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HOMOGENEOUS ASSAY TECHNOLOGIES IN DRUG SCREENING: QUENCHING RESONANCE ENERGY TRANSFER (QRET) TECHNIQUE

by

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To my family

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Homogeneous assay technologies in drug screening: Quenching Resonance Energy Transfer (QRET) technique

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ABSTRACT

Information gained from the human genome project and improvements in compound synthesizing have increased the number of both therapeutic targets and potential lead compounds. This has evolved a need for better screening techniques to have a capacity to screen number of compound libraries against increasing amount of targets. Radioactivity based assays have been traditionally used in drug screening but the fluorescence based assays have become more popular in high throughput screening (HTS) as they avoid safety and waste problems confronted with radioactivity. In comparison to conventional fluorescence more sensitive detection is obtained with time-resolved luminescence which has increased the popularity of time-resolved fluorescence resonance energy transfer (TR-FRET) based assays.

To simplify the current TR-FRET based assay concept the luminometric homogeneous single-label utilizing assay technique, Quenching Resonance Energy Transfer (QRET), was developed. The technique utilizes soluble quencher to quench non-specifically the signal of unbound fraction of lanthanide labeled ligand. One labeling procedure and fewer manipulation steps in the assay concept are saving resources. The QRET technique is suitable for both biochemical and cell-based assays as indicated in four studies:1) ligand screening study of β_2 -adrenergic receptor (cell-based), 2) activation study of G_s-/G_i-protein coupled receptors by measuring intracellular concentration of cyclic adenosine monophosphate (cell-based), 3) activation study of G-protein coupled receptors by observing the binding of guanosine-5'-triphosphate (cell membranes), and 4) activation study of small GTP binding protein Ras (biochemical). Signal-to-background ratios were between 2.4 to 10 and coefficient of variation varied from 0.5 to 17% indicating their suitability to HTS use.

Keywords: high throughput screening, quenching resonance energy transfer (QRET), time-resolved luminescence, cell-based assay, biochemical assay, G-protein coupled receptor, small GTP binding protein

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TIIVISTELMÄ

Terapeuttisten kohteiden ja johtolankayhdisteiden määrä on kasvanut ihmisen genomi-projektin sekä kehittyneen yhdisteiden valmistuksen ansiosta. Jotta kasvaneet yhdistekirjastot kyettäisiin seulomaan kasvaneelle kohdemolekyylien ryhmälle tarvitaan uusia tehokkaampia seulontamenetelmiä. Radioaktiiviset menetelmät ovat olleet yleisesti käytössä lääkeseulonnassa. Turvallisuus- ja jäteongelmat ovat kuitenkin rajoittaneet radioaktiivisten menetelmien käyttöä tehoseulonnoissa. Nykyään fluoresenssiin pohjautuvat menetelmät ovat suosiossa, koska niiden käytössä ei ole samanlaisia turvallisuus- ja jäteongelmia. Aikaerotteinen luminesenssi on tavanomaista fluoresenssia herkempi menetelmä. Tästä syystä aikaerotteista fluoresenssiresonanssienergiansiirtoa hyödyntävien määritysten suosio on kasvanut viime aikoina.

Tässä väitöskirjatyössä kehitettiin luminometrinen yhtä leimaa hyödyntävä määritysmenetelmä: sammutusresonanssienergiansiirto. Työn tarkoituksena oli kehittää aikaerotteista fluoresenssiresonanssienergiansiirtomenetelmää yksinkertaisempi menetelmä. Kehitetty menetelmä hyödyntää liukoista sammuttajaa sammuttamaan sitoutumattomien lantanidileimattujen ligandien signaalia. Vain yhden leimausprosessin vaativa ja vähemmän työskentelyvaiheita sisältävä määritys säästää aikaa ja kustannuksia. Kehitetty määritysmenetelmä soveltuu sekä biokemiallisiin että solupohjaisiin määrityksiin. Tämä on todistettu neljässä tehdyssä tutkimuksessa: 1) solupohjainen β_2 -adrenergisen reseptorin ligandien seulontatutkimus, 2) solupohjainen G_s- ja G_i-proteiinikytkentäisten reseptorien aktivaatiotutkimus (solunsisäisen syklisen adenosiinimonofosfaatin konsentraatiomääritys) 3) solumembraanipohjainen G-proteiinikytkentäisten reseptorien aktivaatiotutkimus (guanosiini-5'-trifosfaatin sitoutumismääritys) ja 4) biokemiallinen pienen GTP:tä sitovan proteiinin Ras aktivaatiotutkimus (leimatun GTP:n sitoutumismääritys). Signaali-tausta suhteet määrityksissä olivat välillä 2,4-10 ja variaatiokerroin vaihteli 0,5 prosentista 17 prosenttiin, mikä havainnollisti määritysmenetelmien soveltuvuutta tehoseulontaan.

Avainsanat: tehoseulonta, sammutusresonanssienergiansiirto, aikaerotteinen luminesenssi, solupohjainen määritys, biokemiallinen määritys, G-proteiinikytkentäinen reseptori, pieni GTP:tä sitova proteiini

CONTENTS

ABBREVIATIONS

ADP	adenosine diphosphate
AlphaScreen	amplified luminescent proximity homogeneous assay screen
AMP	adenosine monophosphate
APC	allophycocyanin
β_2-AR	β_2-adrenergic receptor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CHO	chinese hamster ovary cell line
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CV	coefficient of variation
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EA	enzyme acceptor
EC_{50}	half maximal effective concentration
ED	enzyme donor
EDTA	ethylenediaminetetraacetic acid
EFC	enzyme fragment complementation
EGTA	ethylene glycol tetraacetic acid
E_{max}	maximum effect
FDA	US food and drug administration
FP	fluorescence polarization
FRET	fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanosine exchange factor
GMP	guanosine monophosphate
GPCRs	G-protein coupled receptors
GTP	guanosine-5'-triphosphate
HEK 293_i	human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
HTRF	homogeneous time-resolved fluorescence
HTS	high throughput screening
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	half maximal inhibitory concentration
IFNγ	interferon gamma
IP3	inositol 1,4,5-trisphosphate

LANCE	lanthanide chelate excite
LP	luminescence polarization
LRET	luminescence resonance energy transfer
M	mean
MS	mass spectrometry
PBS	phosphate buffered saline
PEI	polyethyleneimine
PMSF	phenylmethanesulfonyl fluoride
QRET	quenching resonance energy transfer
S/B	signal-to-background ratio
SD	standard deviation
S/N	signal-to-noise ratio
SNP	single nucleotide polymorphism
SOS	son of sevenless
SPA	scintillation proximity assay
TBP	trisbipyridine
TNFα	tumor necrosis factor alpha
TR-FRET	time-resolved fluorescence resonance energy transfer
TR-LRET	time-resolved luminescence resonance energy transfer
UDP	uridine diphosphate
uHTS	ultra high throughput screening
96w MTP	96-well microtiter plate

LIST OF ORIGINAL PUBLICATIONS

I Eija Martikkala, Mirva Lehmusto, Minna Lilja, Anita Rozwandowicz-Jansen, Jenni Lunden, Takenori Tomohiro, Pekka Hänninen, Ulla Petäjä-Repo and Harri Härmä (2009)
Cell-based β_2-adrenergic receptor–ligand binding assay using synthesized europium-labeled ligands and time-resolved fluorescence.
Analytical Biochemistry, **392**:103–109.

II Harri Härmä, Anita Rozwandowicz-Jansen, Eija Martikkala, Heini Frang, Ilkka Hemmilä, Niko Sahlberg, Vidal Fey, Merja Perälä and Pekka Hänninen (2009)
A New Simple Cell-Based Homogeneous Time-Resolved Fluorescence QRET Technique for Receptor-Ligand Interaction Screening.
Journal of Biomolecular Screening, **14**: 936–943.

III Eija Martikkala, Anita Rozwandowicz-Jansen, Pekka Hänninen, Ulla Petäjä-Repo and Harri Härmä (2011)
A Homogeneous Single-Label Time-Resolved Fluorescence cAMP Assay.
Journal of Biomolecular Screening, **16**: 356–362.

IV Eija Martikkala, Stefan Veltel, Jonna Kirjavainen, Anita Rozwandowicz-Jansen, Urpo Lamminmäki, Pekka Hänninen and Harri Härmä (2011)
Homogeneous Single-Label Biochemical Ras Activation Assay Using Time-Resolved Luminescence.
Analytical Chemistry, **83**: 9230–9233.

In addition, unpublished data is presented.

1 INTRODUCTION

Drug discovery pipeline is a multistep process starting from early-stage research of target and discovery of lead molecules and ending to drug marketing after approval of the drug. This process is time and money consuming with high risk of failure. The average time to develop a new pharmaceutical product is from 12 to 15 years and costs around 900 million $ (DiMasi et al., 2003). Statistically the process is more likely ending to failure than successfully to a novel drug. Only one compound from 5,000 compounds screened in early-state discovery will become to market. Often failures occur at early states of pipeline but lately the productivity of late-state drug development, both phase II and III, has decreased (Arrowsmith, 2011a; Arrowsmith, 2011b). The decreasing of the productivity especially at the late-state causes enormous waste of funds. Consequently ability to screen out failures from the masses of the compound at early states would be desirable. Usually the trend in drug industry has been to concentrate to well-known targets and prepare medicine to wide patient groups to minimize the risk of failure. According to the US Food and Drug Administration (FDA) drug approvals list of year 2011, the new evident trend has started to shift from primary care blockbuster mindset to specialty products like medicines for small subpopulation of cancer patients with specific genotype and unknown orphan targets (Mullard, 2012).

High throughput screening (HTS) evolved to fulfill the needs of pharmaceutical industry in the early state of drug discovery pipeline. The basic techniques of HTS were developed after the penicillin was discovered and start to use in human treatment. The use of HTS in lead discovery has enabled screening of large compound libraries against multiple target molecules in relatively short time and at relatively low costs. Development of technologies including robotics, liquid handling, high-quality detectors and high-performance computing together with assay miniaturization has boosted the productivity of the screening campaigns. The speeding of screening has not increased the productivity of drug screening in expected way. Scientists have now realized importance of the quality aspect in primary screening to reduce the number of compounds in downstream pipeline. Cell-based assays have become more popular as they can give more information about the interaction of target molecule with the complete regulatory network. With cell-based assays other aspects to be considered, like cytotoxicity of the drug compound or low membrane permeability, can be noticed in early-state of drug discovery process (Xia, Wong, 2012).

G-protein coupled receptors (GPCRs) are large varied group of cell-surface receptors which mediate their intracellular actions by a pathway involving activation of a heterotrimeric guanine nucleotide binding protein (G-protein). In human genome there are over 800 genes encoding these receptors (Fredriksson et al., 2003) and increased amount of disease causing mutations have been found in them (Thompson et al., 2005). Agonists of these receptors vary from multiple hormones to neurotransmitters. Members

of this superfamily can be divided according to their sequence to five main families: glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin (Fredriksson et al., 2003). According to their α-subunit similarity they can be divided to four subfamilies: G_s, $G_{i/o}$, $G_{q/11,}$ and $G_{12/13}$ (Simon et al., 1991). A common structural feature of all these receptors is the seven membrane spanning region reflecting their similar mechanism of action. GPCRs are an important target class for pharmaceutical industry covering around 30% of all marketed drugs and 30% of all targets studied so far are GPCRs (Hopkins, Groom, 2002). Multiple screening methods are developed for GPCRs studies including both ligand binding and functional assays. The choice of the screening method, especially with functional assays, depends on the class of GPCRs (Greasley, Jansen, 2005). Guanine nucleotide exchange is generic event in receptor activation of all GPCRs and assays observing exchange of GTP are commercially available. The recognition of the receptor activation in next step of GPCRs signal route is already dependent on the G-protein preference of the receptor (G_s, $G_{i/o}$, $G_{q/11}$ or $G_{12/13}$).

