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Joint application of an ARC-probe and antibody in homogeneous TR-FRET assay for determination of the concentration of protein kinase Pim2

Master thesis (30 ECTS)

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Table of contents

1 Abbreviations

ADP	adenosine diphosphate
ARC	conjugate of nucleoside-analogue and oligopeptide
ATP	adenosine 5′-triphosphate
BSA	bovine serum albumin
CDR	complementary determining region
DNA	deoxyribonucleic acid
DOL	degree of labelling
DTT	dithiothreitol
ELISA	enzyme linked-immunosorbent assay
ERK	extracellular regulated kinase
Fab	antigen-binding fragment
FA	fluorescence anisotropy
Fc	constant fragment
FDA	Food and Drug Administration
FP	fluorescence polarization
FRET	Förster resonance energy transfer
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
Ig	immunoglobulin
$\boldsymbol{K_d}$	equilibrium dissociation constant
LOD	limit of detection
Ln-chelate	Lanthanide-chelate
mAb(Pim2)AF-647	monoclonal anti-Pim2 antibody labeled with Alexa Fluor 647 dye
MAPKKK	mitogen activated protein kinase kinase kinase
MAPKK	mitogen activated protein kinase kinase
MAPK	mitogen activated protein kinase
MEK	mitogen/extra-cellular signal regulated kinase
MSK1	mitogen-and stress-activated protein kinase-1
mTOR	mammalian target of Rapamycin

NaCl	sodium chloride
PKAc	cAMP-dependent protein kinase
PKs	protein kinases
RIA	radioimmunoassay
SEC	size exclusion chromatography
STPKs	serine/threonine like protein kinases
TPKs	tyrosine like protein kinases
TR-FRET	time-resolved Förster resonance energy transfer
TRL	time resolved luminescence
cAMP	cyclic adenosine 3′,5′- monophosphate

2 INTRODUCTION

Protein kinases (PKs) catalyse the transfer of a gamma phosphoryl group from ATP to a protein substrate, thus changing the activity, solubility, aggregation, and sub-cellular localization of the protein. This centres PKs as critical components of cellular signal transduction network, regulating cell growth and survival. Dysregulation of PK activity can often be the cause of illnesses, such as cancer, diabetes, and cardiovascular diseases, rendering PKs as important targets in drug discovery research. This has led to search for new inhibitors. On the other hand, the development of selective and sensitive detection assays for characterizing PKs in biological samples is also an important research target. Widely used detection assay, the enzyme-linked immunosorbent assay (ELISA) is a heterogeneous approach with several steps of washing and reagent incubations. Such method tends to be relatively time-consuming and cost ineffective. For high-throughput analysis, homogeneous assays based on fluorescence techniques are preferred for research and diagnosis.

Förster resonance energy transfer (FRET) is a process in which a photoluminescence donor molecule in the excited state transfers energy through dipole-dipole coupling to an acceptor fluorophore, when the acceptor and donor are brought into close proximity. Time-resolved luminescence (TRL) relies on the use of long-lived fluorescent lanthanides such as europium (Eu) and terbium (Tb) to minimize the effect of background fluorescence. Time-resolved-FRET (TR-FRET) combines the TRL and FRET principles. In TR-FRET, lanthanides chelates are typically used as donor species in combination with acceptor molecules such as Alexa Fluors™.

The immunoglobulin G (IgG) is a bivalent molecule that can bind two identical antigens through its antigen-binding fragment (Fab). We proposed that if a protein kinase is in complex with an ARC-type donor probe, TR-FRET can be measured utilizing dye-labelled antibody as an acceptor. Because TR-FRET increases the signal/noise ratio, detecting specific PK directly in bodily fluids can be done *via* our proposed approach.

3 LITERATURE REVIEW

3.1 Protein kinases

Multiple evidence strands suggest that there are approximately 19 000 human protein-coding genes (Ezkurdia et al., 2014), of which over 500 encode protein kinases (PKs) (Manning, et Al., 2002). The family of PKs is one of the largest, making up about 2.8% of all the human proteins (Lander, et al., 2001). PKs are a super-family of enzymes that catalyse the transfer of gamma phosphoryl group from ATP onto a hydroxyl group of serine, threonine or tyrosine residue in the target protein substrate (Fabbro, et al., 2015).

$$MgATP^{-} + protein–OH \rightarrow protein–O:PO_3^{2-} + MgADP + H^{+}$$

Phosphorylation by PKs involves 30% of the human proteome and regulates majority of the intracellular signaling pathways (Cohen, 2001). Most PKs consist of at least two domains; the catalytic domain serves to bind and phosphorylate target proteins and the regulatory region interacts directly with ancillary proteins that through an allosteric action modulate the activity of the catalytic domain (Medicine & York, n.d., 2002). Based on the origin of the amino acid being phosphorylated on the targeted protein, eukaryotic PKs can be classified as tyrosine-targeted protein kinases (TPKs), serine/threonine-targeted protein kinases (STPKs),and dual specificity protein kinases (phosphorylating both, tyrosine and serine/threonine residues).

Phosphorylation mediates signalling cascades, inducing a whole series of subsequent cellular responses (Bairlein, 2010). For instance, in all eukaryotes, mitogen-activated protein kinase (MAPK) pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation, and stress responses. First, STPKs mitogen-activated protein kinase kinase kinases (MAPKKKs) activate MAPKKs (MEK1 and MEK2) through phosphorylation on two serine/threonine residues in a conserved S/T-X3-5-S/T motif, and second, the dual-specificity MAPKKs phosphorylate MAPKs (ERK1 and ERK2) on threonine and tyrosine residues in the T-X-Y motif (Nakagami, et al., 2006). Activated MAPKs then translocate to the nucleus to activate transcription factors and by changing gene expression promotes growth, differentiation, or mitosis (Zhang, Liu, & Tu LIU, 2002). In addition, phosphorylation is also involved in regulation of extracellular processes (Walsh, et al., 2005).

The reversible phosphorylation of proteins regulates almost all aspects of cell life, while abnormal phosphorylation is a cause or a consequence of many diseases (Bairlein, 2010).

Mutations in specific PKs and phosphatases can be the origin to a number of disorders and numerous pathogens exert their effects, which results in debalanced phosphorylation states of intracellular proteins. A number of diseases resulting from mutations of particular PKs and phosphatases are brought in Table 1 (Cohen, 2001). In parallel, as PKs possess well-defined and conserved ATP-site, they have emerged as important targets in oncology and drug discovery research aiming at treating many devastating diseases, e.g., cancer, diabetes, autoimmune diseases (Bairlein, 2010).

Table 1: *Example of diseases caused by mutations in particular PKs and phosphatases (Cohen, 2001)*

Disease	PK/phosphatase
Myotonic muscular dystrophy	Myotonin protein kinase
X-Linked agammaglobbulinaemia	Bruton tyrosine kinase
Hirschsprungis disease	Ret2 kinase
Autosomal recessive SCID	Zap70 kinase
X-Linked SCID	Jak3 kinase
Chraniosynostosis	FGF receptor kinase
Papillary renal cancer	Met receptor kinase
Chronic myelomonocytic leukaemia	Tel-PDGF receptor kinase
Chronic myelogenous leukaemia	Abelson tyrosine kinase
Non-Hodkins lymphoma	Alk kinase
Peutz-Jeghers syndrome	Lkb1 kinase
Coffin-Lowry syndrome	MAPKAP-K1b (RSK-2)
Ataxia-telangiectasia	Atm kinase
Li-Fraumeni syndrome	Chk2 kinase
Williams syndrome	Lim kinase-1
Leprechaunism, diabetes	Insulin receptor kinase

3.2 Inhibitors of protein kinases

The idea that PKs could be targeted came up in the late 1980s with the discovery of rapamycin to inhibit the PK mTOR (mammalian target of rapamycin), a member of the phosphatidylinositide 3-kinase family required for interleukin-2-dependent T cell proliferation (Bairlein, 2010). Currently, 33 PK inhibitors have been approved as drugs with most of them being competitive towards ATP (Fabbro et al., 2015). The high homology of ATP-binding

site in the super-family of PKs and competitive binding to other ATP-requiring enzymes complicate the development of selective inhibitors (Fabbro, 2015). Low selectivity between ATP-binding sites increases the likelihood of side effects. However, detailed structural studies have revealed differences in the ATP binding-site that can be exploited to increase the selectivity of the inhibitor (Roskoski, 2015).

3.2.1 Conjugates of a nucleoside-analogue and oligopeptide

Conjugates of a nucleoside-analogue and oligopeptide (ARCs) are bisubstrate inhibitors that consist of two fragments joined by a flexible linker. The two fragments, the nucleoside and the peptide moiety, mimic ATP and protein substrate of a bisubstrate enzyme such as PK. Hence, an ARC binding simultaneously to both substrate domains creates more interactions with the PK for increased affinity and selectivity compared to the single-site inhibitor of PK. It has been shown that ARCs inhibit several basophilic AGC-group PKs with nanomolar to subnanomolar potency (Lavogina et al., 2009). The bisubstrate property of ARCs has been confirmed by displacement of ARCs from complex with PK by both ATP- and protein substrate-competitive inhibitors, and by the co-crystal structures of ARCs and PKAc (Pflug et al., 2010; Uri et al., 2010).

The application of a bisubstrate fluorescent probe for characterization of both ATP- and protein substrate competitive inhibitors of PKs have been described in assays based on fluorescent polarization, time-resolved luminescence, and Förster resonance energy transfer (FRET) (Enkvist et al., 2011; Uri et al., 2010; Vaasa et al., 2009).

3.3 Antibodies

Antibodies are Y-shaped antigen-binding protein molecules secreted by the immune system to identify and neutralize foreign objects and pathogens, such as bacteria, viruses, fungi, parasites, and toxins. Also known as immunoglobulins, antibodies are manufactured by white blood cells called B-lymphocytes (B-cells). Once secreted in blood, antibodies act as effectors of the humoral immunity by targeting specific antigens for elimination of pathogens. Antigens, on the other hand, are molecules containing antigenic determinants or epitopes against which the immune system responds by production of specific antibodies. Each type of antibody defends the body against a specific type of antigen.

Structurally, antibody molecules are made up of four peptide chains consisting of two identical 25 kDa light chains (L) and two identical 50 kDa heavy chains (H) (Lebakken et al.,

2010). The L and H chains are bound to each other by disulphide bridges (Figure 1). The first 100-110 amino acids (variable region) of the amino terminal region of the L or H chain differ amongst antibodies of different specificity. The amino acid sequence beyond the variable region is constant in all antibody isotypes and is termed constant (crystallisable) region. Five heavy chain isotypes are distinguished (α, μ, δ, γ, and ε) which determine the antibody class or isotype: IgG (γ), IgA (α), IgM (μ), IgE (ε), and IgD (δ). Each class can have either the kappa (κ) or lambda (λ) type of light chain at a time. The antigen binding site of an antibody is located at the top of each of the two outstretched arms. Each site is defined by 6 loops called complementary determining regions (CDR). Three are found on the heavy chain (H1, H2, and H3) and 3 on the light chain (L1, L2, and L3). These protein loops complement the shape and amino acid sequence of specific antigens. As a result, they determine to which specific antigens the antibody can and will bind.

