Diplomarbeit

MAP Kinase Kinase Kinase Assay Development for High Throughput Screening

by

Markus Wunder

University of Applied Science, FH-Giessen-Friedberg

Department of Hospital and Medical Engineering, Environmental Technology and Biotechnology

Degree course Biotechnology

Giessen, Germany

Supervisor:

Prof. Dr. J. Hemberger and Prof. Dr. H. Brunnhoefer

BASF Bioresearch Corporation

Department of Biochemistry

Worcester, MA, USA

Supervisor:

Yajun Xu, Ph.D. and Hamish Allen, Ph.D.

01/05/2001

Für

Erna und Ludwig

STATEMENT

The practical work on this project, interpretation and discussion of the data and writing of the thesis was carried out entirely by myself, except when otherwise stated.

The thesis may be included in the library of University of Applied Science, Fachhochschule Giessen-Friedberg. This thesis should be treated as confidential before being filed in the library of University of Applied Science, Fachhochschule Giessen-Friedberg.

Markus Wunder, 01/05/2001

Markus Wunder
Ulmenweg 43
68167 Mannheim
- Germany -
Phone: +49-(0)177-5216343

ACKNOWLEDGMENTS

I would like to thank Winnie Wong and Yajun Xu for giving me the opportunity to come here and work on this project.

I want to thank Yajun Xu and Hamish Allen for their supervision, teaching, and their always-open offices, which gave me the chance to bother them whenever I had problems.

A big thanks goes to the whole staff of the department of Biochemistry for helping and entertaining me.

I also want to thank the staff in Germany Anja Schantz-Eberle, Peter Eckard, and Claus Markert for their help during my time there.

Special thanks go to Christine Grinnell, Maureen Fujimori, Yong Jia, Dave Bannach, Hamish Allen, and Mitchel Galanek that they gave me the chance to entertain them by making them read the script, or parts of it.

scare ≠ scarce

peek ≠ peak

angel ≠ angle

adopted ≠ adapted

serious ≠ series

stuff ≠ staff

A very special thanks to Dorothy Jacques and Bob Duff, Cynthia Johnston , and Benson Waweru for being good friends.

And finally I would like to thank Bill Gates, and his great spell check program with its very helpful suggestions:

automation = atomization

SUMMARY

The goal of this work was to develop a novel mitogen-activated protein kinase (MAP K) cascade assay suitable for high-throughput screening (HTS). Three major steps were necessary for development and transfer of the assay. Firstly, the assay was developed and optimized at BASF Bioresearch Corporation, Worcester, USA. Within this step the kinase cascade, consisting of three kinases in a row, MAP KKK $\rightarrow$ MAP KK $\rightarrow$ MAP K, was established and optimized. The MAP KKK phosphorylates and activate the MAP KK, which in turn phosphorylates and activate the MAP K. As method for detecting the stage of phosphorylation homogeneous time resolved fluorescence (HTRF, Packard, USA) was chosen. Secondly, the screening campaign was organized, and the assay was transferred to the HTS group at Knoll AG, Ludwigshafen, Germany. Thirdly, the assay method was adapted to the conditions in the German laboratory and miniaturized from the 96-well to the 384-well format. To introduce the assigned biology-lab-technician, as well as the staff of the group in Germany which would later run the assay, a seminar was given. This seminar covered the theory as well as the practical applications of the assay.

A further part of the work was to develop two different follow up assay systems. Follow up assays, has to determine whether a compound active in the primary screen is true or not. False negative and false positive compounds need to be sorted out by these assays. First, a scintillation proximity assay (SPA) setup was tested, and second, a splitted MAP kinase cascade was used. The splitted cascade was optimized and could be used for determination of the enzyme in the cascade which was actually inhibited.