Small GTPases (also called small GTP binding proteins or small G proteins) are monomeric G-proteins. Their molecular masses vary from 20 to 40 kDa and they can be divided to at least five different families: Ras, Rho, Rab, Sar1/Arf, and Ran. They have an important role in regulation of multiple cell functions. Small GTP binding proteins have two activities: GDP/GTP binding and hydrolyzing GTP to GDP (GTPases activity). Small GTP binding proteins can present in either inactive GDP-bound or active GTP-bound form. GDP/GTP exchange is an extremely slow reaction because of slow dissociation of GDP from GDP-bound form. This reaction is stimulated by regulator GEF (guanosine exchange factor). GTPases activity of small GTP binding proteins is low and it is stimulated by GAPs (GTPase-activating protein) (Takai et al., 2001). Small GTPases and especially Ras protein have become very attractive targets for drug discovery because of their strong connection to cancer. Mutations of Ras have been commonly found in tumors as 30 percent of all human cancers contain constitutively activated Ras protein and Ras oncogenes have been established to be involved in early step of cancerogenesis (Oxford, Theodorescu, 2003; Bos, 1989).

Drug screening has an ongoing need to improve the screening techniques. This thesis reports the development of a simple single-label and sensitive time-resolved luminescence utilizing technique: quenching resonance energy transfer. The technique has been applied to screen targets important in drug screening: G-protein coupled receptors and small GTP binding proteins.

2 REVIEW OF LITERATURE

This chapter concentrates on the use of high throughput screening in drug discovery. Only homogeneous (separation free) assay technologies, excluding label-free methods, are introduced and the discussion focuses on:

1. the concept of HTS
2. HTS design and development processes
3. HTS detection technologies
4. commonly used commercial HTS assays

2.1 High Throughput Screening

High throughput screening (HTS) is a technique to find chemical modifiers for different biological targets. In HTS, a validated biological assay is used to test the abilities of the group or libraries of chemical samples to modify the chosen biological action (Janzen, 2008). Discovery of penicillin (Fleming, 1929) and its use as the first human antibiotic launched development of the HTS basic techniques. This revolutionary compound was discovered as a result of systematic sequentially testing of thousands of microbial cultures which show the effectiveness of a systematic approach of sequentially testing. Beside HTS has arisen a new path of HTS, ultra high throughput screening (uHTS). Amount of compounds screened per day with HTS range from 10 000 to 100 000 compounds, while in uHTS in excess of 100000 data points are generated per day (Mayr, Bojanic, 2009).

Increasing number of therapeutic targets and potential chemical compounds for drug use has driven pharmaceutical and biotechnology companies to use HTS. HTS is used for primary screening in lead discovery. HTS is especially useful in lead discovery cases where knowledge about the targets and compounds interacting with them is limited. HTS use in drug discovery process usually covers the phase from target research to lead discovery and includes the assay development and validation, implementation and data captures, storage and analysis (Macarrón, Hertzberg, 2009).

Relevance, reproducibility and repeatability of the screen are the common requirements for an HTS assay (Janzen, 2008). To ensure the biologic relevance and robustness of the screen, choices of the method and the target are critical. Low variability and high signal-to-background ratio of the screen help to avoid unwanted false hits. Also sufficient throughput and low costs are required (Macarrón, Hertzberg, 2009). The assay optimization is needed to fulfill all these requirements of the screen in the best possible way.

2.2 Assay design and development

Design and development process is a multi-stage process, which starts with a selection of targets. Chosen target has the influence on the choice of the assay format and optimization process. Miniaturization and automation are important parts of assay development process. At the end of the development process, assay quality is evaluated. These steps of the HTS assay design and development process are discussed briefly in the next chapters.

2.2.1 Target selection

Validity of the target and chemical tractability and screenability are three things to consider when the target is chosen. Targets with high validity are usually low risk targets but their novelty is poor. Novelty and validity are in inverse relationship to each other. Disease relevance should be the main criteria in target selection but also technical factors need to be considered for the HTS process. During the time HTS has been used, certain target classes have been noticed to be more chemically tractable than others. G-protein coupled receptors (GPCRs), ion channels, nuclear hormone receptors, and kinases are well chemically tractable while targets acting via protein-protein interactions are usually less successful. A problem with the protein-protein interactions is that chemical libraries usually do not include sufficient compounds to affect these interactions. Development of the field of natural products might help drug development with this problem by providing larger and more complex compounds. Screenability, the technical probability of developing robust assay, is nowadays concerning fewer problems because there are more and better assay methods available. After all there are differences between targets. For example ion channels are technically more difficult target for the HTS screen than GPCRs or proteases (Macarrón, Hertzberg, 2009).

2.2.2 Assay formats

Rough classification of the HTS assays fall into two classes: biochemical and cellular. Biochemical assays utilize isolated proteins while whole cells are used in cellular assays. For both assay formats multiple technologies are available for the HTS use. Subdivision of these assays can still be done according separation requirements. Assays requiring separation steps, such as washing or filtration, are known as heterogeneous and assays not requiring any separation steps as homogeneous assays (Janzen, 2008). In some cases concept ‘homogeneous’ might be employed to refer only a single step assay. However, in this study we use the concept ‘homogeneous’ for separation free assays. Homogenous assay format is favored in HTS because they are simpler to use and easy to automate (Hemmilä, 1991).

2.2.3 Assay optimization

Studying the biological systems in artificial conditions can run into the problems in stability. Activity of the test compounds might suffer from the artificial environment. The stability requirements for HTS are higher than in research assays generally. Diluted reagents are used longer periods of time and requirements for signal-to-background ratio and variability are stricter in HTS campaign. To obtain the best possible screening conditions, optimization of the assay should be accomplished during the assay development. The key success factors of the optimization process are the minimizing the use of the time and costs and maximizing the assay quality (Figure 1). Multiple factors can be tested during the optimization process including buffer composition, pH, temperature, ionic strength, and osmolarity. Table 1 presents more factors to be considered during the optimization. The initial knowledge of the system might help to reduce the number of the factors essential for the optimization.

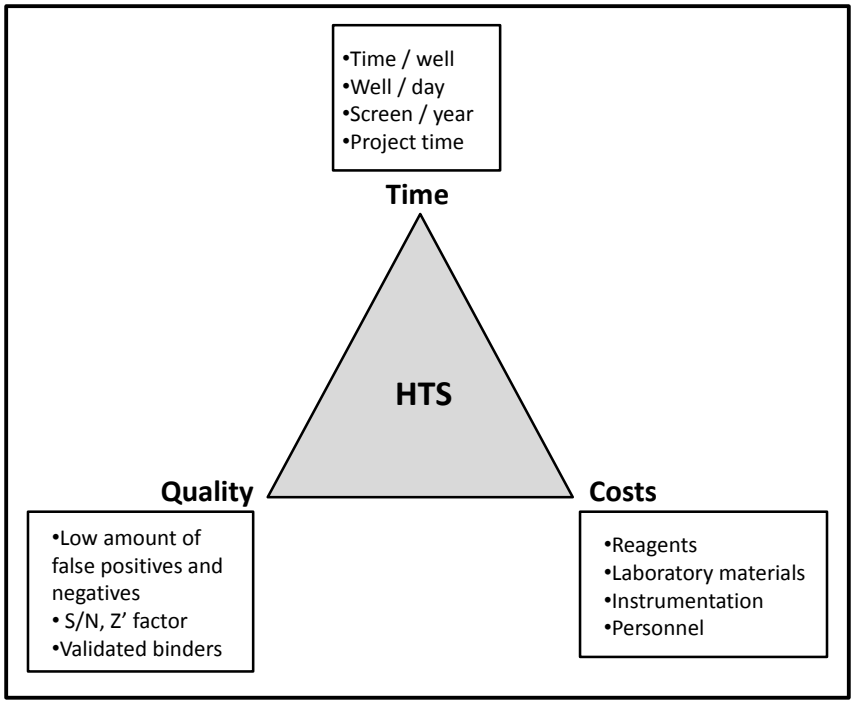

Figure 1. "Magic triangle of HTS" for successful optimization process of the HTS assay (Modified from Mayer, Bojanic, 2009).

Table 1. Group of factors to be considered during the optimization process (Macarrón, and Hertzberg, 2009).

Optimization factor	Examples
Monovalent ions	Na^+, K^+, Cl^-
Divalent cations	Mn^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}
Rheological modulators	glycerol, polyethylene glycol
Polycations	heparin, dextran
Carrier proteins	BSA, casein
Chelating agents	EDTA, EGTA
Blocking agents	PEI, milk powder, BSA
Reducing agents	DTT, β-mercaptoethanol
Protease inhibitors	PMSF, leuceptin
Detergents	Triton, Tween, CHAPS

Synthetic or natural compounds libraries used in the HTS campaigns are most commonly dissolved to dimethyl sulfoxide (DMSO). DMSO might have an influence to biological processes such as binding and it might decrease the activity of the test compounds. The tolerance of the assay to DMSO has to be tested in assay optimization to ensure functionality of the assay in HTS condition. Cell-based assays are usually performed in growth medium with the complex composition and there are plenty of factors to be considered in optimization. Medium, suppliers, selection and concentration of extra proteins are the main factors needed to be optimized. Also cell density, type and coating of the plate, incubation time, temperature and atmosphere are often considered. Extra caution in documenting and reproducing the cell cultures and assay conditions in cell-based assays is needed because there are more variables than in biochemical assays. More attention in the optimization process should be put to consider interactions of different factors than simply study the effects of the factors individually (Macarrón, Hertzberg, 2009).

2.2.4 Assay miniaturization and automation

Pressure to assay miniaturization has evolved from increased number of compounds for testing as well as increased number of molecular targets to be studied (Mayr, Bojanic, 2009). Pressure for miniaturization and automation of assays, to improve the throughput of the screening process, has induced the challenge of developing assays which are robust in a robotic screening environment (Taylor, 2002). In the first half of 1990s, 96-well microtiter plate (96w MTP) was the main HTS format. Later other formats were developed to minimize assay volume. 384w MTP and 1536w MTP are commonly used formats (Figure 2) (Mayr, Bojanic, 2009). Also 3456w MTP format bioassay has been reported (Brandish et al.,2006). However, in the miniaturization of the assay increased exposure to oxygen, higher surface-to-volume interactions and increased error risk, rising from the smaller volume handling and detection, can cause lower signal separation, increasing variability and loss of robustness (Taylor, 2002). For these reasons 3456w

MTP format has not become yet as a commonly used format as it was predicted earlier (Mayr, Bojanic, 2009).

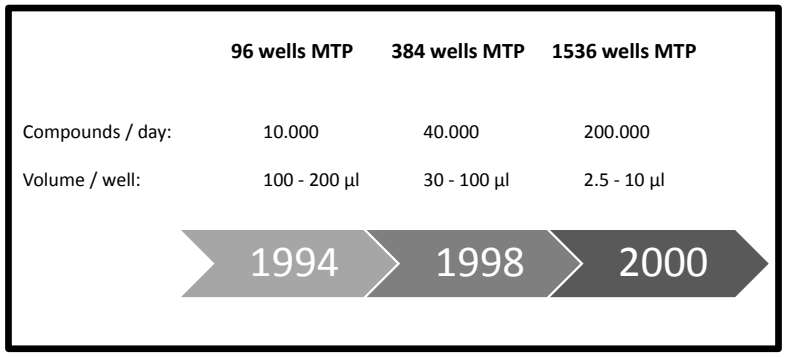

Figure 2. Assay miniaturization development and the effect of the volume miniaturization on effectiveness of the screening process (compounds/day) (Redrawn and modified from Mayr, Bojanic, 2009).