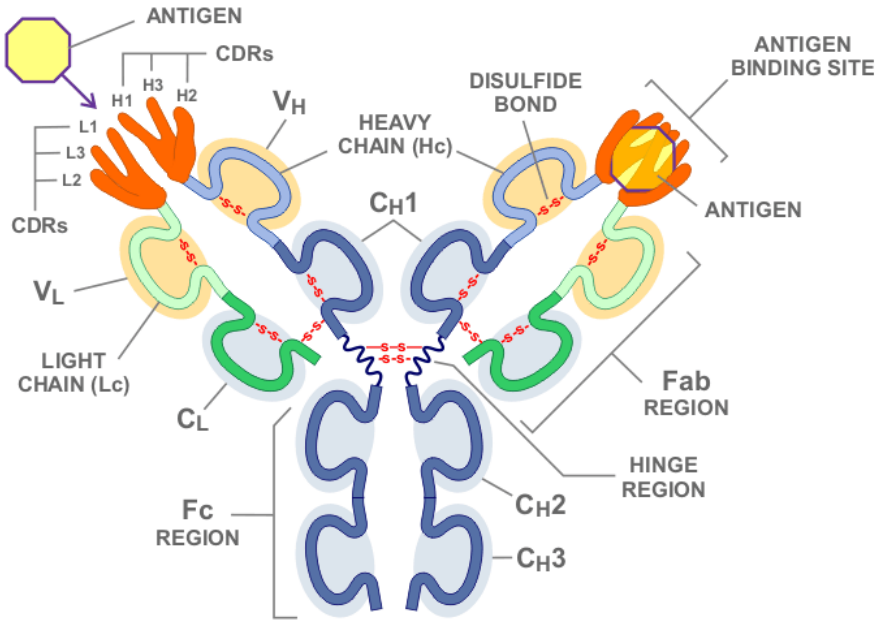

Figure 1: *Structure of an antibody (www.noviimmune.com).*

3.4 Size Exclusion Chromatography

Size exclusion chromatography (SEC), also known as gel permeation or gel filtration chromatography is a semi-preparative chromatography method used to separate analytes according to their size. This method is based on the molecular sieve properties of a porous material within a column.

As a principle, a column packed with micro-particulate cross-linked copolymers (styrene or divinylbenzene) with narrow range of pore size is in equilibrium with the mobile phase for the analyte to be separated. Hence, larger solute molecules surpassing the size of narrow pores will be excluded from the pores, pass *via* the interstitial routes of the column and elute first. Smaller solute molecules that distribute between the mobile phase outside and inside the narrow pores elute at a slower rate. The application of SEC is used for the purification of biological samples by facilitating separation of biomolecules according to their molecular weight.

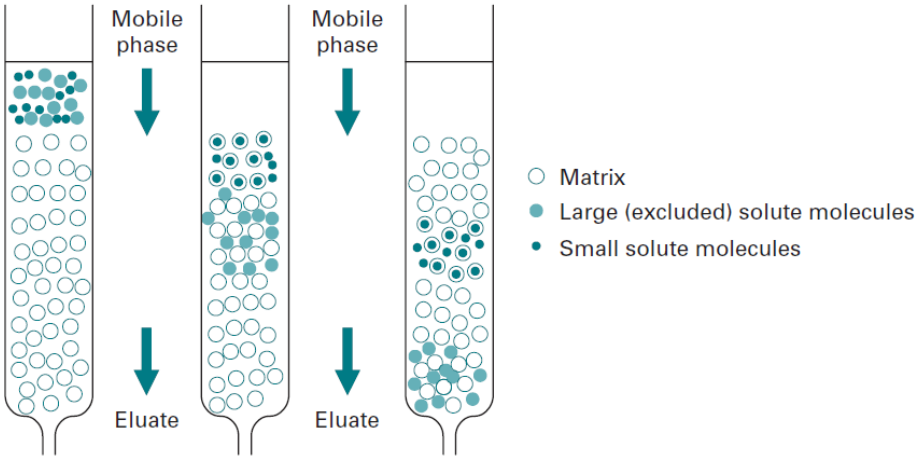

Figure 2: *Separation of different size molecules by exclusion chromatography. Large excluded molecules are eluted first in the void volume* (Keith Wilson & Walker, 2010).

3.5 Immunosorbent Assay

The first description of radioimmunoassay (RIA) was given in 1959 as a method that can be used to elucidate the physiology of peptide hormone insulin (YALOW & BERSON, 1961). In search for an alternative method that does not use a radioactive label as RIA does, the enzyme-linked immunosorbent assay (ELISA) was introduced in the 1970s (Leng et al., 2008). ELISA is a frequently used bioanalytical method in laboratories for the detection and quantification of analytes (mostly antibodies or antigens) from a sample.

There are some main advantages of ELISA compared to other antibody-based assays. First, specific and non-specific interactions can be distinguished through serial binding on a solid support (usually polystyrene multi-well plates) followed by several washing steps to eliminate nonspecific binding. Second, quantification can easily be achieved *via* coloured end product that is correlated to the amount of analyte in the original sample.

On the other hand, because ELISA uses surface binding for its separation, surface unbound materials are removed by several washing steps between each ELISA step. Hence, because ELISA is a heterogeneous approach with several steps of washing (specialized plate washers for improved repeatability are encouraged) and reagent incubations, such method tends to be time-, cost-, and labour-ineffective. Further, there is an aspect of losing the material specifically bound on the solid surface. Therefore, the sensitivity of ELISA may suffer from various intervening steps and heterogeneity of the sample (e.g., blood). Finally, to work out an ELISA for a targeted protein, two primary antibodies which bind to different epitopes on the target protein are needed, hence requiring large amount of well-characterized antibodies.

3.6 Photoluminescence

Photoluminescence is a process in which a substance excited by incident light emits a photon. Photon emission is coupled to the relaxation of an electron from the excited state. Depending on the nature of the excited state from which the light is emitted, luminescence can be subdivided into fluorescence and phosphorescence (Lakowicz, 2006).

3.6.1 Fluorescence/Phosphorescence

The phenomenon of fluorescence describes the emission of light from the singlet excited state (S_1 or S_2) to the ground sate (S_0), mostly occurring with aromatic molecules. The transition from the singlet excited state to the ground state is fast, as the excited electron has an opposite spin compared to the electron in the ground state. This transition is thermodynamically favoured. The electron relaxation from singlet state occurs in the order of 10^{-9} s, thereby fluorescence measurements may be sensitive to the auto-fluorescence of complicated biological systems or materials used in the measurement.

Phosphorescence on the other hand is described as the emission of light from the triplet excited state (T_1) to the ground state (S_0). At the triplet excited orbital, electron shares the same spin with the electron in the ground state. The relaxation of electron from the triplet state to the ground state is thermodynamically unfavoured, resulting in longer lifetime of the photon emission (10^{-6} – 10 s) compared to fluorescence. The Jablonski diagram (Figure 3) illustrates these two phenomenons.

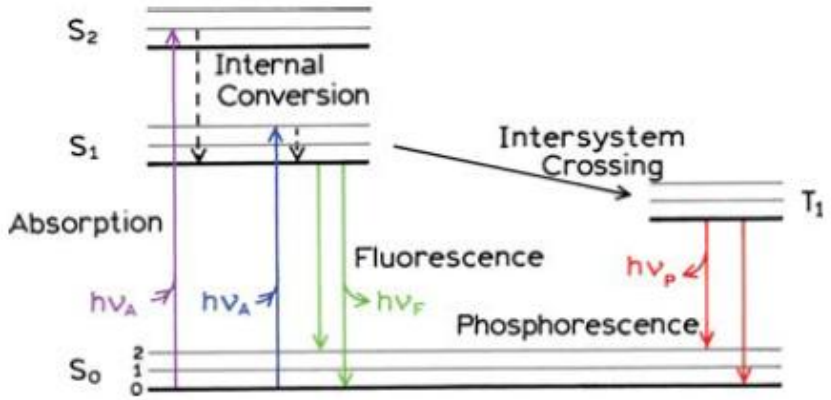

Figure 3: *One form of Jablonski diagram. The singlet ground, first, and second electronic states are depicted by S_0, S_1, and S_2, respectively. At each of these electronic energy levels the fluorophores can exist in a number of vibrational energy levels, depicted by 0, 1, 2, etc.* (Lakowicz, 2006).

3.6.2 Fluorescence polarization and anisotropy

Fluorescence polarization (FP) and fluorescence anisotropy (FA) are two interchangeable techniques (David M. Jameson, 2010). These techniques are used in homogenous assays for their speed, sensitivity, and good signal-to-noise ratio (John C. Owicki, 2000). They are widely used to study molecular interactions, enzymatic activity, and nucleic acid hybridization. The principle of FP and FA is that if excited with polarized light, fluorescently labelled molecule emits light with a degree of polarization that is inversely proportional to its rate of molecular rotation. Based on the measured intensities,

- $FP = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$ Equation 1
- $FA = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$ Equation 2

where $I_{\parallel}$ is the emission intensity parallel to the excitation plane and $I_{\perp}$ is the intensity perpendicular to the excitation plane.

From equation 1 and 2, one can note that the difference between FP and FA lies in the perpendicular intensity term of denominator. FA takes into account the additional perpendicular emission plane oriented along the propagation axis. Hence, use of FA is encouraged since it accounts for contribution of all possible degrees of rotational freedom.

3.6.3 Förster resonance energy transfer

Förster resonance energy transfer (FRET) is a process in which there is transfer of energy between a luminescence donor molecule in excited state to an acceptor fluorophore via dipole-dipole coupling when the two molecules are in close proximity (typically less than 10 nm). The distance between the donor-acceptor and the overlap between donor emission and acceptor excitation spectrums determine the extent of energy transfer (Figure 4) (Lakowicz, 2006). Emission occurs from the acceptor excited state, encompassing a redshift in emission wavelength. The application of FRET technique has increased dramatically in the past decade, mostly in biological applications involving fluorescence microscopy. Amongst others, this technique has been applied in protein-protein interaction, DNA hybridization, and DNA-protein binding studies (Saraheimo et al., 2013). FRET technique reduces signal interference from auto-fluorescence, as the measured signal is the ratio between two channels with respect to the dyes employed, depending only on the distance of the donor and acceptor.