ABBREVIATIONS

ADME - Absorption / Distribution / Metabolism / Excretion
BBC - BASF Bioresearch Corporation
BSA - Bovine Serum Albumin
dpm - Disintegrations per minute
DSK - Dual-Specificity Kinase
DTT - Dithiothreitol
EDTA - Ethylendiaminetetraacetic Acid
EGTA - Ethylene Glycol-bis(b-aminoethyl Ether) N,N,N',N'-Tetraacetic Acid
ELISA - Enzyme Linked Immunosorbent Assay
ERK - Extracellular-Signal Kinase
FCS - Fluorescence Correlation Spectroscopy
FDA - U.S. Department of Health and Human Services, Food and Drug Administration
FP - Fluorescence Polarization
HEPES - (N-[2-Hydroxyethyl]piperazine-N'-[2-ethnesulfonic acid])
HTRF - Homogeneous Time-Resolved Fluorescence
HTS - High-Throughput Screening
LSC - Liquid Scintillation Counting
MAP K - Mitogen Activated Protein Kinase
MAP KK - Mitogen Activated Protein Kinase Kinase
MAP KKK - Mitogen Activated Protein Kinase Kinase Kinase
MBP - Myelin Basic Protein
MEK - MAP/ERK Kinase
PMT - Photomultiplier Tube
RET - Resonance Energy Transfer
SAXL - Streptavidine-Allophycocyanine Complex
SC - Scintillation Counting
Ser - Serine
SPA - Scintillation Proximity Assay
Thr - Threonine
TRF - Time-Resolved Fluorescence
Tyr - Tyrosine

PART A: THEORETICAL BACKGROUND

Drug Discovery

Target Identification

Assay Development

DRUG DISCOVERY

The road from discovery to marketing of a novel drug is long and expensive. In order to minimize time, discovery programs integrate a wide range of disciplines such as biotechnology, genomics, molecular biology, combinatorial chemistry and high-throughput screening into their efforts. Choosing a target is the first crucial step in developing a successful drug. Companies usually specialize in a defined niche of research, such as immunology or cancer, out of which they choose their targets, which evolve out of phatobiochemical mechanism within the body. (Borman, 1999; Kennedy, 1997)

Once the decision on a new target is made inhibitors need to be found which modify the target function to obtain the desired effect. Compounds are assayed, in the magnitude of hundreds of thousands, to identify lead structures with a structure-activity relationship (SAR) in regard to the target chosen. For identifying these leads different methods are used in combination with each other. The most frequently applied techniques are random screening of existing compound libraries, and combinatorial chemistry. Random screening is used to identify hits (lead structures), and structural related families in these hits, out of existing compound libraries. Within combinatorial chemistry these lead structures or parts of them are used as a backbone-structure. Subsequently, they are randomly modified, by adding molecular segments of other agents to improve the activity of the initial backbone. Activity in this case means efficient blocking the function of the Target chosen. These lead compounds have to be novel in order to obtain patents applications for them and achieve a competitive advantage in the drug market. (Valler et al., 2000; Hogan, 1996)

A novel lead compound must fulfill certain criteria, such as solubility, potency, and specificity. After assuring that a compound meets these criteria, it is important to make sure that its effects are stable and reproducible and meet ADME criteria. ADME stands for absorption, distribution, metabolism, and excretion. Next, safety tests are required to identify its possible side effects or toxicity. Safety screens are performed early in the process to eliminate leads that are too toxic. Potential inhibitors should meet certain criteria in order to reach clinical trials and later to be successful drugs:

- Target specificity: The drug should react specifically with the target of interest and nowhere else in the body. The more potent the compound for its intended target, the more likely that it will show potency.
- Bioavailability: The drug must be absorbed ideally after oral ingestion and accumulate in the tissue in reasonable quantities.
- Formulation and drug delivery: The drug should be easy to administer.
- Pharmacokinetics: The drug must function in the body as long as necessary, but not too long to cause side effects.
- Toxicity: The drug should have a minimum of non-specific side effects.

(Broach et al., 1996, modified)

Once significant animal safety testing on various models is completed, the company submits the results with an Investigational New Drug (IND) application to the U.S. Food and Drug Administration, or similar applications to the responsible authority in the host country. The FDA reviews animal and other pre-clinical data and decides whether to allow the IND. Once the IND is approved, clinical trials may begin. (FDA, 2000)

In Phase I clinical trials, the drug is investigated in several different tests of around 20-100 normal, healthy volunteers per test in order to prove the drug is safe and to document possible side effects. Phase II trials involve up to several hundred patient volunteers who suffer from the target disease. These trials are controlled, meaning that the patients are divided into two groups: one that gets the drug, and a negative control that gets a placebo or the standard therapy. To make the results more reliable, the groups are randomly assembled and the drug is administered in a double blind fashion. This means neither the patients nor the physicians who are administering the drug know who is getting drug or placebo. Phase II trials take from several months up to two years to be completed. Phase III trials involve several hundred to several thousand patients and take 1-4 years to complete. Within these trials, patients are recruited, tested, and monitored in several hospitals in the country and, in most cases, internationally. All the results go back to the FDA and if everything is in order the drug is approved. Only 20% of INDs survive this procedure. (FDA, 2000)