HTS involves always some automation. Two classes of automation are unit automation and fully automated or integrated system (Menke, 2002; Cohen, Trinka, 2002). In unit automation only a single task is treated with automation. In the division of the unit automation, three groups can be separated according to the function of the single task performed with it: liquid handling, plate storage and incubation and detection instrumentation. Liquid handling includes the pipetting and dispensing of the liquid to the microplates. Plate storage and incubation unit is needed to store a high number of assay plates, handled in each assay run, in proper storing conditions such as humidity and temperature. Detection instrumentation is needed in the final step of the screening process. The microtiterplate readers are available for all different detection techniques used in HTS and those can accept the plates from both manual feeding as well as robotic system. Many instruments are able to measure signals simultaneously from all wells in the plate which saves time and costs. Fully automated systems consist of all three unit automation units. Unit automation can be sufficient for effective screening but this usually required more personnel (Janzen, 2008).

Automation and miniaturization have enabled the HTS using labs to screen hundreds of thousands of compounds in short time period. To enable more efficient HTS screening, the focus is now shifting to reduce the costs and to speed up the assay developing process. Assay optimization has become one of the main bottlenecks of drug discovery in HTS (Taylor, 2002).

2.2.5 Assay quality and validation

Suitability of the developed assays for the screening purpose needs to be evaluated before implementation. Purpose of the validation is to ensure the high quality of the data.

Assays quality needs to be good enough to accurately distinguish the hits from inactive compounds in a sample collection. Before the main validation the statistical evaluation of the assay must be performed. Typical parameters calculated for HTS assay are mean (M), standard deviation (SD), signal-to-background ratio (S/B), coefficient of variation (CV), signal-to-noise ratio (S/N) and a screening window coefficient called the Z´ factor.

The signal-to-background ratio is calculated by using equation 1:

(1) $$S/B = \frac{\mu_S}{\mu_B}$$

(μ_S = signal mean, μ_B = background mean)

Signal-to-background ratio indicates the separation of positive and negative controls. It is a poor indicator of the assay quality because it is not dependent on the variability. It can still be helpful in early assay development to understand the potential of the assay.

Variability of the assay provides information about the assay stability and the precision of liquid handling and detection instruments. Good indication of the variability can be made from the relative measurement of the variability. Equation 2 for calculating coefficient of variation is:

(2) $$CV = 100\frac{\sigma}{\mu}$$

(σ = standard deviation , μ = mean)

For signal-to-noise ratio there is two equations (3 and 4) available:

(3) $$S/N = \frac{(\mu_S - \mu_B)}{\sigma_B}$$

(4) $$S/N = (\mu_S - \mu_B)\sqrt{(\sigma_S)^2\,(\sigma_B)^2}$$

(μ_S = signal mean, μ_B = background mean, σ_S = signal standard deviation, σ_B = background standard deviation)

The latter equation (4) can only be used for the evaluation of HTS performance because the former provides incomplete combination of the signal window and variability. Z´ factor is most commonly the standard parameter calculated when the HTS assay quality is evaluated and S/N ratio is very rarely used.

Equation 5 for Z´factor is:

(5) $$Z' = 1 - \frac{3(\sigma_S + \sigma_B)}{|\mu_S - \mu_B|}$$

(μ_S = signal mean, μ_B = background mean, σ_S = signal standard deviation, σ_B = background standard deviation)

Z´ factor combines two parameters mainly used for the assay quality evaluation: signal window and variability. Z´ factor is dimensionless and its values ranges between 0 and 1. Overlapping of the signal and background populations occurs when the Z´ factor value is 0 and infinite separation of signal and background populations is reached when the value is 1.

Real validation process of the assay before its acceptance for HTS use is performed after the assay has passed its quality control with an acceptable Z´ value. In validation process a representative sample of the screening collection is tested by using the identical protocols and robotic systems with the HTS run. Validation process gives useful information about production on assay performance, interferences from screening samples, evaluation of reproducibility in screening environment and estimation of hit rate and optimal sample concentration which simplify the latter HTS campaign (Macarrón, Hertzberg, 2009; Zhang et al., 1999).

2.3 Detection technologies

Several detection technologies have been developed but not all of them are suitable for HTS use. Detection methods commonly used in HTS include luminescence, absorbance, luminescence polarization, time-resolved luminescence, luminescence resonance energy transfer, scintillation proximity assays, AlphaScreen™, and electrochemiluminescence (Wu, Doberstein, 2006). Broadly, all these methods can be categorized to three main classes: luminometric, radiometric and photometric detection. Short descriptions of each main class of detection methods are given in following chapters.

2.3.1 Luminometric

Luminometric detection techniques are widely used in bioanalytical research. Increased interest towards luminometric techniques has risen from the need of developing safer, sensitive methods to replace radiometric techniques. Luminometric techniques allow fast and sensitive detection which enable their use in HTS. Small sample volumes needed for a luminometric assay are as well desired in HTS (Roda et al., 2003; Lakowicz,1999).

Jablonski diagram, first proposed by prof. A. Jablonski (1933), describes the absorption and emission of light (Figure 3). Absorption, fluorescence and phosphorescence can be illustrated schematically by using Jablonski diagram. Photoluminescence is the emission of light which occurs from electronically excited states (Lakowicz, 1999). Chemiluminescence differs from photoluminescence in the way the excited state is generated. In chemiluminescence the excitation energy is gained from an exothermal chemical reaction. Bioluminescence is a special form of chemiluminescence. In bioluminescence the light producing reaction is catalyzed by an enzyme (Fan, Wood, 2007). Depending on the excitation state luminescence can be divided to fluorescence

and phosphorescence. Fluorescence is emission of light from singlet state. Return of the electron is spin-allowed and occurs fast. Typical fluorescence lifetime is short (10 nanoseconds). Phosphorescence is emission of light from the excited triplet state. The electron has the same spin orientation in excited orbital as in ground state. Because the transitions to the ground state are forbidden, phosphorescence lifetime is longer, typically from milliseconds to seconds (Lakowicz, 1999).

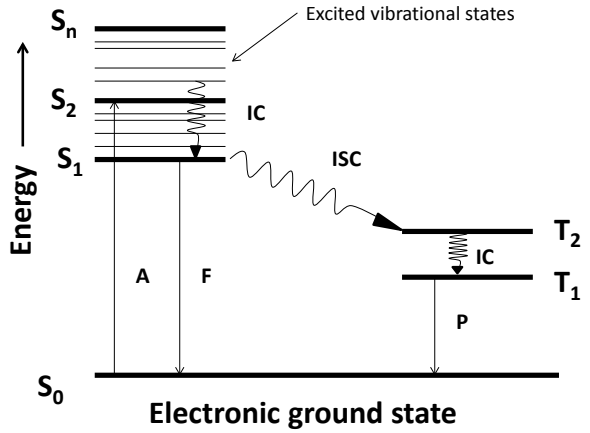

Figure 3. Jablonski diagram (modified from Lakowicz 2006). Abbreviations used in the figures: A = absorption of photons, S = singlet state, T = triplet state, IC = internal conversion, F= fluorescence (emission), ISC = intersystem crossing, P = phosphorescence.

Intensity, quantum yield, and lifetime are important parameters used to characterized fluorescence. The fluorescence intensity is defined as the amount of photons per time unit (s) (Renschler, Harrah, 1983; Rost, 1992).

The quantum yield is the ratio of the number of photons emitted to the number of absorbed photons.

(6) $$Q = \frac{\Gamma}{\Gamma + k_{nr}}$$

(Q = quantum yield, Γ = the emissive rate of the fluorophore, k_{nr} = the rate of non-radiative decay to S_0)

The fluorescence lifetime τ_0 is the time which fluorophore spends in the excited state (Lakowicz, 1999).

Quantum yield and lifetime can be influenced by many parameters. Temperature, pH, viscosity, polarity, hydrogen bonding and presence of quenchers (discussed later in this thesis) are examples of these parameters. Quantum yield and lifetime are usually decreased as temperature is increased. With free fluorophores differences in their temperature sensitivity occur. The change of temperature might change the intensity,

quantum yield and lifetime of fluorescence or have no effect on them. Binding to macromolecules might also change the temperature sensitivity of the fluorophores. Temperature dependency or independency of the fluorophore should always be taken into account during the assay design to avoid unwanted quenching effects. On the other hand temperature sensitivity of the fluorophore and quenching can be exploited in the assay designing (Valeur, 2002; Albani, 2004).

Chelates of certain lanthanide ions such as europium (Eu^{3+}), terbium (Tb^{3+}), samarium (Sm^{3+}) and dysprosium (Dy^{3+}) have special chemical and spectral properties compared to other organic species and metal ions. In a lanthanide chelate, lanthanide ion forms complex with one or more organic molecules containing the chelators or antennas. Lanthanide ions possess very weak luminescent properties but complexing of the lanthanide ion with a suitable ligand will reveal a luminescent characteristic of the lanthanide ion (Figure 4). Intersystem crossing of the absorbed energy from the first excited state of the ligand to the ligand triplet state can be followed by transfer of the energy to the first excited state of the lanthanide ion (Dickson, et al., 1995). Energy can still be transferred from the lanthanide ion to the acceptor molecule (Figure 4). Commercial technologies such as HTRF and Lance utilize this luminescence energy transfer process which will be discussed more in detail below (Pope, 1999).

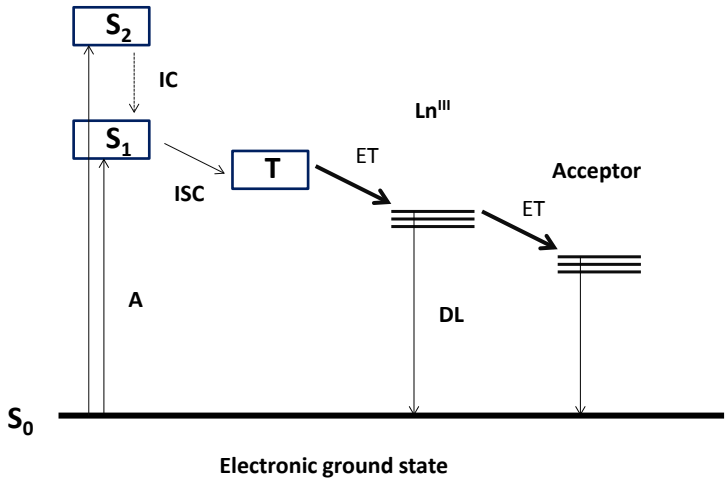

Figure 4. Luminescence of lanthanide chelates and luminescence resonance energy transfer. Abbreviations used in the figures: A = absorption of photons, S = singlet state, T = triplet state, IC = internal conversion, ISC = intersystem crossing, ET= energy transfer, DL = delayed luminescence (Modified from Bünzli, Eliseeva, 2010; Lakowicz, 1999).