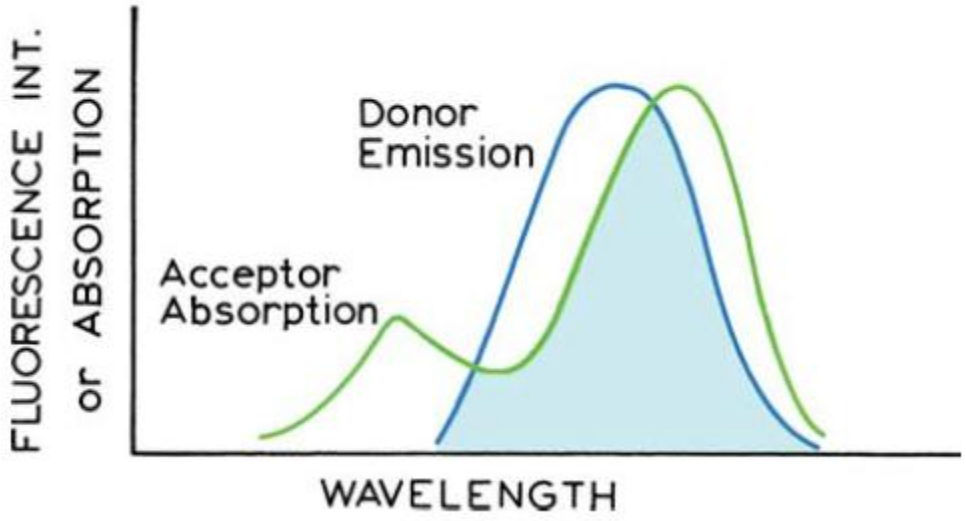

Figure 4: *Spectral overlap for Förster resonance energy transfer (FRET):Donor Emission in blue and Acceptor Absorption in green* (Lakowicz, 2006).

3.6.4 Time-resolved luminescence and lanthanide chelates

Time-resolved luminescence (TRL) utilizes long-lifetime luminescence emission to increase the signal-to-noise ratio (Figure 5). The use of long-lifetime donor fluorophore together with pulsed-laser excitation are effective means to reduce some of the interferences that might arise in the fluorescence technique employed (Qin, et al., 2003). The lanthanides are unique fluorescent metals with the ability to display emission in aqueous solution. Their emission has a decay time of 0.5-3 ms and the lanthanides have a low absorption coefficient of less than 10

$M^{-1}cm^{-1}$ (Lakowicz, 2006). Because of this low absorption coefficient, excitation of the lanthanides can be acquire by coupling them to an energy sensitizer such a chelated organic ligand that will absorb the energy and transfer it to the lanthanide central ion (Vuojola & Soukka, 2014). Methods that combine TRL with FRET (TR-FRET), frequently use lanthanide (Eu^{3+} or Tb^{3+}) chelates (or cryptates) as a donor exhibiting long-lifetime luminescence. Lanthanide-chelates display unique excitation and emission characteristics that make them ideal donor species for TR-FRET-based methods. The luminescence lifetime of most fluorophores is in the range of 1-100 ns that is in stark contrast with 200-1500 µs for lanthanide chelates.

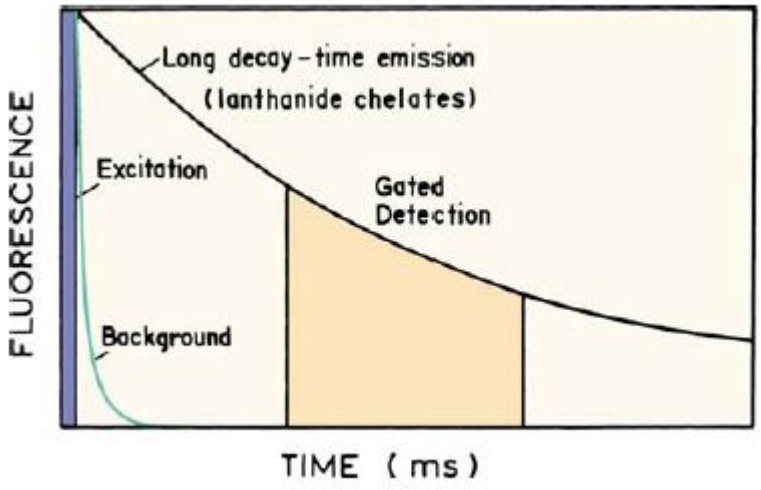

Figure 5: *Principle of time-resolved detection in lanthanide immunoassays. The gated detection is the range where the measurement can be performed with high signal/noise ratio* (Lakowicz, 2006).

Another property of the lanthanide chelates is the multiple distinct emission bands within emission spectra that coupled with narrow band-pass filters can be used for differentiation of donor and acceptor emission intensities (Kevin R. Kupcho, et al., 2007). This has found use in the homogeneous TR-FRET assay (Wang, Yuan, et al., 2006).

Emission of Tb^{3+} chelates characterized by four distinct bands with maximum absorption peaks centred at 490 nm, 546 nm, 583 nm, and 620 nm (M. Jeyakumar et al., 2009). Because these sharp emission bands span a wide range of spectral wavelengths and emission is negligible between and beyond these peaks, terbium chelate emission can be paired with an acceptor that has excitation spectrum that overlaps with one of the terbium emission peaks.

3.7 Triple complexes described by Time-resolved-FRET technique

During the past decades, growing interest in the development of PK assay technologies has mirrored the interest in PK as potential drug targets. Gerard Mathis in 1995 described the application of TR-FRET technology to assays of biological activity. The principles of the assay allow a double discrimination of the emitted signal through spectral and temporal selectivity. The cage-like structure of the complex, ion pairing around europium, as well as double wavelength detection, fully shield the assay from perturbations of media (Mathis, 1995). Over the past decades, researchers have been working on the development of fluorescence based methods capable of assaying targets that span the kinome. In this regards, a non-activity-based assay to characterize PK inhibitors that depended on displacement of an Alexa Fluor-647 conjugate of staurosporine (a "tracer") from a particular PK was developed and described in 2001 (Cohen, 2001). In this assay, PK inhibitors were characterized by a change in fluorescence lifetime of the tracer when it was bound to a PK relative to when it was displaced by an inhibitor (Lebakken et al., 2010). Further developments led to reconfiguration of the assay to a TR-FRET format that simplifies instrumentation requirements and allows for the use of a substantially lower concentration of PK than was required in the assay format based on fluorescence-lifetime (Lebakken et al., 2010).

MATERIALS AND METHODS

3.8 MATERIALS

3.8.1 Reagents

The reagents used in the experiments were purchased from the manufacturers as follows: DTT was obtained from Fluka, NaCl from Riedel de Hæn, HEPES hemisodium salt, BSA, and Polysorbate 20 (Tween 20) from Sigma Life Science, HCl from Lachner, $Na_3PO_4 \cdot 12H_2O$ from Kebo AB, PKAc type α from Biaffin, MSK1 from Cell Signaling, and Alexa Fluor 647-labeled monoclonal anti-Pim2 [mAb(Pim2)AF647] antibodies (DOL5, DOL10, DOL15, and DOL20) were obtained in collaborative work. His-PKAc-His was produced in-house by Ganesh babu Manoharan.

All of the ARC-type inhibitors were synthesized in research group of Asko Uri (Institute of Chemistry, University of Tartu).

3.8.2 Equipment

NanoDrop 2000c was used to determine the concentration of the fluorescent compounds, Pim2 total concentration, and the degree of labelling for mAb(Pim2)AF647.

FA and TR-FRET measurements were performed using PHERAstar microplate reader (BMG Labtech). Corning black low-volume round-bottom 384-well NBS plates were used in the plate reader experiments. The plates were incubated with THERMOstar (BMG Labtech) before data collection with PHERAstar.

Surface experiment was performed on ArrayIt SuperAmine 96-well plate. The intensity of mAb(Pim2)AF647 specifically immobilised on surface was measured with fluorescence scanner LiCOR Odyssey CLx (685 nm diode laser).

Size exclusion chromatography was carried out using Yarra 3µ SEC-2000 (300*7.8 mm) column in HPLC (Shimadzu), Readings were performed by using two detectors: SPD-M20A (Prominence diode array detector) for absorption (280-653 nm) and RF-10AXL (Shimadzu) for fluorescence (excitation at 635 nm and emission at 673 nm). The data from TR-FRET and FA measurements was analysed with GraphPad Prism Software 5.00.

Mettler Toledo S20 SevenEasy equipped with InLab Routine Pro electrode was used for determining pH of the buffer solutions used.

3.9 METHODS

3.9.1 Size Exclusion Chromatography

A phosphate buffer to be used as mobile phase was prepared and filtered. 19.006 g of $Na_3.PO_4 \cdot 12H_2O$ was weighed and dissolved in 500 ml of milliQ water, the pH was adjusted to 6.8. The buffer was stored at 4 ^oC.

The following day, SEC for 4 differently labelled anti-PIM 2 antibody batches were ran sequentially using Yarra 3µ SEC-2000 (300·7.8 mm) column. The flow rate and pressure were set to1 µL/min (at 7.5 MPa on average). Two detectors, SPD-M20A (prominence diode array detector) for absorption (280-653 nm) and RF-10AXL (Shimadzu detector) for fluorescence (excitation at 635 nm and emission at 673 nm) were used for data recording.

3.9.2 Degree of labelling determination for the antibody

Degree of labelling (DOL) represents the average number of dye molecules per antibody. Four batches of monoclonal anti-Pim2 antibody [mAb(Pim2)AF-647] each labelled to a different degree with Alexa Fluor 647 were analysed. For each batch, absorbance maximal of the dye at 650 nm and the protein absorbance at 280 nm were determined respectively while preparing appropriate dilution in cases were absorbance is found to be higher than 1 absorbance units. The absorbance of the respective dye (Alexa Fluor 647) at 280 nm was used to correct the protein absorbance. The Lambert-Beer law and the following formula were used for the determination of the DOL of each batch of the antibody.

$$\mathrm{DOL} = \frac{A_{max} / \varepsilon_{max}}{A_{prot} / \varepsilon_{prot}} = \frac{A_{max} \cdot \varepsilon_{prot}}{(A_{280} - A_{max} \cdot CF_{280}) \cdot \varepsilon_{max}}$$

Where:

A_{max} is absorbance of Alexa Fluor 647 at wavelength of maximum absorbance (653 nm)

$\mathcal{E}_{max}$ is extinction coefficient of Alexa Fluor 647 at wavelength of maximum absorbance

A_{prot} is absorbance of antibody at 280nm

$\mathcal{E}_{280}$ is extinction coefficient of antibody at 280nm

CF_{280} is the correction factor for accounting the absorbance of Alexa Fluor 647 at 280 nm

3.9.3 Investigation of mAb(Pim2)AF647 specificity

Recombinant kinases (HisPKAcHis, MSK1, PKAc, and Pim2) were used in surface experiment. 400 nM solutions of these PKs were made considering total concentration, from which three 0.2 µL were pipetted onto an amino-functionalized glass surface as spots. PK spots were allowed to dry, and the surface was blocked with a 3% BSA solution. On the amino coated surface, 100 µL of a 10 nM mAb(Pim2)AF647 was applied. After 15 min, the antibody solution was removed, the surface was washed 3x, dried and measured with fluorescence scanner LiCOR Odyssey.