Following this time consuming and expensive process (about 10 years and costs of around US$200-600 million) one can easily understand that everything a

pharmaceutical company can do to accelerate this process will be done. As the number of compounds and the number of targets increases there is a increasing demand for screening, which is used in hit detection and, more and more, in early ADME. High-throughput screening is an established field born out of the necessity to shorten drug development time. (FDA, 2000)

High Throughput Screening (HTS)

High throughput screening is the process by which large numbers of compounds can be tested, in an automated way, for activity as inhibitors (antagonists) or activators (agonists) of a particular biological target (Broach et al., 1996). The primary goal is to identify high-quality lead structures, and to supply information necessary to the optimization process. Leads are representatives of one or more different families of structurally related compounds, generated during a screening campaign. The goals of high-throughput screens are specificity, high reproducibility, low costs (~$1/well), and speed, therefore it will be as far automated as possible.

There are several steps required to transfer a new assay from a small-scale laboratory test system to a high-throughput screening system. A robust assay's results give the same result in the hands of any researcher. First, one has to determine optimal assay conditions. The target protein must be well characterized with respect to purity, concentration, specific activity, and kinetics. The choice for the detection and automation schemes is dictated by the assay itself. For example, whole cell assays are more complex than enzyme assays and need additional steps, such as separation. For automation, one is best served by the "kiss" rule: Keep It Simple, Stupid. The final step is the validation of the HTS. Here, assay stability is assured by investigating day-to-day variations, by using compounds within the test, and by comparing the variation between the different runs. Any screening has to avoid false negative compounds (compounds active against the target but not scoring in the test), and false positive ones (compounds scoring in the assay but not active against the target). False negative compounds can be crucial, especially in cases where not enough other hits are generated in the screen. False positives also have an impact, as it is cost and time intensive to rule them out. For this reason, the assay should be designed as straightforwardly as possible, and suitable controls must be added (0%

inhibition / 100% inhibition) (Broach et al., 1996). Follow-up assays have to be designed so that the activity of hits from the primary screen can be confirmed.

Implementation of HTS

An effective and valuable HTS system needs to adopt an appropriate infrastructure. An appropriate infrastructure contains four key elements that should work together as one. These are sample management, assay design, automation, and data management (Broach et al., 1996).

Sample Management

Sample management includes compound management and logistics and plays an important role in HTS. Without converting the compound library, traditionally in vials, into a format manageable for automation, an effective HTS cannot be performed. Electronic data handling systems and libraries transferred into microtitration plates are essential for high throughput. By automatically labeling them with bar codes, every compound can be tracked without problems. Sample preparation, e.g. solubilizing or storage, can compromise the integrity of samples. This risk must be balanced against the risk of sample transfer becoming a bottleneck. (Valler et al., 2000)

Miniaturization to reduce storage size, costs, and increase throughput is ongoing from the traditional 96-well plate format towards higher densities on the same footprint, such as the 384- and 1536-well plates. The 96-well microplate format has been the standard format used to array compounds for screening. A typical assay well in a 96-well plate uses about 100 µl test solution, but reactions in a 1536-well plate only use 2-10 µl. Thus, 10 times more reagents are needed in a 96-well plate. The amount of compounds in many cases is limited and reagents are cost-intensive. Therefore the 384-well format is increasingly popular, followed by the 1536-well plate. Points to consider here are evaporation, as well as effective mixing in these low volume plates. Another approach is the rapid and direct change from a 96-well format to the nanoliter high-density formats, like the sheet screening from Abbot Laboratories (Karet, 2000). This involves substantial investments in new equipment and manpower, as well as time to implement and validate the system. (Houston et al., 1997)

Assay Design

The HTS system relies strongly on the complexity of the assay method. Here the key elements are assay protocol optimization, adaptability to automation, and availability of high quality reagents. A simple assay procedure is preferable. To find the best conditions, the assay has to be optimized systematically by varying factors believed to have an effect on the response, such as ATP or enzyme concentration, and with respect to cost and quality, as described in the practical part of this work. (Lutz et al., 1996)

Automation

HTS systems are composed of a series of devices such as pipette stations, readers, and incubators. These devices are physically linked by a transport system, usually a robotic arm that performs a series of tasks within an HTS assay (Brandt et al., 1998). All these devices rely on each other; if only one device fails, the whole assay will fail. Reliability can be broken down into three components: functional quality of the device (how well it functions independently), the ease of the physical interaction between the device and the transport mechanism, and the software routines used to control the device within the system. A system should, following the kiss rule, be able to accommodate different devices from different manufacturers. This makes the system flexible and allows changes for different approaches. Another factor in reliability is the required maintenance of the device, from changing bulbs in a detector to avoiding crystallization in a washer. System reliability depends upon the reliability of each device included in it. Overall, the more complex an HTS system is, the less reliable it is in general. (Brandt, 1998)

