashes	25
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms
Excitation wavelength Acceptor	415(20)
Emission wavelength Acceptor	535(25)
Lag time	0 µs
Integration time	40 µs
Flashes	25
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT

Links

• http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

• http://lifesciences.tecan.com/infinite200pro_readmodes

HTRF® – Homogeneous Time-Resolved Fluorescence[33]

TR-FRET-based assay platform

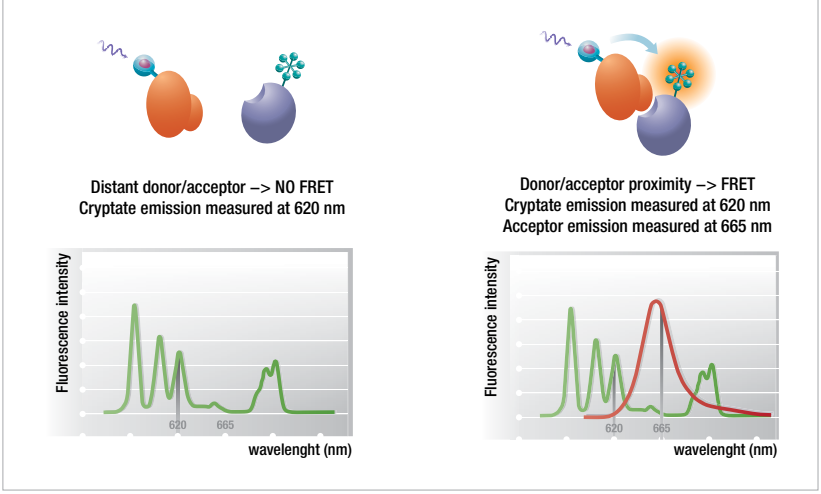

Figure 1: Mechanistic principle of the HTRF technology and fluorescence spectrum

ASSAY OVERVIEW

Technology

TR-FRET – Time-Resolved Fluorescence Resonance Energy Transfer

Provider

HTRF is Cisbio's TR-FRET-based assay platform, which provides a broad range of solutions.

Format, major applications

Biomolecules for detection can either be self-labeled or purchased pre-labeled. Additionally, ready-to-go assays and pre-coupled antibodies are available for major targets, including GPCRs, with second messengers and binding assays, kinases, epigenetic enzymes, protein-protein interactions and biomarkers.

Mechanism

HTRF is based on Eu^{3+} / Tb^{2+} cryptate donors and XL665 or d2 acceptors, which can be coupled to almost any biomolecule desired, including proteins, peptides, DNA and small molecules. The technology is based on no wash assay procedure detecting proximity events between donor and acceptor dyes.

The assay detection is obtained upon dispensing acceptor and donor conjugates to the sample to be assessed (e.g. enzymatic reaction mixture, cell lysate, or supernatant). No washing steps are required (homogeneous assay), and detection is performed after the completion of incubation, by measuring both specific donor and acceptor fluororescence (Figure 1).
To compensate for well-to-well variation, the ratio of both values is calculated (hence ratiometric assay). Donor fluorescence will always be detected and used as an internal control, while an emission signal from the acceptor is only detected if both biomolecules are in close proximity and FRET occurs[33].

[33] http://www.cisbio.com/drug-discovery/htrf-technology

Instrument parameters

				
Reader	**Infinite 200 PRO**	**Spark**	**Infinite 200 PRO**	**Spark**
Assay	**HTRF (Europium)**	**HTRF (Europium)**	**HTRF (Terbium)**	**HTRF (Terbium)**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	320(25)	320(25)	340(35)	340(35)
Emission wavelength Donor	620(10)	620(10)	620(10)	620(10)
Lag time	150 µs	100 µs	150 µs	100 µs
Integration time	500 µs	400 µs	500 µs	300 µs
Flashes	50	50	50	50
Mirror	automatic	automatic	automatic	automatic
Gain	optimal	optimal	optimal	optimal
Z-position	calculated from well	calculated from well	calculated from well	calculated from well
Settle time	0 ms	0 ms	0 ms	0 ms
Excitation wavelength Acceptor	320(25)	320(25)	340(35)	340(35)
Emission wavelength Acceptor	665 (8)	665(8)	665 (8)	665 (8)
Lag time	150 µs	100 µs	150 µs	100 µs
Integration time	500 µs	400 µs	500 µs	400 µs
Flashes	50	50	50	50
Mirror	automatic	automatic	automatic	automatic
Gain	optimal	optimal	optimal	optimal
Z-position	same as Label 1	same as Label 1	same as Label 1	same as Label 1
Settle time	0 ms	0 ms	0 ms	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

Adapta® Universal Kinase Assay and Substrates

Life Technologies' version of the ADP detection assay

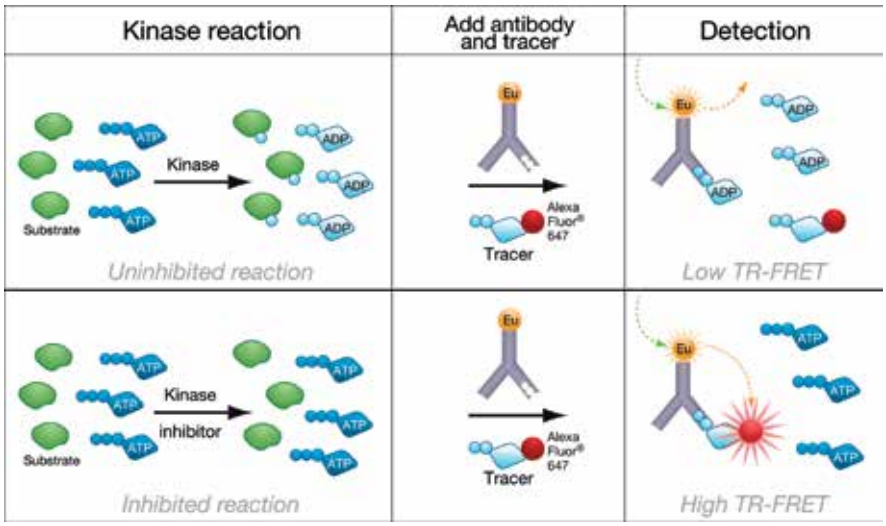

Figure 1: Schematic principle of the Adapta assay

ASSAY OVERVIEW

Technology

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), ratiometric

Principle, provider, major application

Life Technologies' Adapta Universal Kinase Assay Kit is a homogeneous, fluorescence-based immunoassay for measuring the activity of ADP-producing enzymes, mainly kinases. Additionally, the Adapta assay is available for a selection of lipid- and peptide-based substrates. Life Technologies supplies europium-coupled antibody specific for ADP. In contrast to the Transcreener® platform, Adapta is only available for ADP.

Mechanism

The ADP-specific antibody and the tracer are added to the sample. In an inhibited reaction (Figure 1), the monitored kinase produces no ADP and only the added, tracer-bound ADP molecule binds to the antibody, causing a high FRET signal.