3.9.4 Determination of Pim2 active concentration by FA

A characterized probe for Pim2, ARC-3117 ($K_{D,Pim2}$ = 0.7 nM) was initially used for determining active Pim2 concentration. FA measurements described here were carried out using two optical modules depending on the type of fluorescent ligand attached to the ARC:

- Excitation 590(50) nm, emission 675(50) nm for Promofluor-647-labeled ARC.
- Excitation 540(20) nm, emission 590(20) nm for Promofluor-555-labeled ARC.

For FA experiments, the following buffer was used:

50 mM Hepes, 150 mM NaCl, 0,5 mg/ml BSA, 0,005% Tween20, 5 mM DTT (buffer A)

The final volume of the solution in assays were 20 µl, all the concentrations in the following sub-sections are the final concentrations obtained after all components had been added. Before carrying out FA measurements, the solutions were incubated with THERMOstar (BMG Labtech) for 10 minutes at 30 ^{o}C. The samples were measured in 3 cycles using 200 flashes. The intensity adjustment was carried for the well containing free ARC.

3.9.5 Displacement of ARC-3117 from its complex with ARC-1450

A binding assay was first prepared using ARC-3117 labelled with Promofluor 647 by preparing a threefold dilution series of Pim2 kinase starting from 1 µM to which a 4 µL of a 2 nM ARC-3117 solution was added and the plate was incubated at 30 ºC for 10 minutes. The measurement was performed using FP optical module (ex: 590(50) nm, em: 675(50) nm). Second, the affinity of ARC-1450 to Pim2 was examined through a displacement assay performed with ARC-1450. ARC-1450 being a limited resource, 2 µL of a 1 µM solution of it was prepared and 1 µL was added to the well showing optimal concentration of Pim2:ARC-3117 complex from previous result.

3.9.6 Determination of ARC-3159 affinity to Pim2

3-fold dilutions series of Pim2 was prepared in four separate columns starting from 300 nM using buffer A. To these four dilutions series, 4 µL of ARC-3159 (labelled with Promofluor 555) solutions (0.5, 1, 5, and 10 nM) were added respectively. The solutions were measured using the fluorescence polarization module [ex: 540(20) nm, em: 590(20) nm].

3.9.7 Displacement of ARC-3159 from its complex with ARC-1422.

3-fold dilution series of ARC-1422 was prepared starting from 93.75 µM using the buffer A. Pim2 and ARC-3159 (25 nM and 2 nM, respectively) solution was separately prepared and 4 µL of this solution was added to the ARC-1422 dilution series. The solutions were measured using FP module (ex: 540(20) nm, em: 590(20) nm).

3.9.8 TR-FRET ASSAY

TR-FRET assay is carried out such that there should be resonance energy transfer between a long life-time fluorescent lanthanide chelate (terbium chelate) labelled ARC as donor and an organic fluorophore (Alexa Fluor 647) labelled antibody (at various degree of labelling) as acceptor in order to determine molecular interaction and formation of a complex. In this thesis, terbium chelate labelled ARC 1422 (donor) and Four batches of monoclonal anti-Pim2 antibody [mAb(Pim2)AF-647] each labelled at different degree with Alexa Fluor 647 (acceptor) were used.

HTRF measurements described here were carried out using two optical modules depending on the type of fluorescent ligand attached to the ARC:

- HTRF for terbium cryptate and AlexaFluor-647 (excitation 337 (300-360) nm, emission 665(8) nm and 615(10) nm)
- HTRF for TAMRA and AlexaFluor-647 (ex: 337 (300-360) nm, em: 675(50) nm and em2: 590 (50) nm).

For TR-FRET, the following buffer was used:

TRF-buffer- 50 mM Hepes, 150 mM NaCl, 0,005% Tween20 – Buffer B

The final volume of the solutions in TR-FRET assays were 20 µl, all the concentrations in the following sub-sections are the final concentrations obtained after all components had been added. Before carrying out TR-FRET measurements, the solutions were incubated with THERMOstar (BMG Labtech) for 30 minutes at 30 ^{o}C.

The sample measurements were done in 3 cycles using 200 flashes with a delay of 60-250 us post excitation to eliminate interferences.

3.9.9 Determination of mAb(Pim2)AF647 specificity in homogeneous solution

BSA (1 µM), PKAc (300 nM), and Pim2 (300 nM) 3-fold dilution series were prepared using buffer B. 4 µL of a fixed concentration of ARC-1422 and DOL5 mAb(Pim2)AF-647 (8 nM and 1 nM respectively) complex was added to the dilution series. For Pim2, another series was prepared including 5 mM DTT in the buffer. The final solutions were incubated for 30 min at 30ºC. HTRF measurement with filters EM 665(8) and EM 615(10) was used. The delay was set to 60 µs and the signal acquisition time to 400 µs. The formation of a triple complex was investigated by finding the ratio for intensities of 665 (8) nm and 615(10) nm.

3.9.10 Determination of contributors to the TR-FRET signal of triple complex.

For triple complex determination with DOL5 and DOL10, 3-fold dilution of Pim-2 was made starting from 300.0 nM using buffer B. A combination of concentrations for mAb(Pim2)AF-647-Dol5 and ARC-1422 was prepared as shown in appendix ii. 4 µL of it was added in each case to the dilution series of Pim2. The measurement of HTRF was carried out using TRF optical module (ex: 337 (300-360) nm, em: 665(8) nm and em2: 615 (10) nm). The delay was set to 60 µs and the signal acquisition time to 400 µs.

3.9.11 Assessment of the Pim2 concentration determination range of triple complex.

The activity of Pim2 was measured as previously explained and the k value obtained was used during the following experiment. For triple complex determination with Dol5 in this case, two fold dilution of Pim-2 was made starting from 8 µM of Pim2 using buffer B. This was prepared repeatedly in five different columns. In each of the five titration series, a 5 µL solution containing ARC-1422 and mAb(Pim2)AF647-Dol5 (2 nM and 15 nM) was added to make a final volume of 30 µL. The following day, two fold dilution of Pim2 was made starting from four different concentrations: 8000 nM, 200 nM (in triplicate), 50 nM (in triplicate) and 5 nM (in triplicate) using buffer B. 5 µL solution of ARC-1422-mAb(Pim2)AF-647-Dol5 (2 nM and 15 nM) were added to each of these dilutions series. The final solutions in each case were incubated for 1 hour at 30ºC. The measurement of HTRF was carried out using TRF optical module (ex: 337 (300-360) nm, em: 665(8) nm and em2: 615 (10) nm). The delay was set to 60 µs and the signal acquisition time to 460 µs.

An inhibition assay was prepared from the above experiment by adding 50 µM of ARC-1415 in the maximum ratio wells of the above mentioned dilution series. Finally, the limit of detection of the TR-FRET assay was determined experimentally both at low and high Pim2 concentration.

4 RESULTS AND DISCUSSION

The aim of this thesis was the development of a novel homogenous assay based on TR-FRET of ARC:PK:mAb triple complex for the detection of Pim2 and characterization of formed triple complex.

First, ARC comprising a lanthanide chelate interacts with the active site of the PK. Second, the ARC:PK complex interacts with the labelled anti-PK antibody through PK:Ab antigenic binding site. The resulting triple complex emits long wavelength TR-FRET signal (Figure 6) excited with light (electromagnetic radiation) in the near-UV range. By selecting appropriate filters detecting emission of the donor and acceptor at their absorbance maximums, resonance energy transfer process can be monitored without spectral interference for the detection of a triple complex. Anti-Pim2 antibody used here possessed Alexa Fluor 647 (acceptor) as the dye, having an excitation spectrum that overlaps with the third and fourth emission bands of the terbium chelate (583 nm, and 620 nm, respectively). The excited state lifetime of terbium chelate (up to several milliseconds) permits measurements of FRET in a time-resolved mode (FRET detection 60 µs after excitation), minimizing non-specific interference from components with short luminescence lifetime, assay plate plastics, and from direct excitation of Alexa Fluor 647.

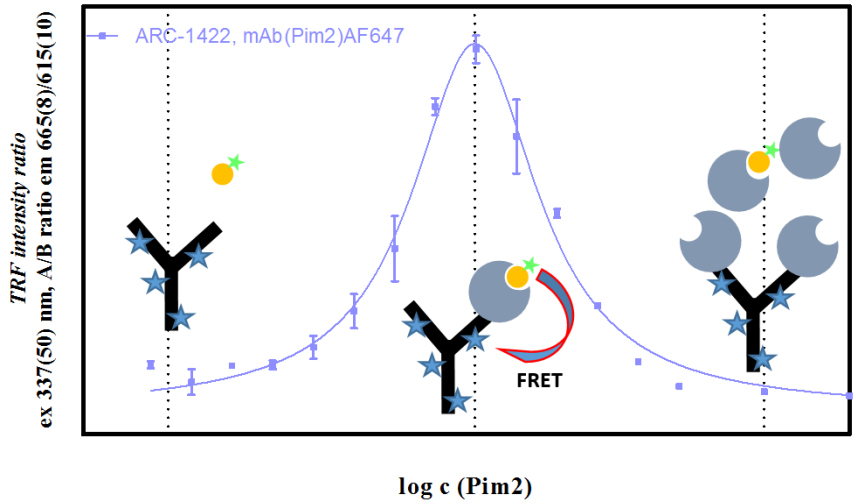

Figure 6: *Scheme for the assay setup. ARC comprising lanthanide chelate as donor (in yellow) and anti-PK antibody labelled with fluorescent dye Alexa Fluor 647 as acceptor (Y-shape) are separated in the absence and excess of PK, resulting in low TR-FRET ratio. Optimal concentration of PK induces the formation of the triple complex, bringing together FRET pair and an increase in TR-FRET ratio is registered.*

In this thesis, the monoclonal anti-Pim2 antibody coupled to Alexa Fluor 647 [mAb(Pim2)AF647] was used. It is important to note that high photostability is a key characteristic of Alexa Fluor dyes. Alexa Fluor 647 has its excitation maximum at 653 nm and emission maximum at 665 nm.