Data Management

Data handling and management are other bottlenecks in HTS. Hundreds of thousands, even millions, of data points are generated. Thus, a reliable and effective data handling software is required. The database must cope with this huge amount of data and has to provide functions for supporting data analysis. Features of databases should include compound inventory and management, compound logistics, automated

data processing, identification of potential hits, and integration of communication interfaces, for exchanging the data. (Brandt, 1998; Valler et al., 2000)

TARGET IDENTIFICATION

BASF Bioresearch Corporation (BBC) focuses on immunology. Its targets emerge from the fields of autoimmune disease and prevention of transplant rejection. BBC develops anti-inflammatory compounds, and immunosuppressive agents to inhibit signal-transduction in immune cells. Cellular cycles are regulated through a variety of different mediators. Some of the most important mediators are kinases. Out of the huge field of kinases, a MAP kinase was chosen as a target. Kinases in general, and the MAP kinases in particular, are discussed below.

Kinases

Protection by the nuclear membrane of eukaryotic cell DNA places multiple physical barriers between the cell's genetic material and the cell's external environment. On one hand, the DNA is secure and interaction with noxious agents, such as nucleases or mutagens, is limited. On the other hand, this barrier makes it more complicated for the genes in the nucleus to sense changes in the cell's environment and to initiate a proper response. To connect the genes with the environment, an elaborate system of biochemical networks has been developed over the course of eukaryotic evolution. These networks, termed signal transduction pathways, rely on multiprotein complexes of receptors on the cell surface, enzymes, and protein and non-protein second messengers to regulate the flow of information from outside of the cell, through the cytoplasm, and finally into the nucleus. One of these mechanisms is the phosphorylation reaction of proteins, catalyzed by kinases, on amino acid residues from Serine, Threonine, or Tyrosine (Figure 1). (Alberts et al, 1994; Heimbrook et al., 1998; Robinson et al., 1997)

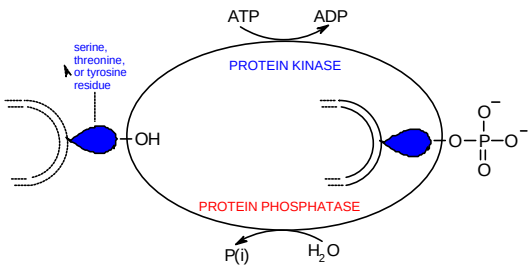

Figure 1: ATP-dependent protein phosphorylation and dephosphorylation as catalyzed by protein kinases and phosphoprotein phosphatases.

This is generally recognized as a fundamental mechanism by which eukaryotic cells respond to changes in extracellular signal molecules or adverse environmental conditions. It also appears to be a major mechanism through which discontinuous cellular processes, such as DNA replication, mitosis, differentiation, and apoptosis are switched on and off. Since phosphorylation is a dynamic process, it necessarily follows that the activity of the activated enzymes has to be reversed in order to protect the cell from pathological reactions and to come back to the stage of basal activity. This job is done by protein phosphatases. Phosphatases are enzymes with the ability to hydrolyze phosphate groups from phosphorylated enzymes (Figure 1) (Alberts et al., 1994). Recent discoveries related to Src kinase suggest that ubiquination may also play an important role in the process of controlling kinase activity (Harris et al., 1999).

The importance of protein phosphorylation is becoming more and more apparent from the ongoing genome projects, with the discovery that 1-2% of the entire human genome may encode protein kinases. Conversely, there may also be as many genes encoding protein phosphatases as there are opposing the protein kinases.

The kinase family can be divided into several subfamilies. They usually use ATP as a phosphate donor. Each phosphate group, added to a hydroxyl group of an amino acid side chain, contains two negative charges, which can cause a conformational change, for example, by attracting positively charged side chains. Such a change occurring on one site can alter the conformation elsewhere in the protein. The active form of ATP is usually in a complex with metal ions like Mg^{2+}, or Mn^{2+} (Figure 2). In most physiological cellular processes it is the metal-ATP complex that reacts, rather than ATP itself (Stryer, 1999).