Active kinases convert ATP to ADP. The free ADP competes with the tracer-bound ADP to bind to the antibody, resulting in a low FRET signal. Hence, the signal intensity is indirectly proportional to the activity of the kinase.

Instrument parameters

Reader	**Infinite 200 PRO**
Assay	**Adapta Assay**
Measurement Mode	FI Top (2 labels)
Excitation wavelength Donor	340(35)
Emission wavelength Donor	620(10)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms
Excitation wavelength Acceptor	340(35)
Emission wavelength Acceptor	665(8)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT

Links

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• http://lifesciences.tecan.com/infinite200pro_readmodes

LanthaScreen™ Kinase Activity Assays[34]

Kinase activity assay with TR-FRET readout

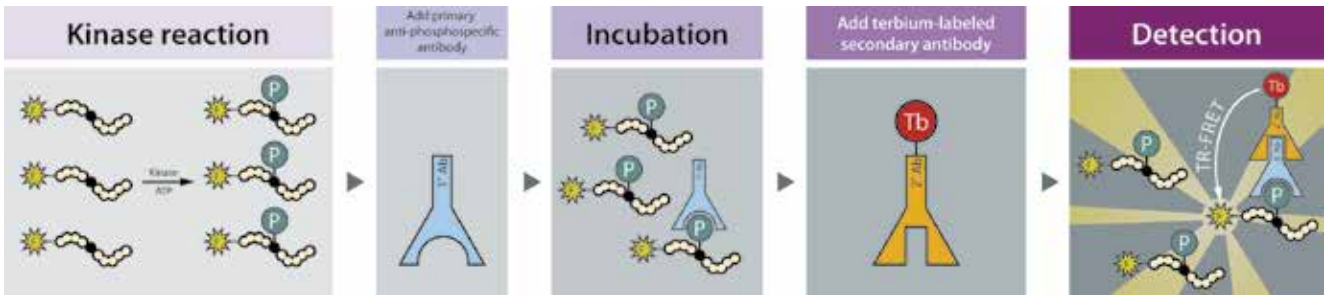

Figure 1: Schematic principle of the LanthaScreen assay

ASSAY OVERVIEW

Technology

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), ratiometric

Principle, provider, major applications

LanthaScreen is a kinase activity assay sold by Life Technologies. Kinases are important cellular enzymes, and their major function is to add phosphate groups to peptide substrates. For researchers, it is important to know how active kinases are in the presence of certain inhibitors. LanthaScreen quantifies kinase activity by measuring the amount of phosphorylated substrate.

Format

Life Technologies supplies a broad panel of fluorescein-labeled substrates and the corresponding lanthanide-labeled antibody specifically for the detection of phosphorylated substrates.

Mechanism

Kinase and fluorescein-labeled substrates are incubated to enable phosphorylation. After incubation, a terbium-labeled antibody is added to the reaction (Figure 1).

Scenario 1 – kinase is active

The substrate was phosphorylated, allowing the phospho-specific antibody to bind. The fluorescein and terbium labels are now in close proximity, resulting in a high FRET signal.

Scenario 2 – kinase is inactive

No phosphorylation occurred, and therefore the antibody could not bind to the substrate. FRET cannot occur. The final result is a dimensionless number that is calculated as the ratio of the acceptor (fluorescein) signal to the donor (terbium) signal.

[34] www.invitrogen.com/lanthascreen

Instrument parameters

Reader	**Infinite 200 PRO**
Assay	**LanthaScreen**
Measurement Mode	FI Top (2 labels)
Excitation wavelength Donor	340(35)
Emission wavelength Donor	495(10)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms
Excitation wavelength Acceptor	340(35)
Emission wavelength Acceptor	520(10)
Lag time	100 µs
Integration time	200 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT

Links

• http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

• http://lifesciences.tecan.com/infinite200pro_readmodes

FP – Fluorescence Polarization.

Binding assay for biomolecules

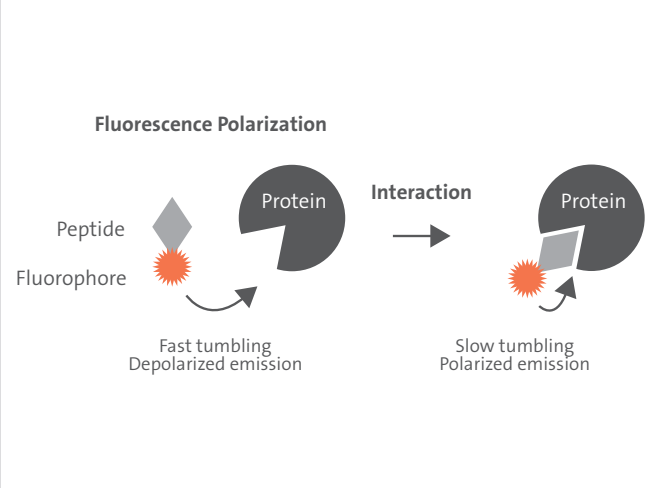

Figure 1: Schematic reprenstation of Fluorescence Polarization

$$mP = 1000 \times \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

Equation 1: Calculation of the polarization value. $I_{\parallel}$ = light parallel to the polarization plane. $I_{\perp}$ = light perpendicular to the polarization plane

TECHNOLOGY

Fluorescence anisotropy is colloquially referred to as Fluorescence Polarization. On excitation with polarized light, the emission from many samples is also polarized. Rotational movements of the excited molecule destroy this correlation. The extent of polarization remaining depends on the size of the molecules measured: the bigger the molecules, the slower they rotate and the higher the conservation of the original polarization. Other influences include solvent viscosity, temperature and the lifetime of the excited state.[35]

The following metaphor is an easy way to explain FP. Imagine a little child playing in a field. While it is free, it can twist and turn as much as it wants in any direction. Once it is 'attached' to its mother's hand, the movements will slow down and get direction. This comparison can be used to show how FP detects molecular interactions (Figure 1). The little child represents the smaller of the interaction partners, and the mother the larger one. A fluorescent probe is attached to the small molecule to observe the turning and twisting movements. As long as there is no interaction between the small and the large molecule, the rotation of the fluorophore is fast and the emitted light depolarized. Once it binds to a larger interaction partner, its movements will slow down and the emitted light will preserve more and more of the original polarization.

Assay design

A major application of FP is the detection of molecular interactions. FP assays require interaction partners to be different sizes, and the smaller molecule to be labeled with a fluorophore. Commercial assays commonly provide these labeled partners or substrates. The final result is a ratio of the polarization values, measured before and after addition of the suspected interaction partner. The polarization is calculated using the equation given below, measuring the intensity of emitted light in perpendicular and parallel planes.

Changes in polarization give information about the creation of interactions and their strength. A higher mP (milli-polarization) value represents a stronger interaction between the two molecules.