4.1 Assessment of antibody purity

Four batches of mAb(Pim2)AF647 each conjugated with Alexa Fluor 647 at different degree of labelling (DOL5, DOL10, DOL15, and DOL20) were used in this thesis. SEC was carried out to characterize and validate the purity of these antibody batches separately.

The obtained chromatograms showed that all monoclonal antibodies, with the exception of DOL20, were free of protein contaminants. The chromatogram of DOL20 contains what seems to be a split peak of antibody as the retention time (as shown on table below) on

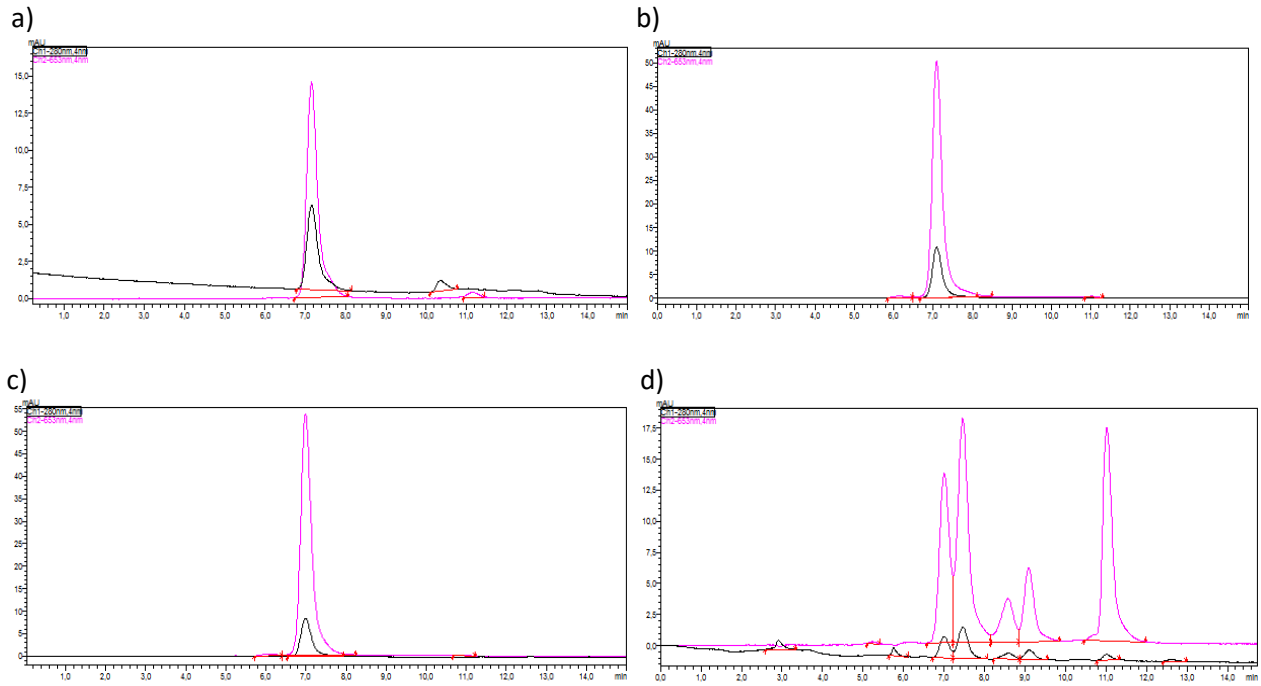

Figure 7: *Absorption chromatograms at 280 nm (black) and at 653 nm (purple) of the mAb(Pim2)AF647 in SEC analysis: a) DOL5, b) DOL10, c) DOL15, and d) DOL20.*

DATA #	Data file name	Retention time	Area	Height
1	mAbPim2AF647_Dol5	7.154	290375	14527
2	mAbPim2AF647_Dol10	7.087	936324	50328
3	mAbPim2AF647_Dol15	6.991	1065502	53578
4	mAbPim2AF647_Dol20	7.001	241620	13641

Table 2*: Data from the SEC analysis of the mAb(Pim2)AF647*

4.2 Determination of degree of labelling of the antibody

The average number of dye molecules (Alexa Fluor 647) per antibody was determined for each of the four batches of anti-Pim2 monoclonal antibodies. Absorbance of the protein at its maximum value (at 280 nm) was measured and the absorbance at maxim of the dye (Alexa Fluor 647) conjugated to the antibody was measured at 653 nm. The correction factor was used to account for the absorbance contribution of the Alexa Fluor 647 at 280 nm. The degree of labelling was then calculated using the formula in 4.2.2. The results obtained are depicted in the table 3.

Table 3: *Degree of labelling of the four batches of antibodies with the fluorescent dye Alexa Fluor 647.Comparison of the measured DOL and that shown on the antibody labels from manufacturer*

Batch	A280 nm	A650 nm	Correction Factor (CF)	A280 of AF647	Degree of labelling	Given Degree of labelling
IgDOL5	**0.074**	**0.200**	**0.037**	**0.007**	**2.5**	**5**
IgDOL10	**0.081**	**0.400**	**0.037**	**0.014**	**5**	**10**
IgDOL15	**0.139**	**0.800**	**0.037**	**0.032**	**7**	**15**
IgDOL20	**0.082**	**0.653**	**0.037**	**0.068**	**9.6**	**20**

The analysis of the measurements showed that the initial degree of the labelling by the manufacturer is overestimated by approximately 2-fold. This results in lower fluorescence intensities during measurements.

4.3 Investigation of mAb(Pim2)AF-647 specificity

For further validation and characterization, the specificity of the mAb(Pim2)AF647 towards Pim2 was examined. Here, four recombinant PKs including Pim2 were spotted onto protein-

adsorbing surface in a single well and solution containing mAb(Pim2)AF647 was applied thereafter. Figure 5 shows that the measured fluorescence intensity at 700 nm was greatly increased for the spots containing the recombinant Pim2 compared to the other PK spots.

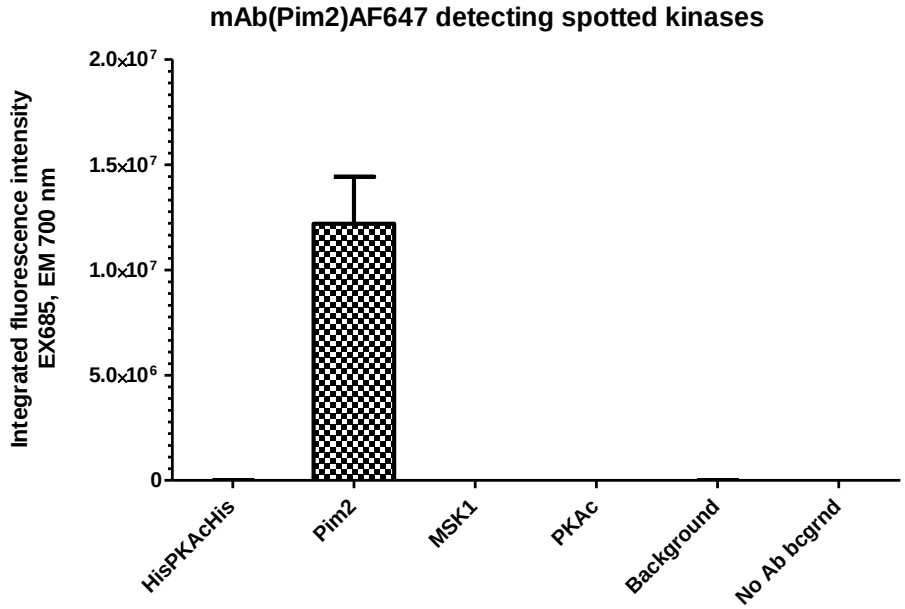

Figure 8*: Specificity of the mAb(Pim2)AF647 towards Pim2. Integrated fluorescence intensity for a selection of PKs spotted on the surface, as detected by visualization with fluorescently labelled Pim2 antibody.*

4.4 Displacement of ARC-3117 from its complex with ARC-1450

First, a binding assay was prepared using ARC-3117 labelled with Promo Fluor 647 by preparing a 3-fold dilution series of Pim2 kinase starting from 1 µM to which a 4 µL of a 2 nM ARC-3117 solution was added and the plate was incubated at 30 ºC for 10 minutes. Second, the affinity of ARC-1450 to Pim2 was examined through a displacement assay performed from the binding assay. The binding assay permitted us to estimate the active fraction of Pim2. From Fig. 9, on the left, it is shown that the binding of ARC 3117:Pim2 was maximal in the well containing 111.1 nM Pim2. In this well, 1 µM ARC-1450 solution was added and from the result depicted in Fig. 9 (on the right) below, it is shown that this concentration of ARC-1450 was not enough to displace all ARC-3117 (total concentration, 2 nM) from its complex. This meant that high concentration of ARC-1450 is required in order to achieve displacement.

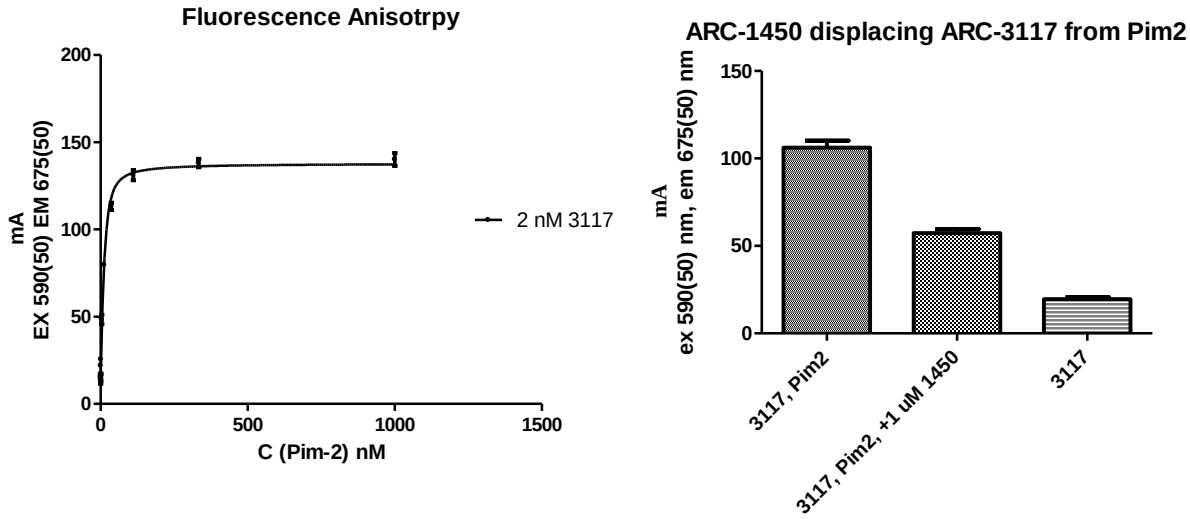

Figure 9: *Binding assay and displacement of ARC-3117 with ARC-1450.*

4.5 Determination of ARC-3159 affinity to Pim2

In order to determine the affinity of ARC-3159 to Pim2, a binding assay was performed using four different concentrations (0.5, 1, 5, and 10 nM) of ARC-3159 comprising promofluor 555 fluorescent dye. These solutions were added to 4 dilution series of Pim2 starting from 300 nM. The result shown in Fig. 10 below gave a K_d of 0.31 nM for the complex ARC-3159:Pim2. This K_d value obtained is smaller than all the concentrations of ARC-3159 used in this experiment. This tells us that ARC-3159 will bind to Pim2 and there is good complex formation between the ARC-3159 and the Pim2, hence, high affinity of ARC-3159 to Pim2.