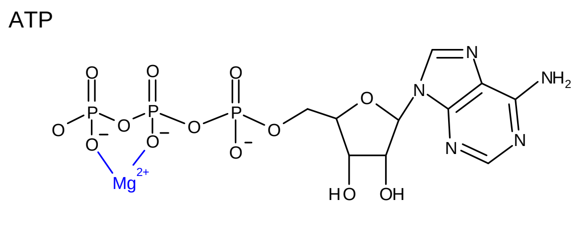

Figure 2: ATP in complex with Mg^{2+}, the most reactive conformation of ATP within the cell (Stryer, 1999).

Magnesium is important for inducing a conformation of ATP, which can be recognized by kinases as a substrate. The eukaryotic protein kinases can, with respect to their

substrates, be divided into two major classes. The first class phosphorylates hydroxyl-groups of Serine and Threonine residues, whereas the second class phosphorylates the same group within Tyrosine residues (Figure 3). However, there are certain kinases that are able to phosphorylate all three amino acids, the so-called dual-specific kinases (DSK), reviewed in Dhanasekaran et al., 1999. The DSK are unique in that they share the consensus kinase motifs of both Ser/Thr and Tyr-kinases and are opposed by dual-specific phosphatases (DSP), reviewed in Camps et al., 2000.

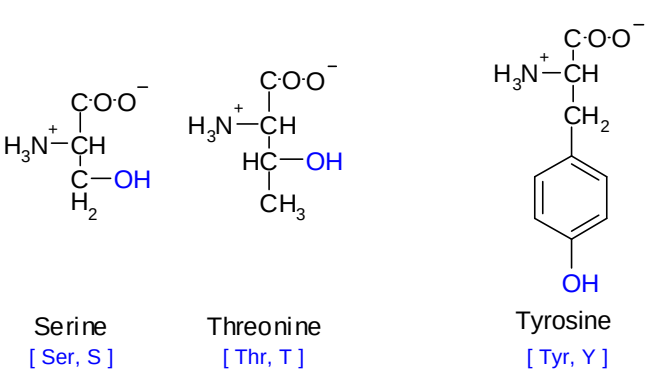

Figure 3: Serine, Threonine and Tyrosine contain hydroxyl groups, which act as substrate for kinases. (Stryer, 1999).

MAP-Kinases

Mitogen-activated protein kinases (MAP K), are members of the Ser/Thr kinase family, and play an important role in signal transduction pathways. They are activated by a range of stimuli such as growth factors, cytokines, cell stress or osmotic pressure. Thus, MAP kinases mediate a number of physiological and pathological changes in cell function (Alberts et al., 1994). A prototypical MAP-kinase-signaling module consists of a minimum of three kinases in cascade. This cascade, MAP KKK ? MAP KK ? MAP K seems to be highly conserved in eukaryotic cells. One member of the MAP kinase family is the extracellular-signal-regulated kinase (ERK). Activation of MAPKs, like ERK, requires phosphorylation of Threonine and Tyrosine, which are separated by one amino acid in the protein sequence. This activation is done by a dual-specific kinase (MAPK/ERK kinase, MEK). MEK is, in turn, phosphorylated by an MEK kinase (MEKK) at a Ser/Thr residue, also called MAP kinase kinase kinase.