Major applications

- PolarScreen™ Assays
- Transcreener® Assays
- Predictor™ hERG assay

[35] Lakowicz, J. R. (1999). Principles of Fluorescence Spectroscopy. Kluwer Academic / Plenum Publishers

PolarScreen™

FP-based kinase activity assay; FP-equivalent to LanthaScreen

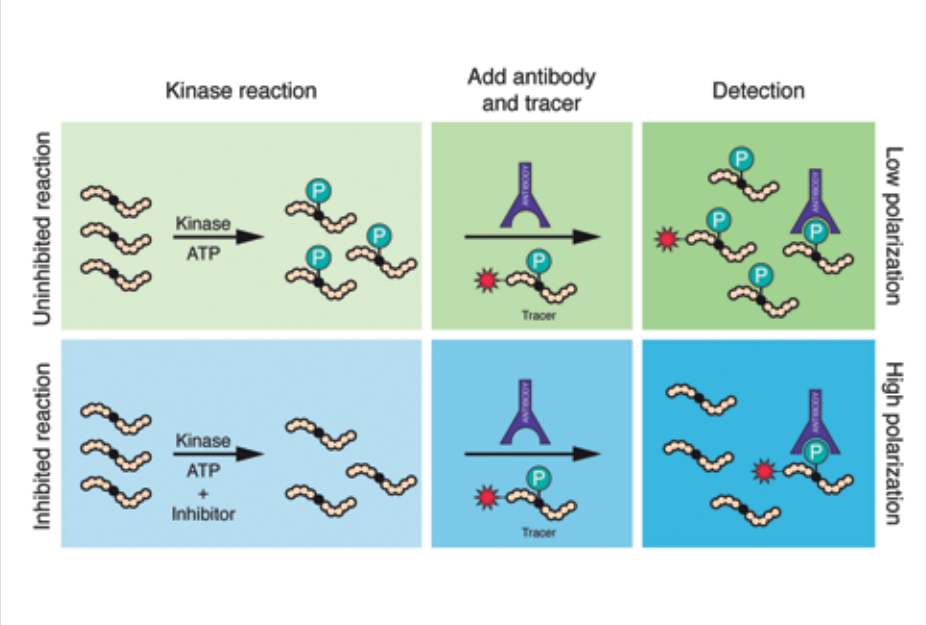

Figure 1: Schematic principle of the PolarScreen assay.

ASSAY OVERVIEW

Technology

Fluorescence Polarization (FP)

Provider, major application

PolarScreen[36] is Life Technologies' version of a FP-based kinase activity assay. Life Technologies offers a panel of phospho-specific antibodies which detect peptide substrates when phosphorylated by protein kinases.

Mechanism

The target kinase is incubated with a dedicated, unlabeled substrate (Figure 1). Antibody – specific for the phosphorylated- phosphorylation site of the substrate – and additional, tracer-bound substrate are added to the sample. If the unlabeled substrate's phosphorylation site remains unphosphorylated, for example due to an inactive enzyme, the antibody will only bind to the added, tracer-bound substrate, causing a low FP signal.

Active kinases will phosphorylate the non-tracer bound substrate, which then competes with the tracer-bound substrate for binding to the antibody, resulting in a high FP signal. Hence, the FP signal is directly proportional to the amount of phosphorylated substrate.

Format

Fluorescence labels are available for green, red or far-red detection. Red fluorescence readouts help to reduce autofluorescence.

[36] https://www.thermofisher.com/us/en/home/industrial/pharma-biopharma/drug-discovery-development/target-and-lead-identification-and-validation/nuclear-receptor-biology/nuclear-receptor-biochemical-assays/polarscreen-competition-assays.html

Instrument parameters

PolarScreen Green	
Reader	**Infinite 200 PRO**
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	485(20)
Emission wavelength Donor	535(25)
Lag time	0
Integration time	20 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

PolarScreen Red	
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	535(25)
Emission wavelength Donor	590(20)
Lag time	0
Integration time	20 µs
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

PolarScreen Far Red	
Assay	**PolarScreen**
Measurement Mode	FP
Excitation wavelength Donor	610(20)
Emission wavelength Donor	670(40)
Lag time	0
Integration time	default
Flashes	10
Mirror	automatic
Gain	optimal
Z-position	calculated from well
Settle time	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

Transcreener®

Nucleotide (ADP, GDP, etc.) detection assay

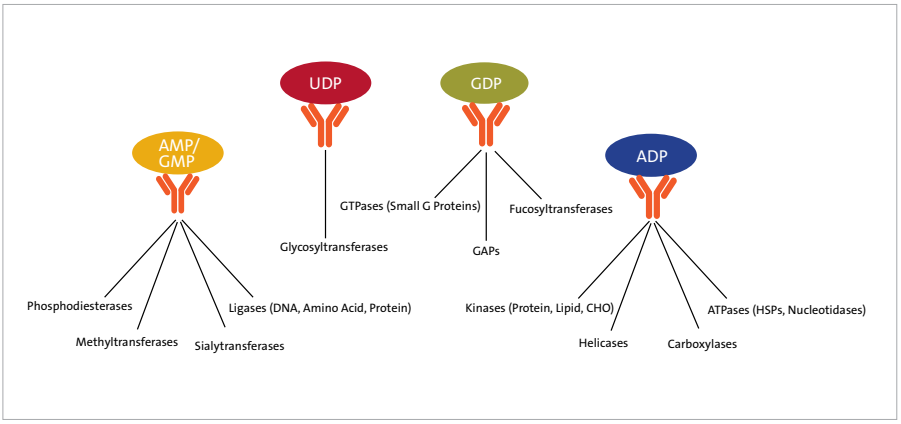

Figure 1: Transcreener targets

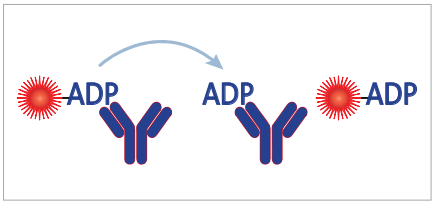

Figure 2: Transcreener principle

Assay	Readout
Transcreener ADP² Assays	FP, FI, TR-FRET
Transcreener AMP/GMP Assay	FP, FI
Transcreener GDP Assays	FP
Transcreener UDP Assays	FP

Table 1: Alternative readouts

ASSAY OVERVIEW

Technology

Fluorescence polarization (FP)

Fluorescence intensity (FI)

Time-resolved fluorescence resonance energy transfer (TR-FRET)

Principle, provider

Bellbrook's Transcreener assays[37] are designed to detect various mono- and dinucleotides using FP, TR-FRET or FI detection mode. Four assays (Table 1) cover thousands of target enzymes, including any kinase, ATPase or GTPase. Transcreener is a universal assay method that can be used across entire families of nucleotide-dependent enzymes. All assays are based on different antibodies that show a high affinity for one specific nucleotide (Figure 1).