Luminometric measurements can be roughly divided to two types of measurements: steady state and time-resolved. Steady state measurements are performed with constant illumination and observation. In time-resolved measurements the sample is illuminated with a short pulse of light (excitation) and intensity or anisotropy decay is recorded with

a high-speed detection system enabling nanosecond timescale measurements. An average of time-resolved phenomena over the intensity decays of the sample is the steady state observation. The steady state measurements are simpler to run and they do not require complex and expensive instrumentations as time-resolved measurements. Regardless of these facts and that the relationship of the steady state and the time-resolved measurements is known, time-resolved measurements are worthwhile to run. Part of molecular information available is lost in time-averaging process of the steady state measurements (Lakowicz, 1999).

Background luminescence interferences are inevitably present in all luminometric measurements. This limits the sensitivity of the conventional fluorometry. In time-resolved luminometry the use of delayed counting window aids to diminish the background signal and enable more sensitive measurements (Figure 5). Delayed measurement required longer decay times from lanthanide labels used in time-resolved luminometry (Hemmilä, Harju 1994). The chelates of lanthanide ions Eu^{3+}, Tb^{3+}, Sm^{3+} and Dy^{3+} have suitable qualities to fulfill the needs of the time-resolved luminescence: long emission duration and reasonable high quantum yield (Soini, Hemmilä, 1994).

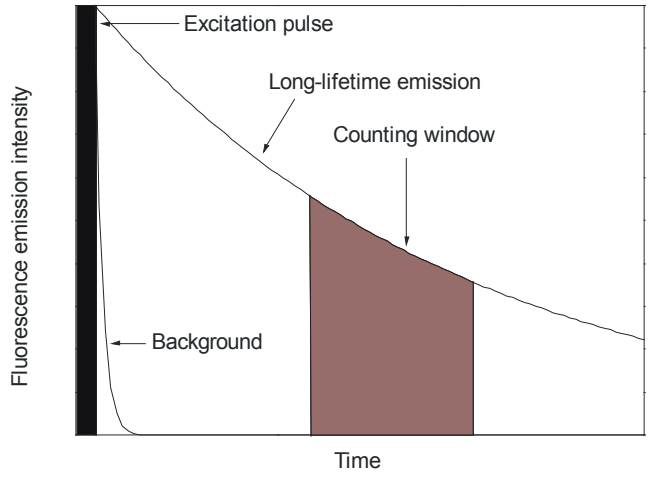

Figure 5. The principle of the time-resolved fluorescence. The use of delayed counting window enables more sensitive measurements (redrawn and modified from Hemmilä & Harju, 1994).

Luminescence polarization

Basis of luminescence polarization (LP) is the difference of luminescent molecular rotation proportional to molecular size when excited with plane polarized light. Small luminescently labeled molecules rotate rapidly during excitation and have low polarization values. Excitation of large luminescently labeled molecules does not cause large changes in their rotation and the polarization values of the large molecules are high. Luminescence polarization is commonly used in binding studies because the binding of the small luminescently labeled molecule to a large molecule changes the rotation of the

labeled molecule and can be easily detected. Other applications of LP are nucleic acid hybridization and enzymatic activities assays. LP assays do not require separation steps or immobilization, measuring of sample is fast and does not harm the sample. Because of these qualities LP has been used as a standard detection method in diagnostic as well as in drug discovery. On the other hand the used luminescent label should have appropriate lifetime to enable the measuring of the rotation. Especially with larger labeled molecules longer lifetimes of the labels (>100 ns) are needed to enable for detection of the slow rotation (Burke et al., 2003). Because utilizing conventional fluorescence the technique is vulnerable to the assay interference during the large compound library screens (Vogel et al., 2008; Riddle et al., 2006). In comparison to TR-fluorescence based techniques LP suffers from a more narrow dynamic range (Härmä et al., 2010).

Luminescence resonance energy transfer (LRET)

In luminescence resonance energy transfer (LRET) the excited state energy of the excited donor molecule (D) is transferred to an acceptor molecule (A). Process occurs typically when the emission spectrum of the donor molecule and the absorption spectrum of the acceptor molecule overlap. Energy transfer is a non-radiative process which does not require photons. Non-radiative energy transfer can occur as a result of two different types of interactions between the donor and the acceptor: Coulombic or intermolecular overlap of orbitals. Förster's mechanism contains a long range dipole-dipole interactions of Coulombic interactions. Distance between the donor and the acceptor molecules have influence on the efficiency of the energy transfer with Förster's mechanism. Förster distance indicates the distance at which the energy transfer efficiency is 50%. Förster distance is typically between 20 and 60 Å (Lakowicz, 1999; Valeur 2002). With lanthanide donors larger distances up to 90 Å have been obtained (Mathis, 1993; Selvin, Hearst, 1994; Bazin et al., 2002). Coulombic short range interactions contain multi-polar interactions. Intermolecular orbital overlap interactions are all short range interactions which occur either via electron exchange (Dexter's mechanism) or charge resonance interactions (Valeur, 2002). With lanthanide chelates both Dexter's and Förster's mechanisms have been proposed to explain energy transfer from ligand to lanthanide ion (Bünzli, Eliseeva, 2011).

RET-based methods are commonly used in biomolecular research to determine distances. RET can be applied to both quantitative and qualitative measurements (Valeur, 2002). Applications of RET include ligand-receptor interactions, conformational changes of biomolecules, receptor oligomerization, protein-protein interactions, protein folding, enzymatic activity, membrane organization and fusion, nucleic acid structures and sequences and DNA-protein interactions, immunoassays and biosensors (Valeur, 2002; Bazin, 2001).

Luminescence Quenching

Luminescence quenching is a process in which luminescence intensity of the sample is decreased. Many external molecules added to the luminescent solution can act as a quencher. Quenching can occur in consequence of a variety of molecular interactions. In dynamic quenching, quencher collides with the fluorophore, while in static quenching a non-luminescent complex with fluorophore is formed. Dynamic quenching is also called collisional quenching. In both types of quenching, decrease in the intensity and the quantum yield occurs. Lifetime is influenced only in dynamic quenching (Lakowicz, 1999; Albani, 2007). In more detail, in collisional quenching two diffused molecules, fluorophore and another molecule, quencher, just collide with each other. Complex of these two molecules is not formed. Quenching occurs during the excited state of the fluorophore. This type of quenching is time dependent. In static quenching a non-fluorescent ground state complex of fluorophore and quencher molecule is formed (Albani, 2004). This complex is after absorption incapable to emit any photon while returned to the ground state (Albani, 2007). In several cases combination of these two different types of quenching, collisional and static, occurs (Lakowicz, 1999; Albani, 2007).

Other types of quenching are thermal and resonance energy transfer based (Lakowicz, 1999; Albani, 2007). Quenching can occur without any interaction while the fluorophore and quencher are more far away from each other. This radiative energy transfer from donor to acceptor molecule depends on the spectral overlap of the donor and acceptor molecules and concentration. As a result of radiative transfer, fluorescence intensity of the donor is decreased (Valeur, 2002). Thermal effect on luminescence was discussed earlier in this chapter.

2.3.2 Radiometric

Radiometric techniques have long history of use. Radioactive labels used are usually produced by using a radioisotope of the atom existing in the compound as a substituent. Radiometric detection has been applied to measure receptor binding, immunoassays, DNA hybridization, cellular assays and metabolic studies (Hemmilä, 1994; Tammela, 2004). Disadvantages of the radiometric assays, such as safety and waste problems as well as challenges met with miniaturization of radiometric assays, limits the use of radiometric technologies (Gribbon, Sewing, 2003). Some homogeneous radiometric detection methods such as scintillation proximity assay (SPA), have still a major foothold in HTS campaigns (Wu, Liu, 2005; Khawaja et al., 2008).

2.3.3 Photometric

In photometric detection light absorption efficiency of the solution of biologic or organic compound (absorbance or transmittance) is measured at a given wavelength λ

(Valeur, 2002). Absorbance is commonly used in different quantitative concentration measurements. Simplicity of the absorbance measurements has increased its use in assays regardless of the limitations of the detection method in sensitivity. To be able to record changes, high concentrations of dyes or amounts of cells are needed. Absorbance measurements are still useful in certain measurements with lower sensitivity requirements but in tests with high sensitivity requirements, fluorescence and luminescence based assays are more useful and thus more used (González & Negulescu, 1998).

2.4 Commercial assay technologies

Many commercial assay technologies are available and novel ones are developed continuously. Majority of the developed methods are homogeneous because they are more suitable for HTS use. In the next chapters selected homogeneous HTS suitable commercial assay technologies are discussed briefly to give an overview of available technologies.

2.4.1 Scintillation proximity assay (SPA)

Scintillation proximity assay is radioactivity utilizing HTS suitable technology which does not require a separation step and is easily automated. SPA was originally developed in 1979 by Hart and Greenwald (Hart, Greenwald, 1979). SPA has been used as a golden standard receptor binding assay in many HTS operations and it can be adapted to radioimmuno-, enzyme and protein-protein interaction assays (Wu, Liu, 2005). SPA technology utilizes specific SPA beads where scintillant is incorporated to the bead matrix. When the radioactively labeled molecule is captured onto surface of the SPA bead emission of the energy from β particles to scintillant occurs effectively and dissipation of the energy of the radioactive decay into the aqueous environment is avoided (Figure 6) . Emitted light can be detected by liquid scintillation counter.

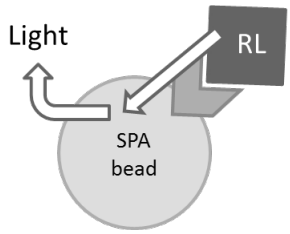

Figure 6. Principle of SPA technique. While radioligand (RL) is captured onto surface of the SPA bead, the emission from β particles to scintillant occurs effectively and light is emitted (Picture redrawn and modified from Sittapalam et al., 1997).

All isotopes used in biological assays are not suitable for SPA because emitted particles might have too long pathlengths and signal is obtained without binding. Isotopes with suitable pathlength for SPA are ^{3}H and ^{125}I (Sittampalam, 1997). Two products

of scintillant coated microplates are available: Flasplate® from NEN™ Life Science Products (Boston, MA) and the Scinitstrip® plate from PerkinElmer Wallac Oy (Turku, Finland). Cytostar-T™ scintillating microplates developed by Amersham Life Sciences (Cardiff, Wales) were specially designed for cell studies. Nowadays SPA reagents and Cytostar-T product line rights belong to PerkinElmer who bought the rights in the end of 2009 (Sittampalam, 1997; GE Healthcare Life Sciencis, 2011).

2.4.2 LANCE™

Lanthanide chelate excite (LANCE) technology is a homogeneous time-resolved fluorescence resonance energy transfer (TR-FRET) based assay technology of PerkinElmer. Technology utilizes two labels: LANCE Europium chelate as a donor fluorophore and a suitable acceptor fluorophore such as U*Light*™ dye, allophycocyanin (APC) or Cy5. Transfer of the energy from donor to acceptor can be detected after excitation of the europium label at 340 nm, if the distance between donor and acceptor is sufficiently short (Figure 7) (PerkinElmer LANCE, 2011).

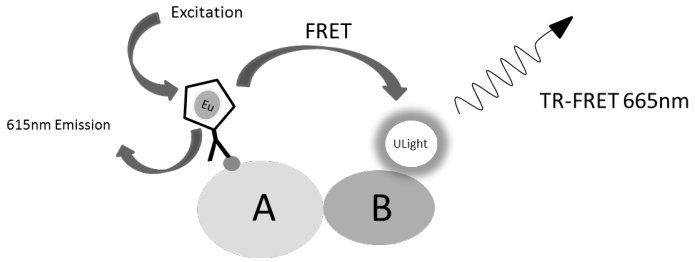

Figure 7. Principle of LANCE technique. While immunolabeled (with Eu-labeled antibody) molecule A is in interaction with molecule B (U*Light*™ labeled), energy transfer between donor (Europium chelate) and acceptor (U*Light*™) can occur (Figure redrawn and modified from PerkinElmer LANCE® TR-FRET assays (2013)).