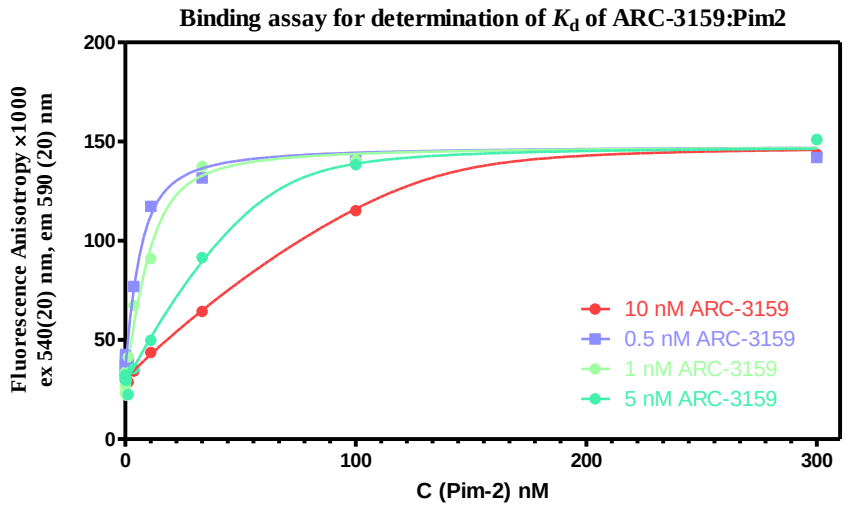

Figure 10: *Binding assay for determination of K_d of ARC-3159:Pim2 complex.*

4.6 Displacement of ARC-3159 from its complex with Pim2 by ARC-1422

For a displacement assay, a 3-fold dilution series of ARC-1422 was prepared starting from 93.7 µM. Fixed concentrations of Pim2 and ARC-3159 (final total concentration of 25 nM and 2 nM, respectively) were prepared and added to each well of the dilution series of ARC-1422. The plate was incubated at 30 ºC for 10 minutes. The measurement was performed and the dissociation coefficient of ARC-1422 expressed as K_d was obtained (K_d=0.4 nM)

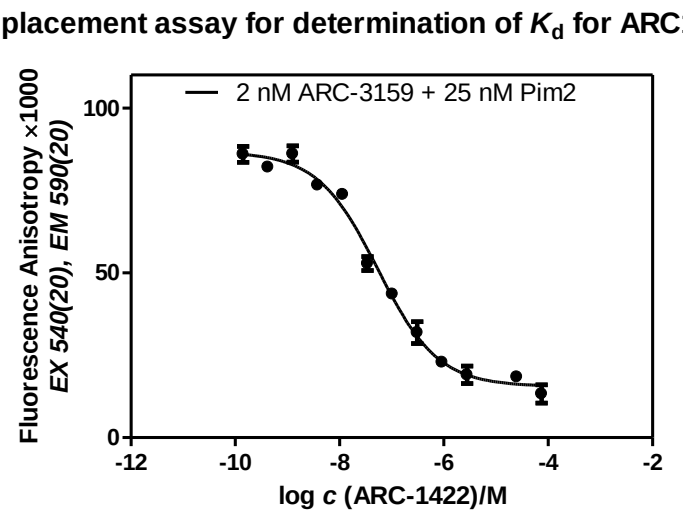

Figure 11: *Displacement of ARC-3159 from Pim-2 complex with ARC-1422.*

This result showed that ARC-1422 has subnanomolar affinity towards Pim2 (K_d = 0.4 nM for ARC-1422 towards Pim2). Therefore ARC-1422 comprising a Tb-chelate was found to be a potential probe for further experiments in TR-FRET format.

4.7 Determination of mAb(Pim2)AF647 specificity in homogeneous solution

The diagnostic samples usually consist of a complex material. Therefore, non-specific binding of the mAb(Pim2)AF647 to BSA and PKAc was examined. In parallel, to account for the effect of disulfide-reducing reagents present in the sample, the effect of 1 mM DTT to the TR-FRET signal was assessed. 3-fold dilution series of Pim2, PKAc, and BSA were prepared and fixed concentration of ARC-1422:mAb(Pim2)AF-647- (DOL5) (8 nM and 1 nM, respectively) was added to the series. From Fig. 12 it can be seen that the increase in TR-FRET ratio is specific and the setup described can be used for the examination of triple complex in solution by employment of ARC-1422.

DTT at 5 mM concentration would dissociate the heavy and light chains of the antibody by reducing the disulfide bonds. There was insignificant change in the maximum TR-FRET ratio,

which is probably due to the presence of labels near paratopes (antigen recognition sites on antibody) which contribute to the overall FRET. However the width of the peak reduced (from 0.92 to 0.57) and the variability of the data increased in the presence of DTT.

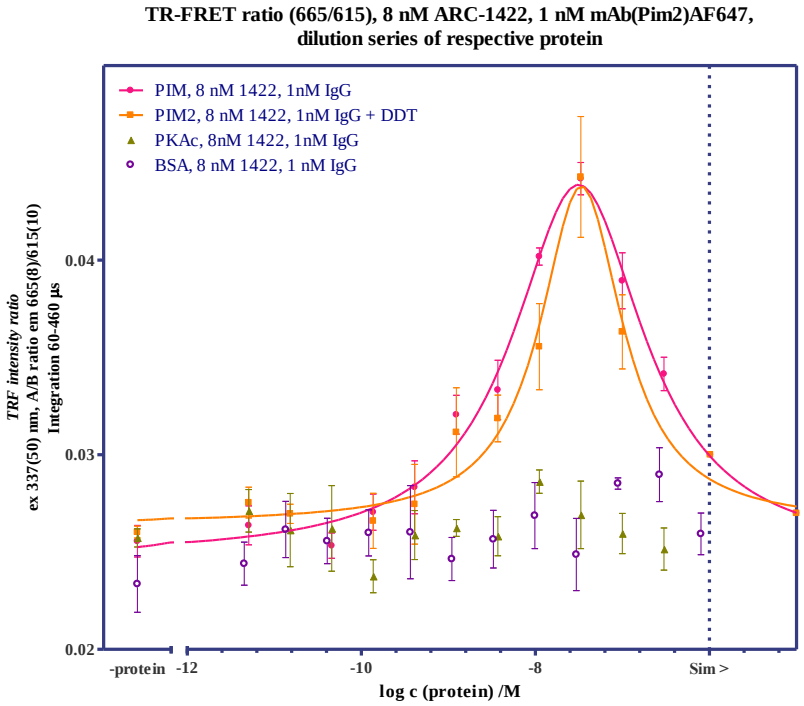

Figure 12: *TR-FRET measurement for the determination of non-specific binding of mAb(Pim2)AF647 to PKAc and BSA and determination of the effect of DTT.*

4.8 Determination of contributors to the TR-FRET signal of triple complex

Triple complex formation with the antibody DOL5 and DOL10 was examined at different concentrations (see in appendix ii) of the assay reagents (Fig. 13).

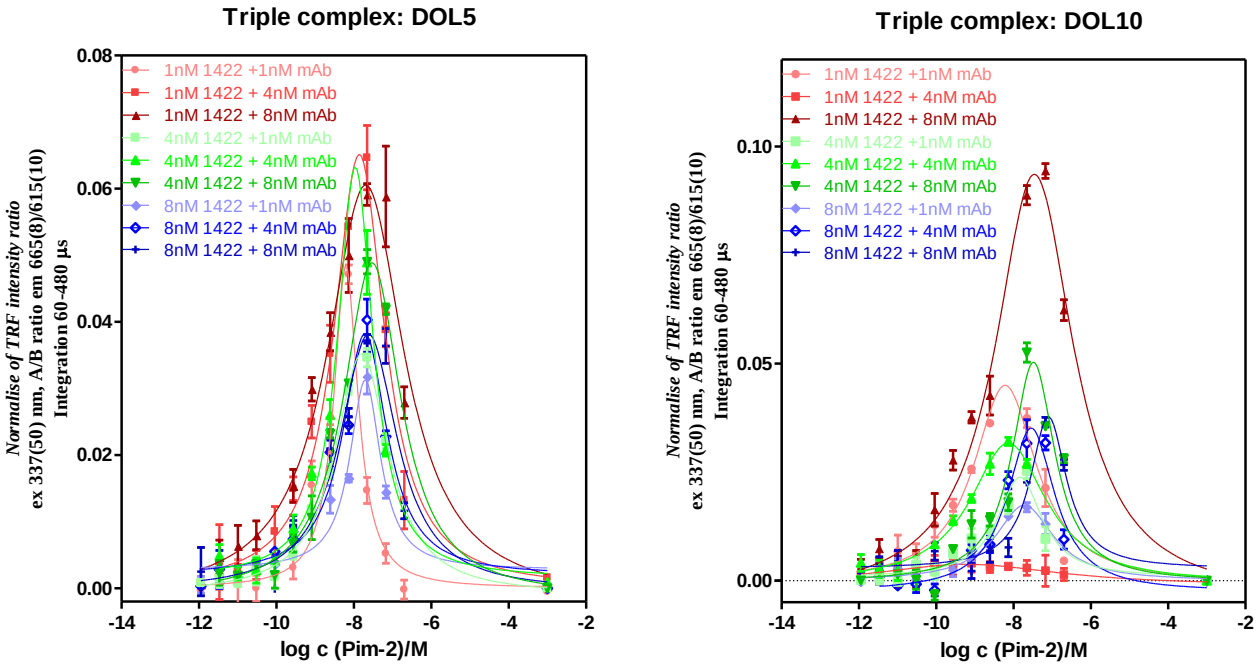

Figure 13: *Normalization of TR-FRET signals for the minimum signal to 0 in triple complex formation of ARC-1422, Pim2, and differently labelled mAb(Pim2)AF647: DOL5 (left) and DOL10 (right).*

Measurement results on Fig. 13 show that the assay can be optimized according to the particular need. Increasing the concentration of ARC-1422 tends to decrease the amplitude of the peak, thereby reducing the sensitivity towards Pim2. Assay sensitivity to ARC-1422 concentration is addressable to the overlap of the donor emission spectrum and the optical filter of the acceptor channel. Increase in the concentration of mAb(Pim2)AF647 brings about the widening of the peak, which is explained by the higher probability of the Pim2 to be bound to the antibody. Comprehensive approach for the performance of experiments would be the combination of low concentration of ARC-1422 and a high concentration of antibody.