Mechanism

The mechanism is the same for all Transcreener assays. The antibody is preloaded with the corresponding nucleotide, which is conjugated to a tracer molecule. All assays use a far red tracer that minimizes compound interference. For example, in the ADP² FP assay the detection mixture comprises of Alexa 633 ADP and a highly selective ADP monoclonal antibody. The Transcreener ADP² FP assay measures the progress of any enzyme that produces ADP by displacing the tracer by ADP thereby causing a decrease in fluorescence polarization. (Figure 2)

[37] https://www.bellbrooklabs.com/technical-resources/transcreener-faq/

Instrument parameters

Reader	**Infinite 200 PRO**	**Spark**
Assay	**Transcreener FP**	**Transcreener FP**
Measurement Mode	FP	FP
Excitation wavelength	610(20)	620(10)
Emission wavelength	670(25)	670(25)
Integration time	default	default
Flashes	10	30
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	50 ms	100 ms

Transcreener FI		
Assay	**Transcreener FI**	**Transcreener FI**
Measurement Mode	FI Top	FI Top
Excitation wavelength	580(20)	580(20)
Emission wavelength	620(20)	620(20)
Lag time	n.a.	n.a.
Integration time	20 µs	default
Flashes	25–100	30–100
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	50 ms	100 ms

Transcreener TR-FRET		
Assay	**Transcreener TR-FRET**	**Transcreener TR-FRET**
Measurement Mode	FI Top (2 labels)	FI Top (2 labels)
Excitation wavelength Donor	320(25)	320(25)
Emission wavelength Donor	620(10)	620(10)
Lag time	150 µs	150 µs
Integration time	500 µs	500 µs
Flashes	10	30
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	0 ms	0 ms
Excitation wavelength Acceptor	320(25)	320(25)
Emission wavelength Acceptor	665(8)	665(8)
Lag time	150 µs	150 µs
Integration time	500 µs	500 µs
Flashes	10	30
Mirror	automatic	automatic
Gain	optimal	optimal
Z-position	calculated from well	calculated from well
Settle time	0 ms	n.a.

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

Alpha – Amplified Luminescent Proximity Homogeneous Assay.

FRET equivalent with reduced distance limitations and amplification

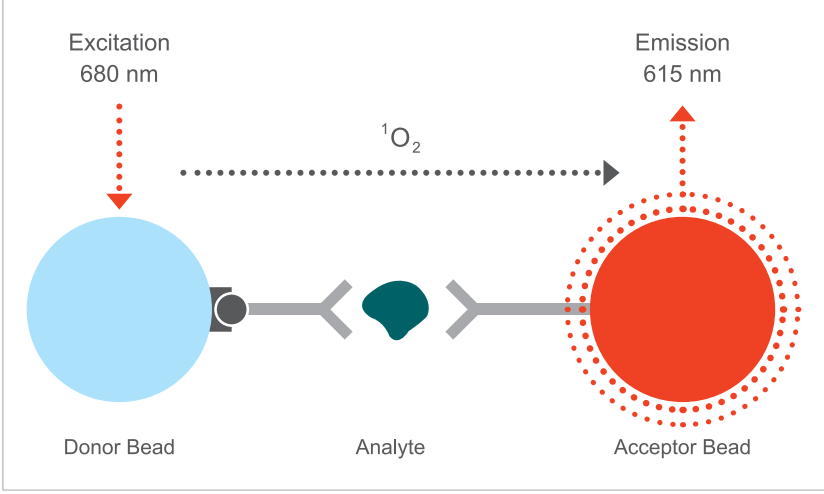

Figure 1: Schematic function of the Alpha technology.

TECHNOLOGY

Mechanism

Alpha[38] is a technology invented by PerkinElmer. The principle is similar to (TR-)FRET, since the Alpha Technology also relies on the interaction of an acceptor and a donor to yield a signal (Figure 1). However, instead of using simple fluorophores, chemically reactive beads are used, and the chemistry that produces the signal is also different. The main advantage of AlphaScreen® and AlphaLISA® is that the distance between the interaction partners can be up to 200 nm, compared to 10 nm for (TR-)FRET. Furthermore, there is an amplification effect which increases the sensitivity of the assay. Background is reduced because the emission wavelength is lower than the excitation wavelength.

Assay design

AlphaScreen (Amplified Luminescent Proximity Homogeneous Assay) is a bead-based screening technology developed by PerkinElmer for fast and reliable detection of biological interactions. The AlphaScreen chemistry employs donor and acceptor beads that can be linked to various types of biologically relevant molecules. The phthalocyanine photosensitizer molecules contained in the AlphaScreen donor beads convert ambient oxygen into large quantities of singlet oxygen when excited by a high energy light source at a wavelength of 680 nm. The singlet oxygen molecules are able to cover a distance of up to 200 nm during their half-life of approximately 4 µs. If AlphaScreen acceptor beads are in close proximity to the donor beads, due to a biological binding event between their coupling partners, the singlet oxygen molecules are able to initiate a cascade of energy transfer steps in the acceptor beads, ultimately resulting in the generation of a strong light emission in the range of 520-620 nm. Due to the amplified signal generation, even small amounts of biological analytes can be detected.

AlphaLISA is a homogeneous, no-wash alternative to conventional ELISA assays based on PerkinElmer's bead-based Alpha (Amplified Luminescent Proximity Homogeneous Assay) technology. AlphaLISAs can be set up as sandwich or competitive immunoassays to detect and quantify molecules of interest in biological samples. High energy excitation of photosensitizer molecules within the AlphaLISA donor beads at 680 nm converts

[38] http://www.perkinelmer.com/catalog/category/id/alphatech

ambient oxygen to singlet oxygen, which is able to react with the chemistry in the acceptor beads if these are in close proximity. A cascade of energy transfer steps ultimately results in the generation of a strong output signal at 615 nm, indicating specific binding between the molecules attached to the two bead types.

The fluorophores embedded in the AlphaLISA acceptor beads produce a narrower bandwidth signal than the acceptor beads used for classical AlphaScreen assays. This makes AlphaLISAs less prone to signal interference at wavelengths of <600 nm, increasing the sensitivity and robustness of the assay. The no-wash nature of this assay makes it easy to use, and the use of dedicated AlphaLISA optics permits the analysis of target molecules in blood and serum by drastically reducing the effect of hemoglobin within a sample.

The Alpha Technology's versatility offers the possibility to assay many biological targets, including enzymes, receptor-ligand interactions, low affinity interactions, second messenger levels, DNA, RNA, proteins, protein-protein interactions, peptides, sugars and small molecules[38].

Major applications

• AlphaScreen / AlphaLISA

SUPPORT

Links

• http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

• http://lifesciences.tecan.com/infinite200pro_readmodes

AlphaScreen / AlphaLISA

Alpha Technology assay platforms

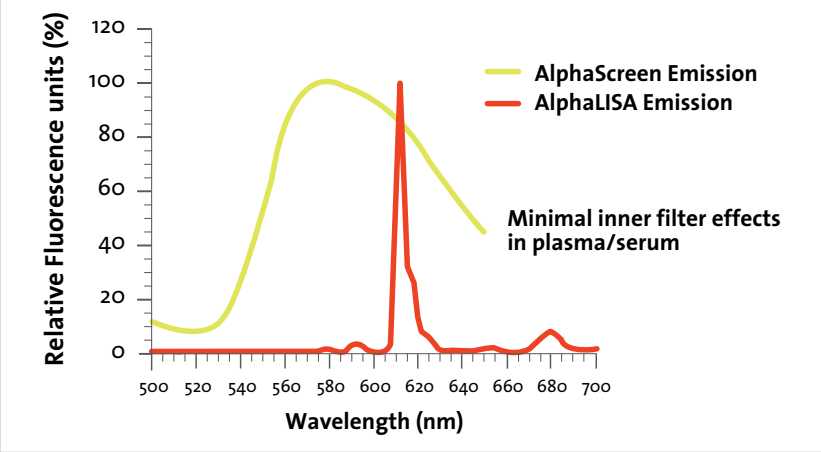

Figure 1: Emission spectra of TAR (AlphaScreen) and europium (AlphaLISA) acceptor beads.