Two different Eu-chelates are used in LANCE assays: W1024 and W8044 having different properties and used under different assay conditions. W8044-Eu chelate gives higher signal and has improved stability over W1204 which allows harsher assay conditions. In the presence of high concentration of EDTA it has more spectral cross-talk and W1024-Eu chelate is a preferred choice under benign assay conditions (Hemmilä, 1999). LANCE fits for 96-, 384-, and 1536-well formats and it is suitable for automation and for HTS use. LANCE has been applied for kinases studies, cAMP assays, protein-protein interaction assays and for epigenetic assays. PerkinElmer has also customer services for labeling needed molecules.

2.4.3 HTRF®

Homogeneous time-resolved fluorescence (HTRF) is TR-FRET based assay technology of Cisbio Bioassay. HTRF utilizes lanthanides with long lifetime such as europium and

terbium. In HTRF labels europium is combined with cryptates which allows increased stability (Cisbio HTRF, 2011). The Eu trisbipyridine cryptate (TBP Eu^{3+}) was the first lanthanide cryptate used as a donor in the HTRF technology. Allophycocyanin (APC) fulfilled the most of the requirements for optimal acceptor and was chosen to be FRET pair of TBP Eu^{3+} (Mathis, 1993). Later on Allophycocyanin has been modified and this modified version of allophycocyanin, XL 665, is now used in HTRF assays (Cisbio HTRF, 2011). Nowadays the second donor used in HTRF is terbium cryptate Lumi4-Tb developed by Prof. K Raymond's group at Berkeley (Cisbio HTRF, 2013; Decorge et al., 2009).

The principle of the technique is based on fluorescence resonance energy transfer between donor and acceptor. When two molecules, other labeled with TBP Eu^{3+} donor and other with allophycocyanin or XL 665 acceptor, are brought to close proximity, energy transfer between donor and acceptor occurs and light is emitted (Figure 8). In the first developed HTRF assay two monoclonal antibodies against prolactin were labeled with TBP Eu^{3+} and allophycocyanin (Mathis G. Clin. Chem. 1993). HTRF has then applied to immunoassays as well as cell-based assays. Applications of HTRF include assays for second messangers (cAMP or cGMP), metabolites (IP3), hormones (insulin, estradiol, glucagon) and molecules of immunoreactions (cytokines, TNF α, IFN γ). (Cisbio HTRF, 2011)

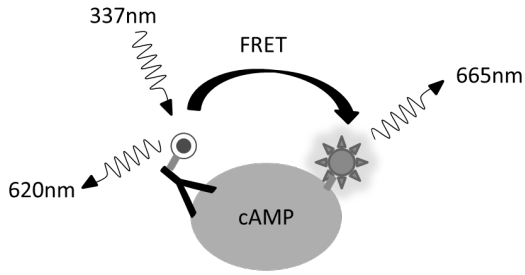

Figure 8. Principle of HTRF technique. Cyclic AMP assay principle is presented in the figure. When the anti-cAMP antibody labeled with a cryptate donor is able to bind to cAMP labeled with acceptor d2, energy transfer occurs between donor and acceptor (Figure redrawn and modified from Degorce et al., 2009).

2.4.4 Glo

Glo is a homogeneous, bioluminescence based assay technology from Promega Corporation (Madison, Wiskonsin). Glo utilizes the light producing property of the thermostable luciferase enzyme (Ultra-Glo™ Recombinant Luciferase) to generate a stable "glow-type" luminescent signal. Luciferase catalyzes the light producing reaction of lucifering and oxygen (Figure 9). Other reactions using ATP have influence on light producing reaction and can be detected. Glo technology has multiple applications including reporter gene assay, cell viability assay, different assays for enzymes such as kinases, proteasomes and caspases, cAMP assay and ADP assays (Promega Glo, 2011).

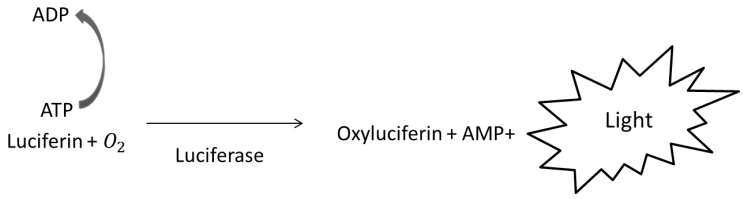

Figure 9. Light producing reaction of luciferin and oxygen catalyzed by luciferase (Figure redrawn and modified from Glo cAMP assay kit manual).

2.4.5 HitHunter™

HitHunter™ is an enzyme fragment complementation (EFC) based assay technology developed by DiscoveRx Corp. EFC is a commonly used HTS technology in diagnostic screening but HitHunter™ was developed for drug screening applications. Many different enzymes have been utilized in the EFC technology but HitHunter™ utilizes the complementation of β-galactosidase fragments. In the HitHunter™ technology complementation of a small α fragment peptide (prolabel or enzyme donor, ED) with a mutated enzyme with ω deletion (enzyme acceptor, EA) generates signal by forming an active enzyme able to hydrolyse the substrate (Figure 10). The possibility to use different substrates enables either chemiluminescent or fluorescent detection. Generated signals can be measured using fixed cells *in situ* or in cell lysates. Without complementation separate enzyme acceptor and prolabel fragments are enzymatically inactive leading to a low background signal. HitHunter™ technology has been applied for many assays and a variety of molecules, peptides and proteins. Enzyme donor can be chemically conjugated or recombinantly fused to a small molecule or protein of interest. Assays for cAMP, tyrosine and serine-threonine kinases, nuclear hormone receptors and, proteases are developed based on chemical conjugation of enzyme donor. Protein expression can be studied with the protein recombinantly fused to the enzyme donor. The technology is adaptable for different plate density formats, and as a homogeneous assay it is easily automated which proves the HTS feasibility of the assay (Eglen, 2002).

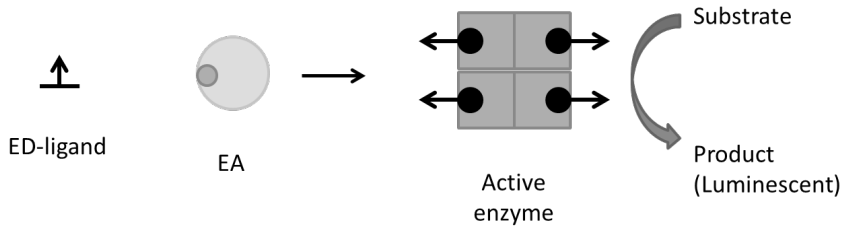

Figure 10. Principle of HitHunter technique. Without complementation separate enzyme acceptor (EA) and prolabel (ED) fragments are enzymatically inactive. While complemented, an active enzyme is obtained and a luminescent product is formed (Eglen, 2002).

2.4.6 AlphaScreen

AlphaScreen (amplified luminescent proximity homogeneous assay screen) is homogeneous bead-based proximity assay technology developed by PerkinElmer (Eglen et al. 2008). The assay technology utilizes oxygen channeling chemistry which was originally developed by Ullman and collegues at Syva Diagnostics for diagnostic detection assay platform - LOCI (Ullmann et al., 1994; Ullmann et al., 1996). PerkinElmer bought the rights to develop technology for research and drug discovery applications use in 1999. Short diffusional distance of singlet oxygen is exploited to initiate a chemiluminescent reaction in the proximity of chemical generation. Two differently coated polystyrene beads, designated as donor and acceptor beads, which form a pair in the presence of analyte is utilized in the assay (Figure 11). To be able to generate the signal, the distance between donor and acceptor beads can maximally be 200 nm. Donor beads include a photosensitizing agent, phthalocyanine. The photosensitizing agent excites ambient oxygen to singlet state when irradiated at 680 nm. Highly amplified response is obtained in contact with the acceptor beads. All AlphaScreen assays use similar acceptor beads containing three dyes: thioxene, anthracene and rubrene. The light energy produced in reaction of thioxene with singlet oxygen is transferred via anthracene to rubrene which emits the light at 520 to 620 nm. Highly sensitive assays utilizing low concentration of beads can be developed. Lifetime of singlet oxygen is short, so to be able to produce signal, binding of the acceptor and donor beads is required. Acceptor beads containing europium are also available. This modified assay technology named as AlphaLISA provides intense and spectrally defined emission at 615 nm which enables more sensitive measurements in biological fluid matrices (serum, plasma) including assays. Non-specific binding is reduced by using latex-based hydrogels containing reactive aldehydes in AlphaScreen and AlphaLISA assays for beads coating. Wide range of applications has been developed to the technology. Signal transduction molecules assays including cAMP (Gabriel, 2003; Li, 2003), inositol [1,4,5] triphosphate (Gray, 2003), protein kinases (Warner,2004; Guenat, 2006) and proteases have been developed. Technology has been applied in proteomics (Poulsen, 2007), and genomics to screen nuclear hormone receptors (Wilson, 2003), transcription factors (Wu, 2003), and single nucleotide polymorphisms (SNPs) (Syvänen, 2001) as well. Sensitivity to intense light or long exposure to ambient light is the main disadvantage of the AlphaScreen technology. Problem can be avoided by adjusting the assay. Other problems might appear with screening library compound able to scavenge the radical oxygen and that way sequester singlet oxygen. Requirement for a high-energy laser excitation source and limitations in readers suitable for this assay technology limits the use of the technology (Eglen, 2008).

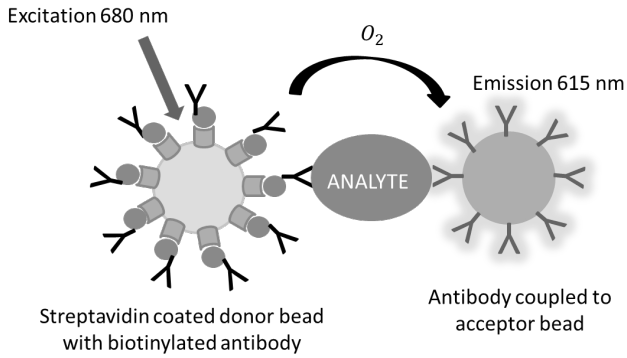

Figure 11. Principle of AlphaScreen technique. While phthalocyanine containing donor beads are excited with laser (680 nm) ambient oxygen is converted to singlet oxygen. If the acceptor bead is in appropriate distance, singlet oxygen molecules react with chemicals in the acceptor beads to produce a luminescent response. Emission at 615 nm occurs when the acceptor beads contain europium (Figure redrawn and modified from Eglen et al. 2008).

2.4.7 Fluorescence polarization (FP)

Fluorescence polarization (FP) technique based assays were earlier in market from multiple companies (BellBrook Labs, Perkin Elmer and Amersham Biosciences) (Zhang et al., 2012) but their amount have decreased lately. BellBrook Labs has a product platform of Transcreener® HTS assays which utilize three separate readout techniques including FP. Transcreener® HTS assays platform includes four assays: ADP^2, AMP/GMP, GDP and UDP. Fluorescence polarization detection based on the differences noticed in rotation speed of fluorescently labeled complex while excited with plane polarized light. Transcreener platform detection based on interaction of two reagents: antibody and tracer. In fluorescence polarization readout system high polarization values are obtained when tracer-labeled nucleotide is in interaction with the unlabeled antibody (Figure 12). In the presence of free nucleotides, output of enzymatic reaction, low polarization values are obtained while the tracer-labeled nucleotide is displaced. Transcreener® FP assays are simple to run and easy to automate (BellBrook Labs Transcreener, 2013). While utilizing conventional fluorescence, it is suffering from assay interferences especially in large compound library screens as FP-based assays usually do (Vogel et al., 2008; Riddle et al., 2006).