Table 4: *Numerical results of the concentration variation of reagents leading to triple complex formation (ratio of ARC-1422:mAb)*

Ratio:	1:1	1:4	1:8	4:1	4:4	4:8	8:1	8:4	8:8
DOL5									
Amplitude	0.04847	0.06518	0.06236	0.02855	0.0364	0.03866	0.03623	0.06182	0.05022
Center	-8.205	-7.859	-7.693	-7.705	-7.712	-7.621	-7.797	-7.962	-7.528
Width	-0.3796	0.801	1.21	0.4832	0.6941	0.9036	0.8424	0.5511	0.9039
A·W	-0.0184	0.052209	0.075456	0.013795	0.025265	0.034933	0.03052	0.034069	0.045394
DOL10									
Amplitude	0.04579	0.004973	0.09793	0.0175	0.03778	0.03501	0.02315	0.03243	0.05075
Center	-8.217	-9.207	-7.46	-7.719	-7.541	-7.096	-7.833	-8.137	-7.483
Width	0.9925	2.971	1.235	0.9107	0.7436	0.5689	0.6981	1.196	0.6686
A·W	0.045447	0.014775	0.120944	0.015937	0.028093	0.019917	0.016161	0.038786	0.033931

Amplitude – peak height; center – concentration at which model achieves maximum; width – calculative width of the peak; A·W – amplitude times width for assessing optimal concentration ratio of the donor:ACCEPTOR.

4.9 Assessment of the determination range of Pim2 concentration in triple complex

The repeatability of triple complex experiment using antibody DOL5 was examined in 5 parallel Pim2 dilution series (Fig. 14). Titration was carried out with 2-fold dilutions instead of 3-fold dilutions and the incubation time was increased to 1 hr. The concentration of ARC-1422 and monoclonal antibody was maintained at 2 nM and 15 nM, respectively.

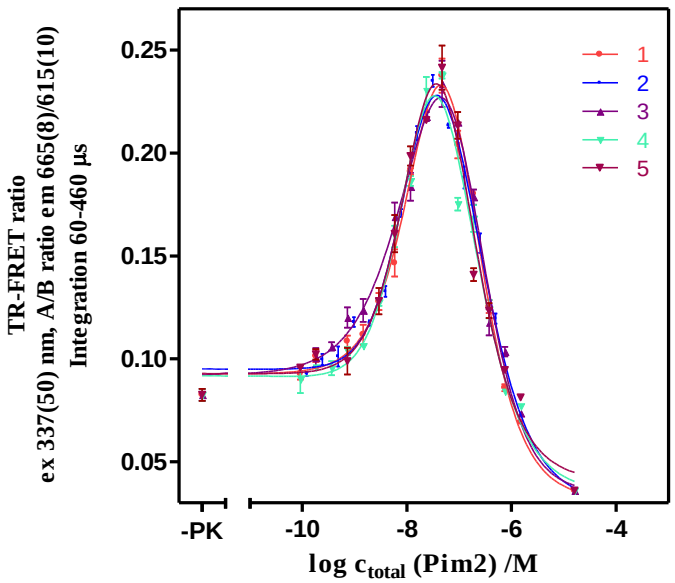

Figure 14: *TR-FRET intensity ratio for the triple complex between ARC-1422:Pim2:mAb(Pim2)AF647.*

The use of relatively high starting concentration of Pim2 revealed that the symmetric model does not apply due to the decrease in ratio of FRET signal in the presence of excess Pim2. This can be attributed to the quenching effect of the protein for the luminescent molecules. Therefore, a bell-shaped model is used for better representation. Considering minimal discrepancy of variability between each series, these results were used for creation of average titration curve (Fig. 15).

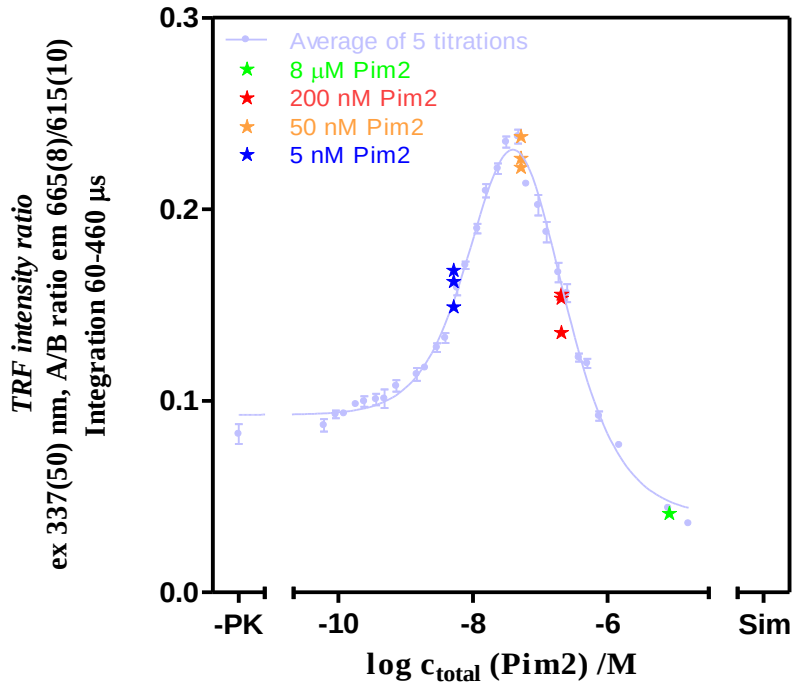

Figure 15: *Average intensity ratio from 5 measurements for building triple complex TR-FRET model for Pim2 concentration determination. Individual concentrations of Pim2 depicted show the TR-FRET ratio obtained for three independent samples of predetermined concentration.*

The obtained curve was tested for conformity using triplicates of 3 concentrations (200 nM, 50 nM, and 5 nM) and 8 µM of Pim2. The final confirmation of triple complex was shown as described in the Appendix under Fig. 16.

4.10 Determination of the detection and quantification limit of the TR-FRET homogenous assay

As shown in section 4.6, ARC-1422 binds to Pim2 with high affinity (K_d = 0.4 nM), lower than the total concentration of probe used in the triple complex experiments.

At low concentration of 5 nM, the probe ARC-1422 is still significantly in excess compared to the concentration of the Pim2 analyte, as shown in Fig. 15. We can practically assume that all

Pim2 molecules are bound to ARC and the binding curve can hence follow a linear regression dependency as seen in Fig. 17. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated based on the standard deviation of the blank sample (SD) and the slope of calibration curve (S) using the equations: LOD = 3.3 X SD/S and LOQ = 10 X SD/S, respectively (Kasari et al., 2012). It is to be noted that these parameters (LOD and LOQ) were determined based on the standard deviation of the blank solution containing the ARC-1422:mAb(Pim2)AF647, because the intensity ratio 665/615 nm for the buffer gave zero value. Also the calibration slope was used for the determination.

The obtained LOD and LOQ were 2.7 nM and 8 nM. These results point to a relatively high sensitivity of the assay. However, with the usage of FRET ratio, the calculative limits are not well describing the data. It can be seen that with the titration of Pim2, it is possible to differentiate subnanomolar concentration of the analyte (Pim2). Employment of other PK specific antibodies in this assay setup can potentially be used to measure the concentration of other PKs to which high-affinity ARCs have been developed.

At higher Pim2 concentrations, the probe is no longer in excess compared to the PK concentration as shown in Fig 15. Here, we can assume that because the Pim2 concentration is too high, there is a high possibility to see a combination of Pim2:mAb complex or a combination of Pim2:ARC complex than a triple complex of the three component. Hence, since the formation of FRET requires that the two chromophores are in close proximity or bound to Pim2. So, at excess Pim2 concentration, the ARC and antibody are not sufficient to bind to every available Pim2 molecule and there is less FRET explaining why the linear regression obtained showed a negative slope (Fig. 17 right). The reciprocal of the values were taken in order to ease the determination LOD and LOQ.

It is to be noted here that for determination of the LOD and LOQ, the standard deviation at highest Pim2 concentration responses and the reciprocal of the calibration slope were used. The LOD and LOQ obtained in this case using above mentioned formulae are respectively 3350 nM and 1104 nM pointing to the fact that Pim2 molecule can be determined even at high excess compared to ARC-1422 and antibody.

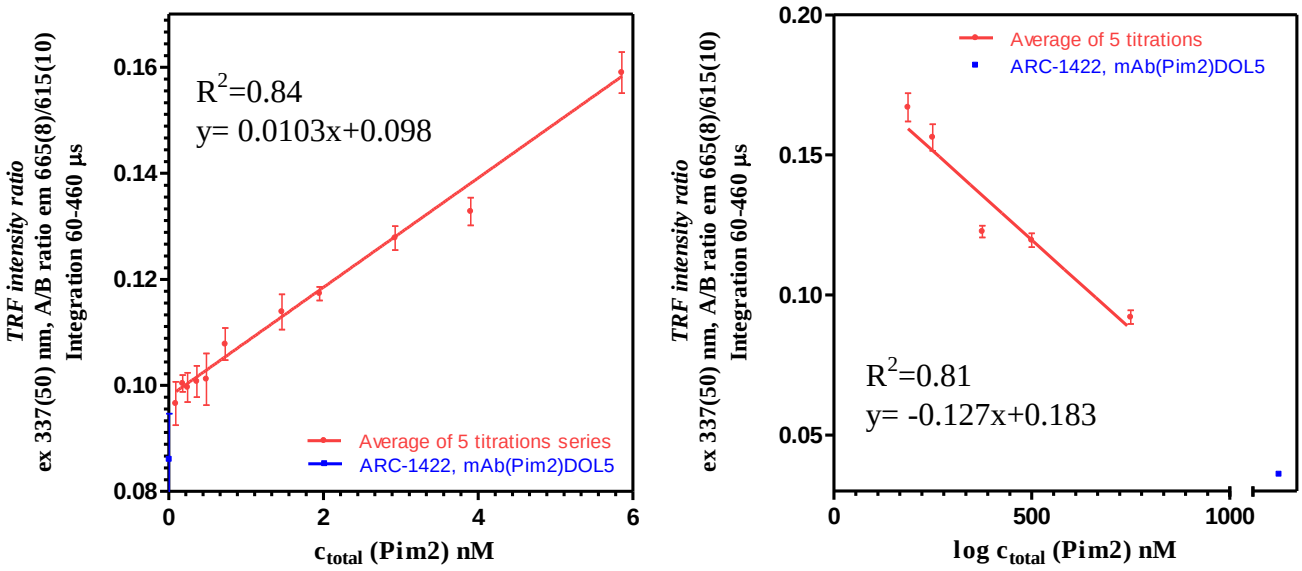

Figure 17: *Linear regression showing TR-FRET at low Pim2 concentration (Left) and at high Pim2 concentration (Right) for the determination of LOD for the assay.*