ASSAY OVERVIEW

Technology

Alpha Technology

Provider, Format

AlphaScreen and AlphaLISA are PerkinElmer's Alpha Technology[39] assay / reagent platforms. PerkinElmer supplies donor beads coupled to streptavidin, and blank acceptor beads for self-labeling. Additionally, acceptor beads can be purchased precoupled to antibodies specific for a broad range of targets.

Principle

AlphaLISA is a development targeting laboratories working with crude blood samples, as the autofluorescence of hemoglobin overlaps with the emission peak (Figure 1) of the AlphaScreen acceptor. The main difference between AlphaLISA and AlphaScreen is that the AlphaLISA emission peak (europium emission) of the acceptor bead is smaller than that of the AlphaScreen assay (rubrene emission).

Mechanism

For a closer description of the assay mechanism, please refer to the Alpha Technology section.

[39] http://www.perkinelmer.com/catalog/category/id/alphatech

Instrument parameters

Reader	**Spark**
Assay	**AlphaScreen**
Measurement Mode	Alpha Technology
Excitation wavelength	n.a.
Emission wavelength	n.a.
Excitation time	100 ms
Integration time	300 ms
Wavelength	520-620 nm
Gain	n.a.
Settle time	0 ms
Temperature correction	activated

Assay	**AlphaLISA**
Measurement Mode	Alpha Technology
Excitation wavelength	n.a.
Emission wavelength	n.a.
Excitation time	100 ms
Integration time	300 ms
Wavelength	610-635 nm
Gain	n.a.
Settle time	0 ms
Temperature correction	activated

Assay	**AlphaPlex**
Measurement Mode	Alpha Technology
Excitation wavelength	n.a.
Emission wavelength	n.a.
Excitation time	100 ms
Integration time 1	300 ms
Integration time 2	300 ms
Wavelength 1	610-635 nm
Wavelength 2	535-560 nm
Gain	n.a.
Settle time	0 ms
Temperature correction	activated

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

LUMI-Luminescence.

Light is emitted from the sample

Figure 1: Firefly

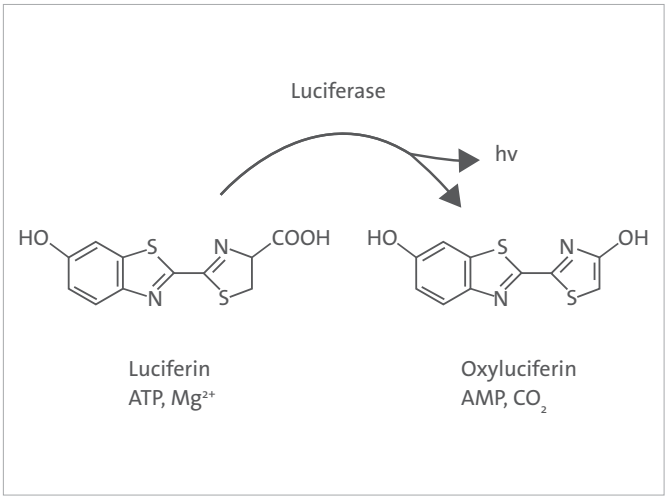

Figure 2: Substrate cleavage by luciferase

TECHNOLOGY

Luminescence is widely known as a reaction that causes the release of light. It can be caused by chemical reactions, electrical energy, subatomic motion, or stress on a crystal. For molecular biology, bioluminescence is the most important of the various luminescence reactions. At the core of this technology is the luciferase enzyme. Luciferases (Firefly, Renilla) (Figure 1) convert a substrate into an excited state (Figure 2). When returning to the ground state, a photon (light) is released (emitted).

One striking difference between fluorescence and luminescence is that luminescence requires no excitation light. This reduces the background to almost zero, resulting in better sensitivity.

Various forms of luminescence can be differentiated.

1. Glow luminescence, which generates stable and measurable light up to several hours, for example the BioThema ATP assay
2. Flash luminescence, which is characterized by rapid, but short-lived, light generation, for example DLR and Aequorin (injectors are required for flash luminescence)
3. Multicolor luminescence, such as BRET[1/2/3/e] and Chroma-Glow™

Major applications

- Dual-Luciferase® Reporter Assay (DLR™)
- Bio Thema ATP detection kit
- BRET (Bioluminescence Resonance Energy Transfer)
- NanoBRET™

Bio Thema ATP detection kit[42]

ATP detection kit based on luminescence

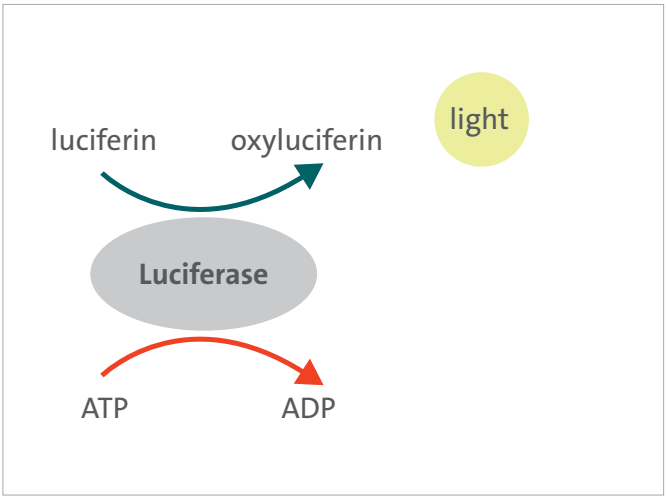

Figure 1: Schematic principle of luminescence caused by a luciferase based on the conversion of ATP.

ASSAY OVERVIEW

Technology

Luminescence (Glow)

Principle, provider

ATP (Adenosine-Tri-Phosphate) is a molecule for short time energy storage and required for almost any reaction in living organisms. Biothema's ATP detection kit measure and quantifies the level of ATP and correlates it to the activity of an enzyme or cells.

Format

The kit consists of a luciferase, the substrate and an ATP standard to quantify the signal of the sample.

Mechanism

The luciferase and substrate are added to the sample. If ATP is present, the luciferase converts the substrate and light is released (Figure 1). If no ATP is present, no light is released. The more ATP is present in the sample the stronger is the signal. In a last step the signal is quantified by comparing it to a standard ATP curve.