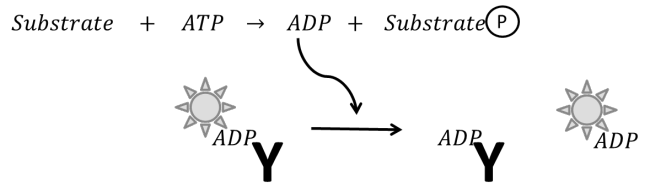

Figure 12. Transcreener® ADP assay is used here as an example of the principle of Transcreener® FP assay. High polarization value is obtained when tracer-labeled ADP is in interaction with the unlabeled antibody. While free ADP (product of enzymatic reaction) is present the tracer-labeled ADP is displaced and low polarization value is obtained (Redrawn from BellBrook Labs Transcreener, 2013).

2.4.8 Summary of the commercial assay techniques

All commercial assay techniques presented in the earlier chapters have advantages as well as disadvantages as summarized in Table 2, where also multiple applications of the techniques are presented.

Table 2. Applications, advantages and disadvantages of commercial assay techniques (Data collected from references used in the chapter 2.4).

Assay	Applications	Advantages	Disadvantages
SPA	cellular function screening for analytes such as prostaglandins, steroids, second messenger(cAMP, cGMP), serum markers, salivary samples and drug quantitation.	simple protocol	radioisotopic
LANCE	cAMP, biochemical kinase activity, protein-protein interactions, enzymatic assays, methylation or acetylation states of peptide histone proteins	high sensitivity, use of generic readers	need of dual labeling process
HTRF	GPCR signaling (cAMP and IP-One), kinases, cytokines and biomarkers, bioprocess (antibody and protein production), as well as the assays for protein-protein, protein-peptide, and protein-DNA/RNA interactions	high sensitivity, use of generic readers	need of dual labeling process
Glo	ADP, cAMP, kinase, caspase, P-450	less prone to interference	more complicated protocol in comparison to other techniques (more dispensing steps)
HitHunter	cAMP, cGMP, cortisol,estrogen and progesterone receptor	use of generic readers	more complicated protocol in comparison to other techniques (more dispensing steps)
AlphaScreen	cAMP, IP3, protein kinases, proteases, nuclear hormone receptors, transcription factors, SNPs	high sensitivity	sensitive to intense light/long exposure to ambient light, compound interference
FP	ADP2, AMP/GMP,GDP and UPD	simple to run, easy to automate	assay interference

3 AIMS

The aim of this thesis study was to develop a homogeneous single label utilizing time-resolved luminescence based technique: quenching resonance energy transfer (QRET). The applicability of the technique was then tested in three separate assay applications.

Spesific aims were:

I to develop a heterogeneous time-resolved luminescence utilizing cell-based β_2-adrenergic receptor-ligand binding assay using synthesized Eu(III)-pindolol ligands.

II to develop a homogeneous ligand binding assay for β_2- adrenergic receptor (β_2-AR) by utilizing time-resolved luminescence and soluble quencher (development of quenching resonance energy transfer technique)

III to develop homogeneous GTP binding assay applying the use of time-resolved luminescence and soluble quencher based technique to motilin receptors.

IV to apply the use of time-resolved luminescence and soluble quencher based technique to measure concentration of cAMP with homogeneous assay format.

V to study Ras protein activation with homogeneous assay format applying time-resolved luminescence and soluble quencher.

4 MATERIALS AND METHODS

4.1 Quenching Resonance Energy Transfer (QRET) Technique

The aim of this thesis work was to develop the homogeneous QRET technology and assays applying the technique. The first publication of the thesis (I) describes the development of the heterogeneous receptor-ligand binding assay. The second publication (II) introduce the QRET method. The publications III and IV introduce different novel applications of the QRET method.

4.1.1 Ligand binding assays for β_2-adrenergic receptor

Transfected cell line HEK 293_i overexpressing β_2-AR was prepared and the expression level of β_2-AR in produced cell line was measured by coauthors in the University of Oulu. Three ligands with different lengths spacer arms (no spacer C0, one spacer C6 and two spacers C12) were prepared for experiment by coauthors in our laboratory. These three different ligands were first investigated in radioligand binding assay by coauthors (University of Oulu). All three ligands were labeled with Eu chelate after binding affinity measurements and the luminescent spectra of Eu(III)-C12-pindolol was measured in our laboratory.

Heterogeneous assay

Publication I presents heterogeneous time-resolving luminescence utilizing cell-based receptor-ligand binding assay for β_2-adrenergic receptor. Assay relies on the detection of the lanthanide chelate derivatized non-peptide ligand, Eu(III)-labeled C12-pindolol, binding to the β_2-AR receptors overexpressed in transfected human embryonic kidney cells (HEK 293_i).

Eu ligand displacement binding assays on intact cells were performed in microcentrifuge tubes by incubating varying concentration of propranolol (0-10 µM) with 2 x 10^6 HEK 293_i cells expressing β_2-AR and 10 nM Eu(III)-labeled pindolol ligand 30 minutes at room temperature in a total volume of 110 µl. The assays were done in a reaction buffer, PBS containing 45 nM BSA. In saturation binding assays concentration of Eu(III)-labeled pidolol ligand varied from 0 to 207 nM and non-spesific binding was determinated with 100 µM propranolol. After incubation cells were washed three times with reaction buffer and resuspended to reaction buffer. For signal measurements the reactions were transferred to Nunc C12 low fluor maxi microtiterplate (Wiebaden, Germany) wells (70 µl per well) and signals were measured with Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland).

Homogeneous assay

Publication II presents homogeneous single-label utilizing time-resolved luminescence based technique quenching resonance energy transfer (QRET). Technique is utilized to screen receptor-ligand interaction of β_2-adrenergic receptor. A soluble quencher is added to reaction solution to quench the luminescence of fraction of unbound luminescently labeled ligand while the bound fraction stayed fluorescent.

β_2-adrenergic receptor-ligand binding assays were performed on intact transfected HEK 293_i cells. Cells were earlier prepared and pellets were stored at -70°C. Assays were run at 384-well plates. Agonist or antagonist in DMSO, cell suspension ($5x10^5$cells) and Eu-C12-pindolol ligand (final concentration 3 nM) prepared in PBS containing 0.1 % BSA were added to the wells. Final concentration of DMSO in 60 µl reaction was 2.5 %. Reactions were incubated 30 minutes at room temperatures in the dark with low shaking. After incubation 10 µl of quencher solution, leucoberbelin blue I (final concentration 980 µM) was added and reactions were shaken 1 minute. Luminescence signals were measured with Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland). LOPAC library study was performed with the same assay protocol by adapting it to HTS instrumentation. The library compounds were printed on 384-well plates by using pintool technology and an automated liquid-handling workstation. Ready-to-use assay plates were prepared by printing 100 nl of 0.1 mM or 1mM stock libraries in DMSO to plates. Plates were stored at -20˚C. The Multidrop Combi dispenser was used to add cells, Eu-C12-pindolol and leucoberbelin blue I on the plates. Final concentration of the test compounds were in total assay volume of 60 µl 0.17 µM or 1.7 µM. Signals were read with HTS mode suitable EnVision 2101 multilabel reader. B-score method was used to normalize raw data (Brideau et al., 2003) and a threshold of 2 for signal-to-background ratio was used as a hit criterion.

4.1.2 Functional GPCRs assays

GTP assay

Application of quenching resonance energy transfer (QRET) technique to homogeneous GTP binding assay was first introduced in publication 'Homogeneous GTP binding assay employing QRET technology' (Rozwandowicz-Jansen et al. 2010). This assay utilized luminescently labeled europium-GTP and soluble quencher and activation of recombinant human α_{2A}-adrenergic receptor was detected as a binding of labeled Eu-GTP to $G_{\alpha i}$ in activation which protected the luminescence of the label from quenching. Developed assay was post-processed to improve signal-to-background ratio and reproducibility of the assay. For this purpose assay technique was applied to motilin receptor activation study (unpublished). Motilin receptor activation was detected as the luminescence of the label of labeled Eu-GTP was protected from quenching in binding.

Homogeneous Eu-GTP assay with motilin receptor membranes were carried out in microcentrifuge tubes and transferred to wells of black microtiterplate (384Optiplate F). Reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl, 5 µM GDP, 600 µg/ml saponin), varied concentration of motilin in 50 mM HEPES pH 7.4 (0 to 10 µM) and membrane solution (40 µg of motilin receptor protein) were incubated 30 minutes at room temperature and dark with slow shaking. Eu-GTP (PerkinElmer) was then added to reactions (final concentration 25 nM) and reactions were further incubated 30 minutes. 50 µl aliquots were then transferred from reactions to wells of 384 microtiter plates and Triton X-100 (final 0,01%) and quencher (final concentration 0.31 mM) were added to each well before measurement. Luminescence was then measured with Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland) and signals were followed 80 minutes in every 10 minutes. The results were calculated from the signals obtained after 70 minutes. The equation $xL = x_{bi} + ks_{bi}$, where xL is the smallest measure from which the limit of detection (concentration or quantity) is derived, x_{bi} is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures and k is 3, was used to calculate the limit of detection (IUPAC gold book, 2013).

cAMP assay

In publication III the homogeneous single-label utilizing time-resolved luminescence based functional assay for cAMP was presented. Assay applied developed QRET technology where soluble quencher is used to quench the signal of unbound labeled binding component. Exogenously added Eu-labeled cAMP competed with intracellular cAMP from binding to anti-cAMP antibody. Assay was performed with intact transfected HEK 293$_i$ cells overexpressing human β_2-adrenergic receptor or human δ-opioid receptors received from coauthor in the University of Oulu. Increase in the intracellular level of cAMP could be detected after cell lysis as a decreasing of the luminescent signal.

Cells harvested at growth medium were exchanged to stimulation buffer (PBS, 0.16 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mg ml^{-1}γ-globulin). Assay was performed at black 384-well Optiplate-384F obtained from PerkinElmer, Wallac (Turku, Finland). Stimulation buffer containing metaproterenol 0.1 nM or 100 nM, the cell suspension with varying number of cells (1x10^3, 3x10^3, 6 x10^3, 9 x10^3 or 12 x10^3) and anti-cAMP antibody (3 nM) (Wallac, PerkinElmer) were added to the plate. Cells were stimulated 30 minutes at room temperature at dark with gentle shaking. Detection buffer (1.5 nM Eu-cAMP, 12 mg ml^{-1} Triton X-100 and the quencher in 50 mM HEPES pH 7.4) was added and final reaction mixture was incubated under the same conditions 20 minutes. Luminescent signals were measured with Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland). For the agonist stimulation assay the same assay protocol was used with some modifications. Number of 9 x10^3 cells was used and agonist (metaproterenol, terbutaline or epinerphine) concentration varied from 0.003 to 10000 nM. cAMP calibration curve was measured under these optimized conditions in a biochemical system without cells by using exogenously added cAMP at concentrations from 0.1 to

10000 nM. DMSO tolerance was tested with the same protocol as cAMP standard curve with two concentrations of exogenously added cAMP (0 or 10 μM) by adding varying concentrations of DMSO (0 to 10 % of the assay total volume).