Information sheet

ARC-sondi ja antikeha kooskasutamine proteiinkinaasi Pim2 analüüsiks FRET-põhisel fotoluminestsents-meetodil

Pagkeu Sylvestre Tc

Kokkuvõte

Biomeditsiinilistes rakendustes üha laiemat kasutust leidev Försteri-tüüpi energia resonantsülekanne (FRET) luminofooride vahel toimub siis, kui sobivate fotoluminestsents-omadustega struktuuriühikud paiknevad teineteise lähedal. Käesolevas töös kirjeldatakse luminestsentsi aeg-lahutusega mõõtmismetoodikat, millega mõõdetakse energiaülekande efektiivsust terbiumkelaadilt, mis on ARC-sondi ARC-1422 luminestsentsmärgiseks, orgaanilisele värvile (Alexa Fluor 647), mis on proteiinkinaasi Pim2 antikeha fluorestsentsmärgiseks. Efektiivne energiaülekanne luminofooride vahel on võimalik vaid siis, kui mõlemad luminestseerivad molekulid (ARC-sond ja fluorestsentsmärgisega antikeha) on samaaegselt seotud analüüdiga (aktiivne proteiinkinaas Pim2). Tuvastasime, et antud meetod võimaldab mõõta aktiivse Pim2 kontsentratsiooni laias kontsentratsioonide vahemikus ning teostada analüüsid madala määramispiiriga. Näidati, et FRETi intensiivsus ei ole tundlik kõrvalistele valkudele olemasolule lahuses ja Pim2-indutseeritud FRET suhte suurenemine tõestati ARC-1422-konkurentse inhibiitoriga, mis põhjustas FRET suhte vähenemise. Kirjeldatud homogeenne analüüsimeetod on mitme uudse lihtsustava omadusega võrreldes levinud meetoditega. Meetod on kohaldatav teiste diagnostiliselt oluliste proteiinkinaaside määramiseks.

Võtmesõnad: FRET (Förster resonantsenergia ülekanne); TR-FRET (aeg-lahutusega luminestsentsintensiivsus FRET baasil); ARC (nukleosiidi analoogi ja oligopeptiidi konjugaadid); Ig (immunoglobuliin).

CERCS code: P310 Proteiinid, ensümoloogia

Joint application of an ARC-probe and antibody in homogeneous TR-FRET assay for determination of the concentration of protein kinase Pim2

Pagkeu Sylvestre Tc

Summary

Förster resonance energy transfer (FRET) is a routinely apply photoluminescence technology in high throughput screening in drug discovery. It involves the transfer of energy between two luminescence molecules, the donor and the acceptor in close proximity. In this thesis, we describe the application of the approach exploiting time-resolved measurement of intensity of FRET (TR-FRET) from terbium donor luminophore to fluorescent dye Alexa Fluor 647, for the measurement of the concentration of a specific protein kinase Pim2 in a solution-phase homogenous assay. This method is based on the simultaneous binding of a specific PK to both a luminophore ARC-type probe inhibitor and a fluorescently labelled monoclonal antibody specific to the PK. In case specific IgG labelled with Alexa Fluor 647 acting as acceptor fluorophore is combined in solution with donor luminophore (lanthanide chelate labelled) ARC-probe and the protein kinase of interest, the formation of a triple complex antibody:protein kinase:ARC-probe can be measured by time-resolved measurement of FRET intensity. First, we were able to determine the active concentration of the PK, using a flurescently labelled ARC-type inhibitor of the PK's active site. Second, we were able to prove the detection of a specific PK by formation of high a FRET signal between a luminophore ARC-type probe PK inhibitor as donor and a fluorescently labelled antibody specific to the PK as acceptor. In addition, formation of the triple complex between ARC-type inhibitor:PK:mAb(Pim2) was confirmed by addition of another ARC-type inhibitor in the well were maximal FRET signal ratio was observed. The determined limit of quantification of the assay was 8 nM, that points to high sensitivity of this homogeneous analytical method. The approach described in this thesis, could be used as a rapid medical diagnostic tool for PK detection and analysis.

Keywords: FRET (Forster Resonance Energy Transfer); TR-FRET (Time-Resolved Forster Resonance Energy Transfer); ARC (Adenosine-Oligoarginine Conjugate); Ig(Immunoglobulin)

CERCS code: P310 Proteins, enzymology

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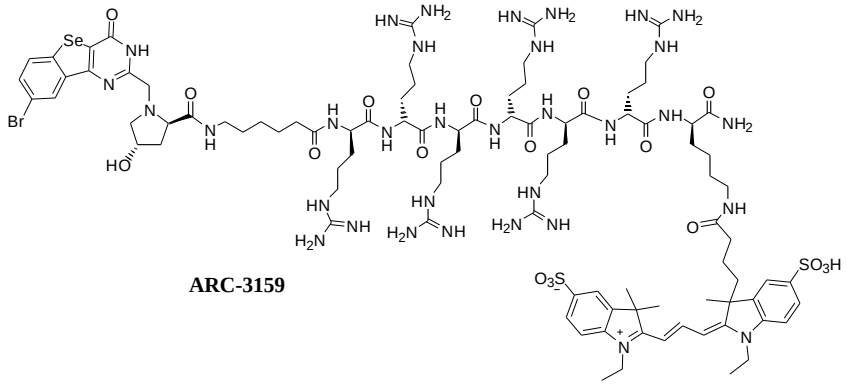
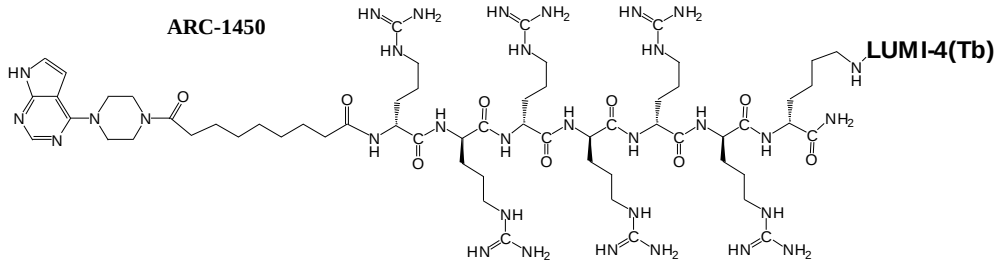
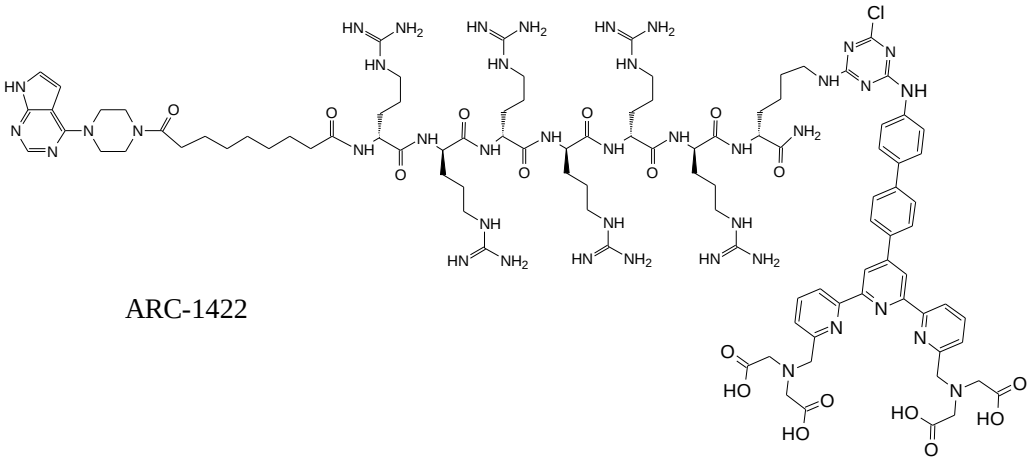
7 APPENDICES



ARC-3159



ARC-1450 **LUMI-4(Tb)**

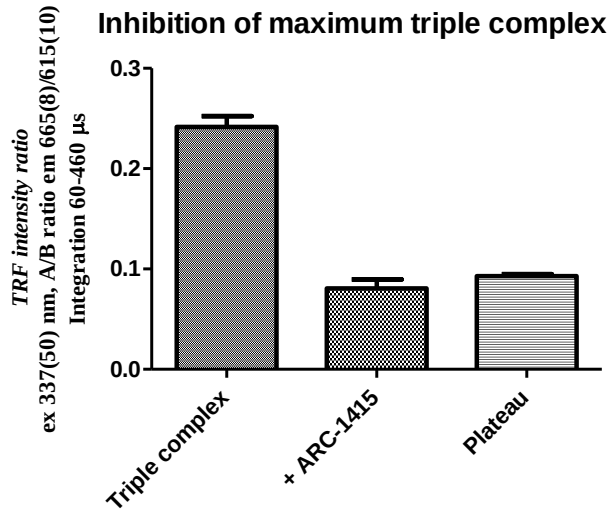


ARC-1422

Appendix 1. *Structures of ARCs (ARC-3159, ARC-1450, and ARC-1422).*

Appendix i: *ARCs used and the functionalized luminescent group attached*

Code	**Labeled**
ARC-3117	Promo Fluor 647
ARC-3159	Promo Fluor 555
ARC-1422	Terbium chelate
ARC-1450	Europium chelate



Appendix 2: *Inhibition of maximum triple complex signal by 50 µM ARC-1415. Addition of ARC-1415 displaced ARC-1422 and reduced the signal to plateau value, showing that the TR-FRET signal is specifically attributed to triple complex of ARC-1422:Pim2:mAb(Pim2)AF647.*

Appendix ii: *Concentration combination used for the triple complex formation with DOL5*

Pim-2 concentration (nM)	ARC-1422 concentration (nM)	mAb(Pim2) AF-647-Dol5 concentration (nM)
300	**1**	**1**
300	**1**	**4**
300	**1**	**8**
300	**4**	**1**
300	**4**	**4**
300	**4**	**8**
300	**8**	**1**
300	**8**	**4**
300	**8**	**8**

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