Major applications

Major applications include ATP detection, cell proliferation, cytotoxicity, enzymatic monitoring.

[42] http://biothema.se/products/kits /

Instrument parameters

Reader	**Infinite 200 PRO**	**Spark**
Assay	**Biothema ATP**	**Biothema ATP**
Measurement Mode	Luminescence	Luminescence
Integration time	1000 ms	1000 ms
Attenuation	automatic	automatic
Settle time	0 ms	0 ms

SUPPORT

Links

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology
- http://lifesciences.tecan.com/infinite200pro_readmodes

Dual-Luciferase® Reporter Assay (DLR™)[40]

Luminescence-based normalizable gene activator assay

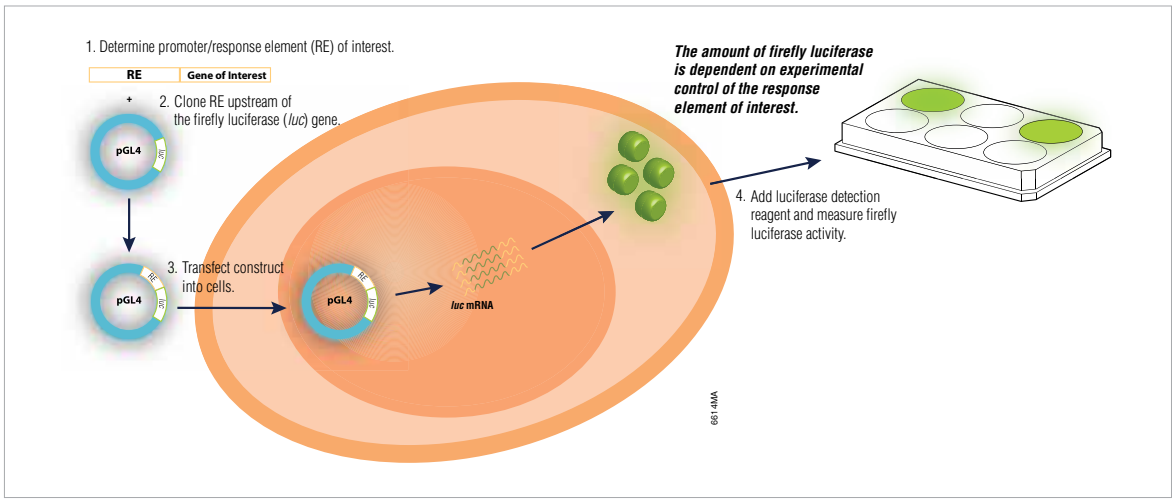

Figure 1: DLR assay mechanism

ASSAY OVERVIEW

Technology

Luminescence (flash)

Purpose, provider, major application

Promega's DLR assay measures gene activation / expression using a luminescence-based readout. One particular challenge of gene activator assays is quantification of the result. The DLR assay resolves this issue by normalizing the output using two luciferases. While one luciferase measures the expression of the gene of interest, the second luciferase measures the expression of a housekeeping gene[41], which is used to normalize the signal. Promega has two series of firefly and Renilla luciferase vectors – pGL4 and pRL – designed for use with the DLR assay systems.

Mechanism

Cells need to be transfected with both luciferase reporter genes (Figure 1). The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a 'glow-type' luminescent signal. After quantifying the firefly luminescence, the reaction is quenched, and the Renilla luciferase reaction simultaneously initiated, by adding Stop & Glo® Reagent to the tube. The Stop & Glo Reagent produces a 'glow-type' signal from the Renilla luciferase, which decays slowly over the course of the measurement.

[40] http://www.promega.com/products/reporter-assays-and-transfection/reporterassays/dual_luciferase-reporter-assay-system/
[41] Houskeeper genes are proteins or enzymes that are constitutionally expressed in most cells as for instance the DNA polymerase

Instrument parameters

		
Reader	**Infinite 200 PRO**	**Spark**
Assay	**DLR**	**DLR**
Measurement Mode	Luminescence	Luminescence
	well-wise	well-wise
Integration time	10,000 ms	10,000 ms
Output	n.a.	counts/sec
Attenuation	automatic	automatic
Settle time	0 ms	0 ms
Injections	Injector A: 100 µl (refill for every injection), wait 3 s	Injector A: 100 µl (refill for every injection), wait 3 s
	Injector B: 100 µl (refill for every injection), wait 3 s	Injector B: 100 µl (refill for every injection), wait 3 s

SUPPORT

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BRET (Bioluminescence Resonance Energy Transfer).

A FRET modification using the donor fluorophore as the light source

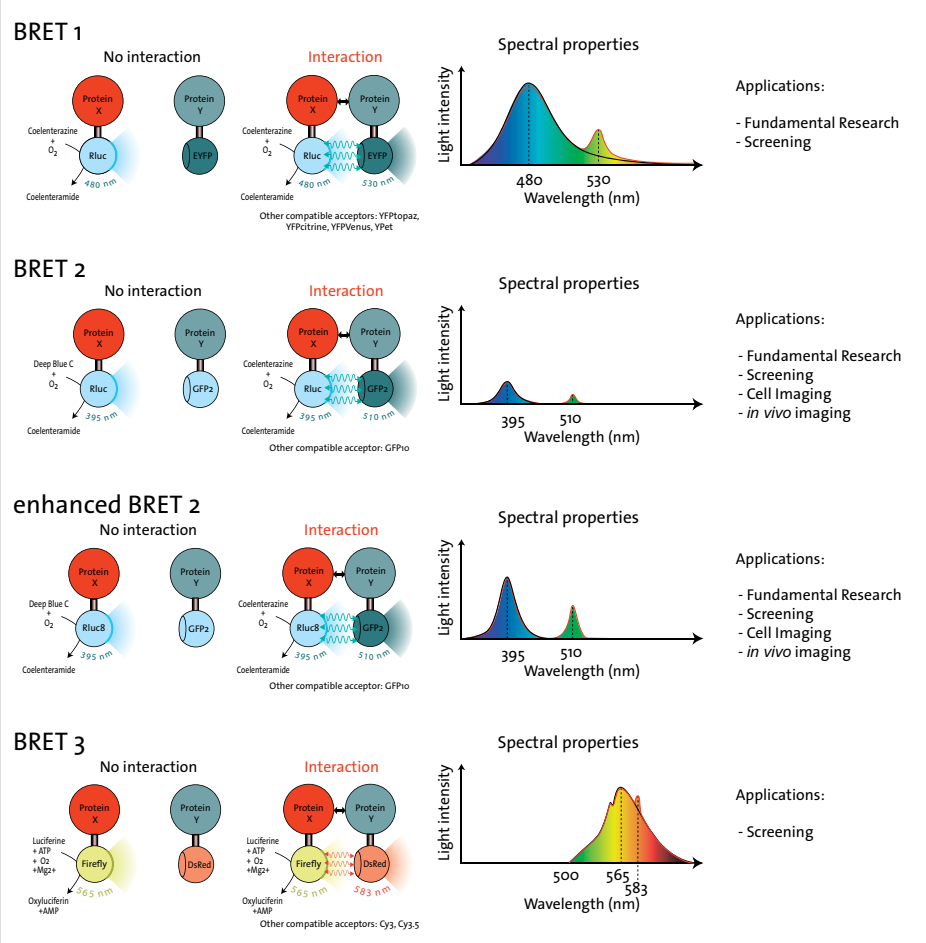

Figure 1: Summary of the most relevant BRET technologies.