Assay parameters of the QRET assay and the commercial cAMP measurement kit Lance cAMP 384 assay from PerkinElmer utilizing time-resolved fluorescence resonance energy transfer were compared. QRET was performed as presented above and LANCE was carried out according to the manufacturer's protocol (LANCE cAMP Assay Kit Manual) by using 0.011 to 10000 nM concentration of metaproterenol. Chemical activation of β_2-AR was stimulated with forskolin at concentrations from 1 to 100000 nM. Decreasing activity of adenylyl cyclase during G_i-coupled receptor activations was detected in test performed by using the intact transfected HEK 293_i cells overexpressing human δ-opioid receptors. Adenylate cyclase activity was stimulated chemically by using 10 μM Forskolin and δ-opioid receptor activation was stimulated with δ-OR agonist SNC-80 (0.03 to 30 nM). The assay conditions were the same as in β_2-AR assays using 9000 cells per reaction.

4.1.3 Biochemical Ras Activation Assay

In publication IV the QRET technique was applied to detect activation of small GTP-binding protein H-Ras. Ras proteins have a role in cell growth and differentiation and Ras oncogenes are involved in the early state of cancerogenesis. Ras activity determination has become an interesting reseach field. In this QRET-based homogeneous *in vitro* assay binding of the terbium labeled GTP to Ras protein protects the luminescence of the label from quenching and activation of Ras protein is detected. Background luminescence is decreased as the luminescence of non-bound fraction of labeled GTP is quenched.

The effect of the Ras protein concentration was tested by using varying concentrations of Ras (0-600 nM). The exchange of guanine nucleotide was activated with His-tagged son of sevenless (His-SOS). Assay was performed in microcentrifuge tubes. Reaction buffer (10 mM $MgCl_2$, 3 mg/mL Triton X-100, 0.1 mg/mL γ -globulins in 50 mM Tris buffer pH 8.0) H-Ras solution and Tb-GTP dilution in reaction buffer (final concentration in reaction 10 nM) were mixed in the tube. His-SOS solution in reaction buffer (final concentration 200 nM) or reaction buffer (control reaction without SOS) was added and reactions were incubated 20 minutes at room temperature in the dark. 40 μl samples were then transferred to OptiPlate384F plate wells. 20 μl of soluble quencher Quench II (final concentration 47 μM) was then added and reactions were further incubated 30 minutes at room temperature in the dark. The time-gated luminescence signal was measured with Victor^2 1420 multilabel counter (Wallac Oy, Turku, Finland).

The varied concentration of SOS protein (0-2000 nM) was used in experiments carried out to detect the effect of SOS protein concentration. Assay was performed as in the effect of Ras protein concentration determination by using 60 nM Ras protein concentration.

Control reactions were carried out without Ras protein. The detection limit was calculated by using equation $xL = x_{bi} + ks_{bi}$, where xL is the smallest measure from which the limit of detection (concentration or quantity) is derived, x_{bi} is the mean of the blank measures, s_{bi} is the standard deviation of the blank measures and k is 3, was used to calculate the limit of detection (IUPAC gold book, 2013).

5 RESULTS AND DISCUSSION

This chapter presents the summary of results and discussions from original publications and some unpublished data.

5.1 Cell-based assays

5.1.1 Heterogeneous β_2-adrenergic receptor-ligand binding assay

Heterogeneous cell-based β_2-AR-ligand binding assay was developed (I). The functionality of Eu-labeled pindolol derivatives was investigated in heterogeneous β_2-AR receptor-ligand binding assay applying intact HEK 293_i cells overexpressing receptors. The cells were incubated in increasing concentrations of β_2-AR antagonist propranolol with 10 nM ligand (Eu-C0-, Eu-C6- or Eu-C12-pindolol). After incubation non-bound ligands were washed and reactions were measured from aliquots of cells without additional enhancement steps. The enhancement steps were unnecessary because they increase only the signal level while signal-to-background ratio remains unchanged. The C0- and C12-pindolol function effectively in displacement assay with nanomolar affinities to the β_2-AR. Eu-C6-pindolol instead did not bind to receptor and displacement of propranolol was not detected below 1 μM concentration. The signal-to-background ratios, the Z'factors and the IC_{50} values for Eu-C0- and Eu-C12-pindolol ligands and the coefficient of variation of these assays are presented in Table 3. In comparison, results of radioligand [^{3}H]dihydroalprenolol binding assay are presented in the same Table 3. The K_i values determinated earlier in radioligand displacement studies and reported for propranolol have been in sub- and low-nanomolar range (Hoffman et al., 2004; Smith et al., 1999; Del Carmine et al., 2002).

Table 3. Comparison of labeled ligands.

	Labeled ligand		
	Eu-C0-pindolol	**Eu-C12-pindolol**	**[^{3}H]dihydroalprenolol**
S/B	5.5	16.0	8.1
Z' factor	0.51	0.77	0.69
IC_{50}(nM)	60	37	33
CV	2 to 17	2 to 17	6.2

Eu-C12-pindolol was chosen to be used in further experiments. Before the saturation binding assay the assay conditions were optimized. The bovine serum albumin was chosen to be added to reaction buffer to prevent the non-spesific binding. The equilibrium dissociation constant (K_d) and receptor density (B_{max}) were then measured in saturation binding assay using optimized assay conditions in increasing concentrations of Eu-

C12-pindolol with or without propranolol (0 or 100 μM). Based on the saturation curve determinated K_d value for Eu-C12-pindolol was 37 nM and receptor number per cell was 237,000.

The use of directly luminescent Eu chelate in the assay reduced the number of steps because no extra enhancement step was required. The need of washing step and reaction preparation in microcentrifuge tubes instead decreases the feasibility of the assay for HTS use. Assay could be potentially modified more HTS suitable by performing the entire assay in microtiter plates by using filter plates and automated instrumentation.

5.1.2 Homogeneous β_2-adrenergic receptor-ligand binding assay

Homogeneous single-label utilizing quenching of time-resolved luminescence based quenching resonance energy transfer format was developed for HTS use. Technique was applied here for cell-based β_2-adrenergic receptor ligand binding assay. The assays were performed with compartments chosen from the earlier study (I): stably transfected HEK 293_i cells overexpressing β_2-AR and Eu-C12-pindolol ligand. The signal-to-background ratio was defined as a ratio between no ligand containing sample and agonist or antagonist containing sample.

Optimization of the number of cells used in reaction was estimated by measuring 11 different cell concentrations. Used cells were earlier induced and stored frozen to enable availability of large cell quantities required in library screening experiments. The number of cells needed for an experiment is dependent from the receptor number on a cell. Receptor density was earlier determined in washing saturation binding assay with Eu-C12-pindolol ligand (I). The number of receptors was 240,000 per cell and calculated K_D value for the ligand was 37 nM. In a radioligand [^{3}H]dihydroalprenolol experiment the K_D value of 1.3 nM was obtained. The experiments were settled on to accomplish with 5 x 10^5 cells per assay according to the results of the assays performed with propranolol (0 and 9 μM) because the favorable signal-to-background ratio for the assay performance, over 8, was obtained. The coefficient of variation for the experiment was under 10%.

In screening libraries compounds are dissolved to solvent, most commonly to DMSO. The tolerance of DMSO differs between cell lines and assay formats. To ensure the usefulness of the developed assay for HTS use, information about the DMSO tolerance is required. DMSO tolerance of β_2-adrenergic receptor-ligand binding assay was tested by carrying out experiment with 11 different DMSO concentrations ranging from 0 to 10% with (9 μM) or without propranolol. The DMSO concentration up to 10% was tolerated. This proved the QRET method to be quite robust. Signal-to-background ratios decreased along the increase in DMSO concentration. The signal-to-background ratios under 4 were obtained when DMSO concentration rose over 5%. These high concentrations of DMSO are disturbing for the cells which limits anyway the use of similar concentrations

especially in functional cell-based assays. Limit of DMSO concentration in this experiment was set to 2.5% with signal-to-background ratio more than 5.

The functionality of the developed QRET based β_2-AR ligand binding assay was tested in dose-response study of four different ligands: two agonist (metaproterenol, terbutaline) and two antagonists (alprenolol, propranolol). K_i values, the signal-to-background ratios and calculated Z' values for ligands are presented in Table 4. The assay CV varied from 6 to 9%. The K_i value obtained for alprenolol was in accordance with result, 11 nM, obtained in [^{3}H]dihydroalprenolol radioligand washing assay.

Table 4. Comparison of four ligands.

	Metaproterenol	Terbutaline	Alprenolol	Propranolol
K_i value (nM)	14	5.9	19	19
S/B	7.0	10.4	7.9	10.0
Z' factor	0.68	0.69	0.76	0.78

The QRET method was examined in a compound library screening study of LOPAC library consisting of 1280 well-characterized compounds to check the HTS suitability of the method. For this study assay was adapted from initial manual protocol to automated system. Two different concentrations of test compounds, 0.17 and 1.7 μM, were printed to 384-well plates. A primary screening using the entire library was carried out with 1.7 μM compound concentration without replicates. Data, normalized by applying the B-score method, resulted in 31 hits including 15 predicted hits (hit rate 2.4%). B-score method is a relative potency score consisting in the numerator of the adjusted raw value and the denominator of measure of variability. The numerator adjustment in B-score accounts both positional effects in the plate and plate-to-plate changes in the mean. A resistant measure of the residual variability in a plate is used in the denominator (Brideau et al., 2003).Threshold value of 2 for signal-to-background was used in hits searching. 31 hits were confirmed manually separately with two different concentrations of test compounds, 1.7 and 0.17 μM, without replicates. Hit number was reduced in these two additional screens to 16 including 15 predicted hits. The detection of one false positive hit (formoterol) gave a false-positive rate of 0.08%.

5.2 Functional assays

5.2.1 Improvements of homogeneous GTP binding assay

The conditions of the homogeneous GTP binding assay using motilin receptor membrane were optimized by modifying the conditions used in published QRET GTP binding assay utilizing α_{2A}-AR. Motilin, the natural peptide ligand of motilin receptor, was used to study GTP binding in the receptor activation process. The motilin standard curve

was run by using eight concentrations of motilin (0 to 10 μM) (Figure 13.). The limit of detection calculated from the signals measured after 70 minutes was 1 nM. The signal-to-background ratio 2.9 was obtained in the experiment. The average coefficient of variation was 5.9%. The improvement in the signal-to-background ratio and the average coefficient of variation were obtained in comparison to earlier published α_{2A}-AR utilizing homogeneous QRET GTP assay (Rozwandowicz-Jansen et al. 2010) where the S/B ratio was 1.9 and the average coefficient of variation was 13.1.

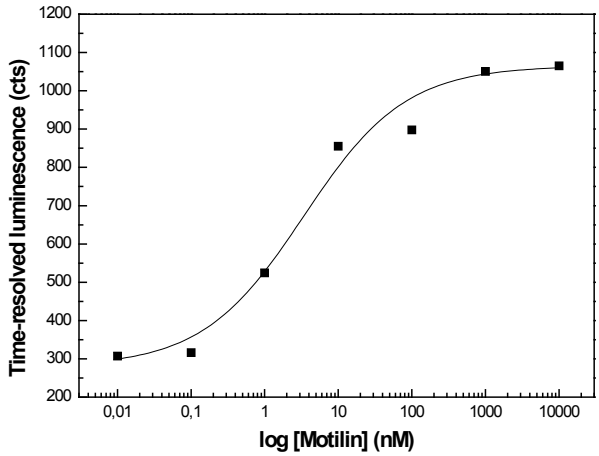

Figure 13. The standard curve of motilin.

5.2.2 Homogeneous receptor activation assay: measuring cAMP

cAMP is an important second messenger mediating many physiological responses. Several homogeneous assay platforms to measure concentration levels of cAMP have been developed and also many commercialized application exist. The aim of this assay developing process was to develop homogeneous, HTS applicable single-label time-resolved luminescence cAMP assay utilizing quenching resonance energy transfer technique. QRET cAMP assay was compared to the commercial TR-LRET based LANCE assay.

An increased level of cAMP was detected as decreasing of the luminescent signal. Calibration curve of cAMP was measured in a biochemical assay without cells under optimized conditions. The assay was carried out with increasing concentration of cAMP (0.1-10000 nM). The EC_{50}, signal-to-background ratio and the average of the coefficient of variation were 44 nM, 12.6 and 5.1%. Assay tolerance of DMSO was tested in a similar manner using a biochemical assay with (10 nM) and without cAMP with varying concentrations of DMSO (0 to 10% of total assay volume). Results proved DMSO tolerance of the cAMP immunoreaction.

Conditions of the cells were noticed to be very critical for cells capability to produce cAMP. Lot of attention should be put to gentle cell harvesting. We began to harvest cells in growth medium to ensure viability of the cells. Cell number was optimized with the cell line overexpressing human β_2-adrenergic receptor with two concentrations of metaproterenol (100 pM and 100 nM). Identical saturation of signal-to-background ratio was detected with those metaproterenol concentrations at over $3x10^3$ cells per reaction cell counts which led us to employ $9 x10^3$ cells per reaction in all subsequent experiments. The number of receptors per cell was earlier determined in saturation ligand-binding assay to be 240000 for the cell line (**I**).

The functionality of the assay was tested with HEK 293_i cells overexpressing human β_2-adrenergic receptor and β_2-AR agonists. Agonists: metaproterenol, terbutaline and epinerphine differ in their efficacies and potencies. Epinerphine is known to be full agonist of β_2-AR whereas terbutaline and metaproterenol work as partial agonists of β_2-AR. EC_{50} and E_{max} values were determinated to all three agonists. The EC_{50} values were 0.056, 3.6 and 2.9 nM and the limit of detection, counted as a 90% from signal, 4.2, 450 and 400 pM for epinerphine, metaproterenol and terbutaline respectively. As expected epinerphine had the highest E_{max} value and the E_{max} values of metaproterenol and terbutaline were 74 and 75% from the E_{max} value of the epinerphine. The coefficients of variation (CV) varied between 1 and 15%.

The QRET and TR-LRET based LANCE assays were compared in forskolin stimulated adenylate cyclase activation test. Varying concentrations of forskolin (1-30000 nM) were used to activate cAMP production in human β_2-adrenergic receptor overexpressing HEK 293_i cells. Increase in the intracellular cAMP concentration was then measured. Assays were run parallel using the same forskolin solution. The EC_{50} value, the limit of detection (LOD), the signal-to-background ratios and the coefficient of variations as well as calculated Z' values for both assays are presented in Table 5. Efficient measurement of cAMP in the cell-based system with both assays was indicated. The functionality of the assays was further compared in cell-based agonist stimulated receptor activation assay. The partial agonist of β_2AR, metaproterenol, was used to stimulate cAMP production in cells. The EC_{50} values, the limit of detection, the signal-to-background ratios, the coefficient of variations and Z' factors for both assays are shown in the Table 5. Comparison of these two assays was justified because they shared the similar detection features: excitation at 340 nM, time-resolved luminescence detection above 600 nM and possibility to use the same instrument for both measurements. The similar results obtained with both assays were not surprising because the same antibody was applied to both assays. Obtained EC_{50} values were in accordance to values from the earlier reported studies (Del Carmine et al., 2002; Hoffman et al., 2004; Swift et al., 2007). The main advantage of the QRET assay is the use of a single label. The time and money are saved when the second labeling procedure is not needed.

Table 5. Comparison of QRET and LANCE assays.

Method	**QRET**		**LANCE**	
Detection	**Luminescence**		**Luminescence**	
Number of Labels	**1**		**2**	
Total incubation time	**50 minutes**		**90-120 minutes[1]**	
	Forskolin	**Metaproterenol**	**Forskolin**	**Metaproterenol**
S/B	5.1	4.5	3.1	3.3
LOD(nM)	6.6	0.35	6.3	0.42
Z' factor	0.72	0.76	0.85	0.95
EC_{50}(nM)	89	3.2	32	1.5
CV(%)	12	0 to 12	1 to 7	1 to 3

[1] Recommendation in LANCE™ cAMP 384 kit manual

Capability of the QRET assay to study an inhibitory G_i-coupled GPCRs was proven. Assay parameters were investigated in agonist activation of G_i-coupled human δ-opioid receptors overexpressed in transfected HEK 293_i cells. Increasing concentrations of a selective opioid agonist, SNC-80, were used in the assay in the presence of 10 μM forskolin to enhance the response. Inactivation of the adenylate cyclase as a response to opioid receptor activation was detected, as expected. The measured EC_{50} value was 0.68 nM and the coefficients of variation (CV) for the assay varied from 1 to 15%. The determined EC_{50} value was in accordance with the values published earlier (Knapp et al., 1996; Lecoq et al., 2004; Piñeyro et al., 2005).

5.3 Biochemical assay: Homogeneous Ras Activation Assay

The effect of Ras protein concentration was studied using a varied concentration of H-Ras (0-600 nM) in His-SOS (200 nM) activated guanine nucleotide exchange assay. The control reactions, whose signals were used as a background signal in signal-to-background calculations, were prepared without SOS. Results showed an increasing signal-to-background ratio up to 200 nM Ras protein concentration. Decreased signal-to-background ratio observed with 600 nM H-Ras was result from increased background luminescence. The highest signal-to-background ratio (2.9) was obtained at 200 nM H-Ras concentration. Because the use of lower protein concentration in the experiments is preferable, 60 nM H-Ras concentration was chosen to employ for subsequent experiments (signal-to-background ratio 2.4). The coefficient of variation in the experiment varied from 2 to 18%.

The functionality of the H-Ras protein activity assay was investigated by testing the effect of concentration of guanine exchange factor His-SOS. Varied concentrations of His-SOS (0-2000 nM) were tested by using 60 nM H-Ras or without H-Ras (control reactions, signals used as a background signal in signal-to-background ratio calculations). Increasing concentrations of His-SOS resulted in the increased luminescent signal. The increase of signal was observed while more Tb-GTP bound to H-Ras. Increasing signal-

to-background ratios up to 7.7 (2000 nM His-SOS) were obtained in the experiment. However, successful activity assay can be carried out with 60 nM His-SOS concentration (S/B 3.4). The detection limit of 6 nM was calculated from the results. The assay CV varied between 0.5 and 17 %.

The signal-to-background ratio 7.7 obtained in this QRET-based H-Ras activity measurement is significantly higher than S/B ratios typically obtained with commonly used Mant-GDP assay (S/B 2 to 3)(Lenzen et al., 1998; Brownbridge et al., 1993). The efficient protection of bound terbium labeled GTP and effective quenching of luminescence of non-bound fraction enable better S/B ratio. Lower concentration of assay components can be used while using highly sensitive time-resolved luminescence detection in comparison to conventional fluorescence based methods such as Mant, BODIPY or Tamra (Lenzen et al., 1998; Soini & Hemmilä, 1979; Jameson et al., 2003; Ford et al., 2009; Eberth et al., 2005). Especially the need of high concentrations of purified protein in the assay brings more costs and therefore savings in purified protein use are desired. Many GTPases activity assays need the use of nucleotide free proteins which means usually an extra purification step (Gureasko et al., 2008). Our QRET method was performed with GDP bound H-Ras protein. The Tb-GTP used in this experiment has been previously used in FRET based assay where Tb-GTP transferred energy to GFP fused with small GTPase Rab21 (Vuojola et al., 2009). In comparison to this method QRET has less limitations because the fused GFP can cause sterical hindrances in reactions with large target proteins such as modulators. The use of QRET method is not limited only to small GTPase H-Ras. Preliminary results have been obtained with Rab21 and Rab5 (data not published).

6 SUMMARY AND CONCLUSIONS

In this thesis five different assays were developed:

heterogeneous:

1. cell-based β_2-adrenergic receptor-ligand binding assay

homogeneous:

2. cell-based β_2-adrenergic receptor-ligand binding assay
3. GTP binding assay for motilin receptor (post processed from earlier α_{2A} -adrenergic receptor assay)
4. single-label time-resolved fluorescence cAMP assay
5. single-label biochemical Ras activation assay

Novel homogeneous technique, quenching resonance energy transfer (QRET), presented first time in publication II was applied to four of these assays. QRET technique has many advantages in comparison to other available techniques. First of all QRET technique is time-resolved fluorescence based technique and exploits the advantages of the TR-fluorescence. Time-resolved fluorescence is known as a sensitive detection technique and it avoids the waste and safety problems confronted with the radioactivity. On the other hand the use of TR-fluorescence for binding studies has usually needed two labeling procedures which cost time and money. With QRET technology the costs can be reduced because only one labeling procedure is needed. QRET technology is also able to avoid the common problems met in FRET-based methods as for example the distance of labeled partners and optimization of FRET signal.

In publication III the developed QRET technique based cAMP assay was compared with commercial LANCE cAMP assay. In Table 6 is shown the results of our comparison study together with the results of comparison study of other commercial cAMP assays published earlier (Gabriel et al., 2003). According to the results summarized in Table 6 alphascreen has the best signal-to-background ratio. The QRET assay has the second best S/B ratio but it is less time-consuming than all the other assays. Cell number and antibody used in assay, always have an influence on assay sensitivity. To achieve the best possible results, the optimized assay conditions need to be used. In the study of commercial cAMP assays, published by Gabriel et al., all parameters, like cell number and reagents ratio, were not optimized separately on all assays which might have an influence on results.

Table 6. Comparison of cAMP assays. Forskolin (FSK) was used as a chemical activator for cAMP production (III; Gabriel et al., 2003).

Assay	Dispensing steps	Incubation time	S/B	FSK EC_{50} (μM)
AlphaScreen	3	2h 15 min	14	0.11
FP	3	1h 45 min	1.8	2.525
HitHunter	5	3h 15 min	3.8	1.703
HTRF	3	2h 15 min	5	0.675
LANCE	3	1.5-2h	3.3	0.032
QRET	3	50 min	5.1	0.089

Applications of commercial methods, presented in Table 6, were presented earlier in this thesis in Table 2. The QRET technique has also multiple applications. In addition to those presented in this thesis work, the QRET technique has been applied to ligand binding assays of delta opioid and estrogen receptors (Kopra et al., 2013; Huttunen et al., 2011). In one application the QRET technique has been combined with DiscoveRex PathHunter assay format to provide a cell-based functional assay which is able to identify β_2-AR ligand binding and downstream response of agonist activation in the same assay (Kopra et al., 2013).

To be applicable for HTS, assay has to fulfil specific requirements. The first of all, assay needs to be simple and fast to run. Possibility to miniaturization is saving reagents and costs and is desired in HTS. Assay should tolerate low concentrations of DMSO because in libraries compounds are usually dissolved to DMSO. Assays which are easily automated are desired. The homogeneous QRET technique base assays are simple and fast to run. The 384-well plate format, commonly used in HTS, is used with QRET assays. The QRET technique has also been proved to tolerate DMSO which demonstrates the applicability of the technique for HTS use. Automation of the QRET based assays would still be required to enable their use in HTS.

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