TECHNOLOGY

BRET is a modification of FRET, the main difference being that the donor fluorophore is substituted by a luciferase. A major application for BRET is interaction studies. The advantage is that no excitation light is needed, and hence the background is much lower. BRET itself is a technology, and therefore can't be commercialized. However, various companies offer proprietary assay components for this technology, for example BRET[1/3/e] are an unlicensed technology, while BRET[2] is a licensed product of PerkinElmer.

The difference between BRET[1] and BRET[2] lies in the selection of the donor and acceptor proteins / fluorophores (Figure 1). This results in a shift of the excitation and emission wavelength.[42]

A new improved chemistry version of BRET, NanoBRET™, combines an extremely bright NanoLuc® luciferase with a means for tagging intracellular proteins with a long-wavelength fluorophore (HaloTag), providing a better dynamic range and sensitivity.[43]

[42] Bacart J, Corbel C, Jockers R, Bach S, Couturier C: The BRET technology and its application to screening assays. Biotechnol J 2008, 3:311-324.
[43] Machleit et al. NanoBRET–A novel BRET platform for the analysis of protein-protein interactions. ACS Chem. Biol., 2015, 10 (8), pp 1797–1804.

Compatible readers

Reader	**Infinite 200 PRO**	**Spark**
Assay	**NanoBRET-based assays**	**NanoBRET-based assays**
Measurement Mode	Dual Color Luminescence	Luminescence Multi Color
Filter 1 / Wavelength Range 1	Blue1_NB	445-470 nm
Integration time	300 ms	300 ms
Filter 2 / Wavelength Range 2	Red_NB	610-635 nm
Integration time	300 ms	300 ms
Settle time	0 ms	0 ms

SUPPORT

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Cell-based assays.

Imaging-based analysis of cell samples

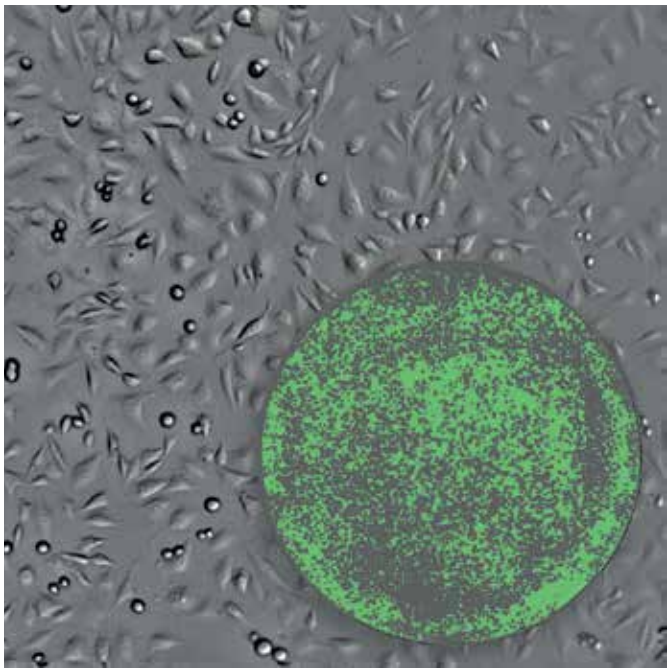

Figure 1: Cell imaging in the Spark reader

TECHNOLOGY

Cell-based assays in microplate formats have become a vital part of life science, with cell growth, proliferation, migration, and cytotoxicity being central research objectives.

Cell counting, viability and confluence assessment are important quality control parameters that are routinely analysed in cell culture laboratories. In this context, imaging-based readouts provide valuable insights and provide the basis for reproducible analysis and accurate interpretation of experimental data.

Automating and standardizing these processes therefore greatly facilitates cell-based workflows and helps to free time in the lab.

SUPPORT

Links

- http://lifesciences.tecan.com/cellbiology
- http://lifesciences.tecan.com/live-cell-imaging

Cell Counting / Viability

Determine the number and health of your cells

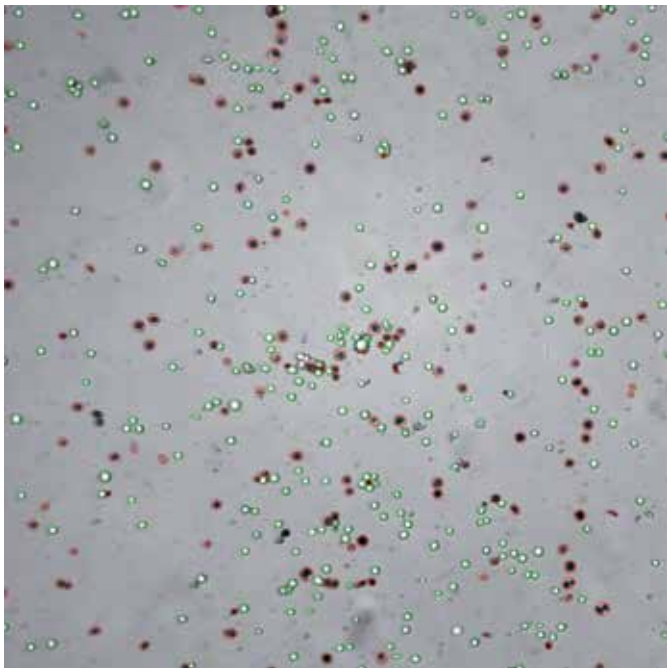

Figure 1: Discrimination of live/dead cells

ASSAY OVERVIEW

Technology

Camera-based analysis

Cell-based assays in a microplate format have become a vital part of life science; proliferation, cytotoxicity and gene expression studies all inevitably use cells as a working tool. Regardless of the research area, cell counting and quality checks are commonly performed before cell seeding or passaging steps. One approach is to identify if cells are alive or dead by staining with trypan blue; only dead cells or cells with a damaged cell membrane will be stained blue, enabling them to be discriminated from viable, healthy cells.

Automated cell counting saves a lot of time in the laboratory and helps to standardize cell culture workflows.

Major applications

- Cell culture quality control
- Cell seeding

SUPPORT

Link

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

Cell Confluence

Check the growth status of your cells

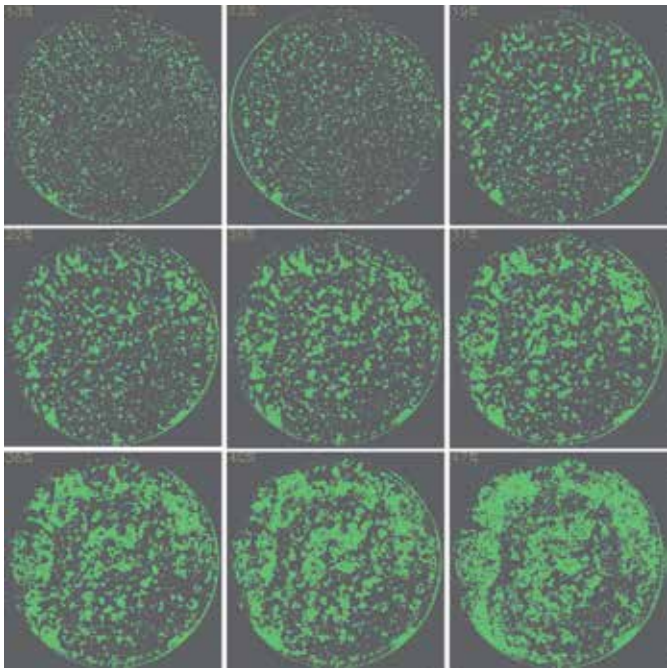

Figure 1: Continuous growth monitoring with the cell confluence function

ASSAY OVERVIEW

Technology

Camera-based analysis

In cell culture applications, confluence assessment is an important quality control parameter that is commonly used to estimate the proportion of adherent cells on a growth surface as an indicator of the cell density inside a well or culture flask. Some cell lines behave differently regarding their growth rate or gene expression depending on the degree of confluence. Consistent determination of cell confluence and estimation of cell numbers are therefore important for reproducible assays and accurate interpretation of experimental data.

Image capture and confluence determination of adherent cells directly in microplates improves crucial scientific parameters like assay variation and cell seeding uniformity. Label-free confluence assessment can also be used as a replacement for costly and time-consuming cell mass analyses like the quantification of protein levels or ATP content. It also enables new application types like cell migration-, wound healing- and clonogenicity assays.

Major applications

- Cell culture quality control
- Cell migration and wound healing studies
- Single cell cloning

SUPPORT

Link

- http://lifesciences.tecan.com/multimode-plate-reader?p=Technology

Technical terms.

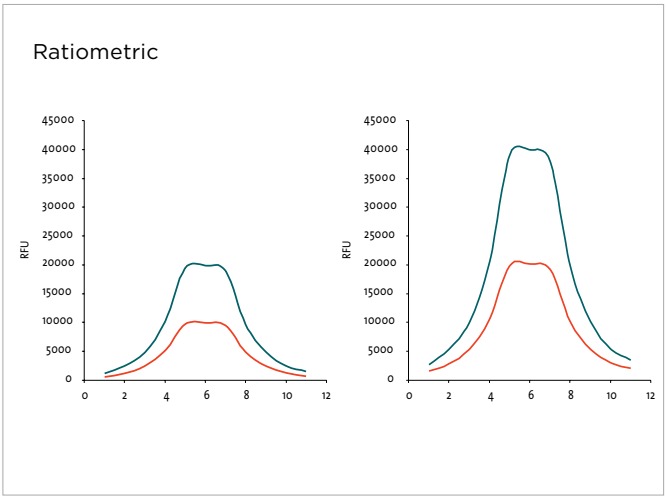

Figure 1: Two assay signals from the same sample. The difference in intensity is caused by varying amounts of added assay reagents

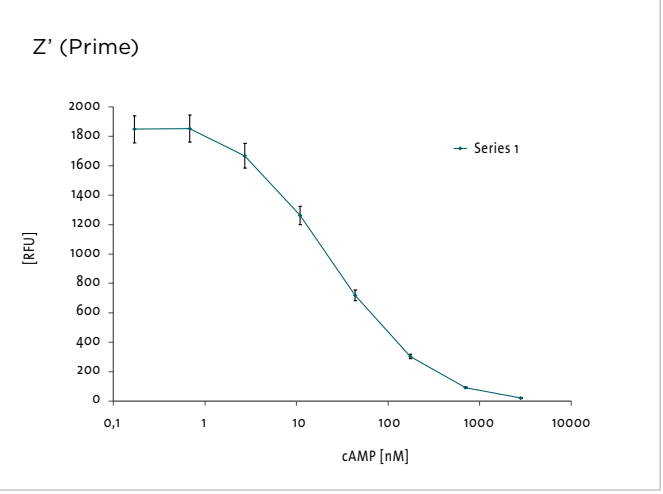

Figure 2: Data acquisition for Z' calculation

Homogeneous

The term homogenous refers to the design of an assay, and means that no washing is required. All the required assay components are added either simultaneously or one after another, but there are no washing steps in between. This simplifies the assay set-up.

Ratiometric

Signal intensities for duplicates of the same sample might differ depending on the amount of assay reagent added. Eventually, this will lead to higher standard deviations of the results. Ratiometric assays are self-referencing because they normalize the result to the concentration of assay reagent added.

For example:

HTRF assays are excited at 320 nm (Eu) and emission is measured at 620 nm and 665 nm.

The emission signal measured at 620 nm (donor) is independent of the interaction, depending solely on the concentration of the added assay reagents, and is therefore used as an internal reference.

The emission signal at 665 nm (acceptor) is used as an indicator of the suspected biological interaction being assessed, and represents the amount of interacting molecules.

To eliminate the influence of the reagent concentration, the emission signal at 665 nm is divided by the reagent concentration measured at 620 nm, eliminating well-to-well deviations.

Ratiometric measurements include FP, FRET, TR-FRET and BRET-based assays.

G-Factor

G ... Gear / Device Factor (eng.)

Filters, mirrors and quartz fibers absorb light, resulting in signal intensity losses. However, the amount of light lost depends on the polarization angle of the light. To compensate for these differences, a gear (G) specific correction factor needs to be calculated. In general, it should be between 0.8 and 1.2.

Z' (Prime)

Z' is a quality criterion for assays and instrument features. It is a dimensionless factor calculated by dividing the standard deviations (SD) of the highest and lowest values by the difference of between the highest and lowest signals (dynamic range) of a curve. In general, Z' may be between 0 and 1, but would ideally be close to 1.

Tecan – Who we are.

Tecan is a leading global provider of life science laboratory instruments for the biopharmaceuticals, forensics, clinical diagnostics and academic sectors, specializing in the development and production of automation and detection solutions, including microplate readers, microarray products and washers.

Founded in Switzerland in 1980, Tecan has manufacturing, research and development sites in both North America and Europe, and maintains a sales and service network in 52 countries. To date, Tecan has distributed approximately 20,000 microplate readers worldwide, and is committed to continuous technological improvements and compliance with the highest global quality standards.

IMPORTANT INFORMATION

Tecan has not independently validated the methods described in the document with all possible sample types or analytical uses and is providing this example method as a convenience to users. Consistent with USP recommendations and good laboratory practice, the user must independently evaluate and validate: (a) the suitability of the method for their use, (b) their ability to process samples of their choosing following the method; and (c) their ability to proficiently perform the method in their facility with their personnel.

For more details, visit the Tecan webpage: **www.tecan.com**

Spark multimode reader is for research use only.

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