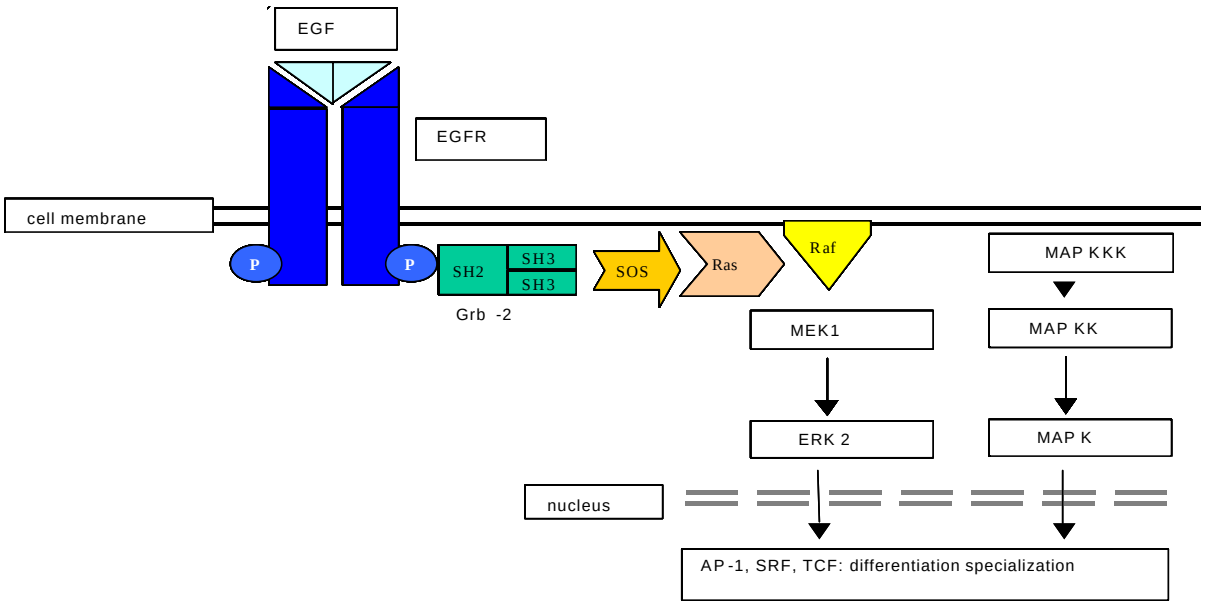

Figure 4: A possible mitogen activated protein kinase (MAP) signaling pathway.

The best understood pathway is the module Raf/MEK/ERK (Figure4) which is regulated upstream by Ras, a small GTP (Guanosine 5'-triphosphate) binding protein. Growth factor ligands, in this case epidermalal growth factor (EGF), cause the EGF receptor to autophosphorylate. The phospho-tyrosine residue of the autophosphorylated receptor binds the SH2 (Src homology-region 2) domains of adaptor proteins, in this case Grb 2 (growth factor receptor bound protein 2). Grb2 itself binds a guanosine nucleotide exchange factor (SOS, son of sevenless). SOS, now located in the plasma membrane, has access to Ras. There, it converts Ras from its GDP (Guanosine 5'-diphosphate) bound inactive state to its GTP bound active state. The activated Ras now targets Raf to the membrane where Raf is subsequently activated. Raf (MAP KKK) stimulates MEK 1 (MAP KK), by phosphorylating Ser-217 and Ser-221. The DSK MEK 1 subsequently phosphorylates ERK 2 (MAP K) at Thr-183 and Tyr-185. The nuclear targets of this enzyme are transcription factors in the nucleus such as Elk-1 or AP-1 (Heimbrook et al, 1998; Robinson et al., 1997; Seger et al., 1995; Alessi et al., 1995; Burack, 1997).

Inappropriate stimulation of the previously discussed signaling processes due to interference in the individual components in the pathway can cause disordered cell replication, tumor formation, or cell death. Identification of the critical components in these pathways to create new drugs is a necessity. Ras oncogenes, for example, are

one of the most frequently mutationally activated genes in many human tumor types (Bos, 1989). As all members in the chain depend highly on each other, it is reasonable to assume that selective inhibition of a MAP KKK, MAP KK, or MAP K will block mitogenic signals from Ras and may lead to a new drug for the cure of cancer. For this reason, components of the MAP cascade modules provide viable targets for developing new drugs in order to inhibit growth factor dependent activation of MAP KKKs, MAP KKs, and MAP Ks, therefore, pathological cell proliferation. (Heimbroock et al., 1998)

The MAP KKK chosen as a potential target is activated by cytokines, in stress and growth factor induced MAP-cascade modules. The goal is to discover a new anti-cytokine drug for rheumatoid arthritis (RA) by blocking pro-inflammantory cytokine signaling. Its involvement in this signaling makes this kinase an interesting and potential target. Because it is a member of the MAP KKK family, the MAP KKK Raf can be used as a blueprint. Since Raf is already well described in the literature, for example, Alessi et al., 1995, conditions used for the Raf cascade could easily be adapted for the target cascade. In the following section, the steps needed to develop a well-defined MAP kinase assay are described.

MAP KINASE KINASE KINASE ASSAY DEVELOPMENT

Overview

In order to successfully develop and optimize a MAP kinase assay, certain criteria must be considered (Figure5). After the Target is expressed and purified its substrate has to be determined. If no substrate can de discovered, peptide libraries have to be created and screened. If one is lucky and a direct substrate can be determined the next step is to screen the phosphorylated peptide with antibodies. Antibodies are a valuable tool in phosphorylation detection. Is there no commercially antibody available, either an alternative assay method has to be found or an antibody has to be made. Once all tools for the assay development are in place the assay development can start.

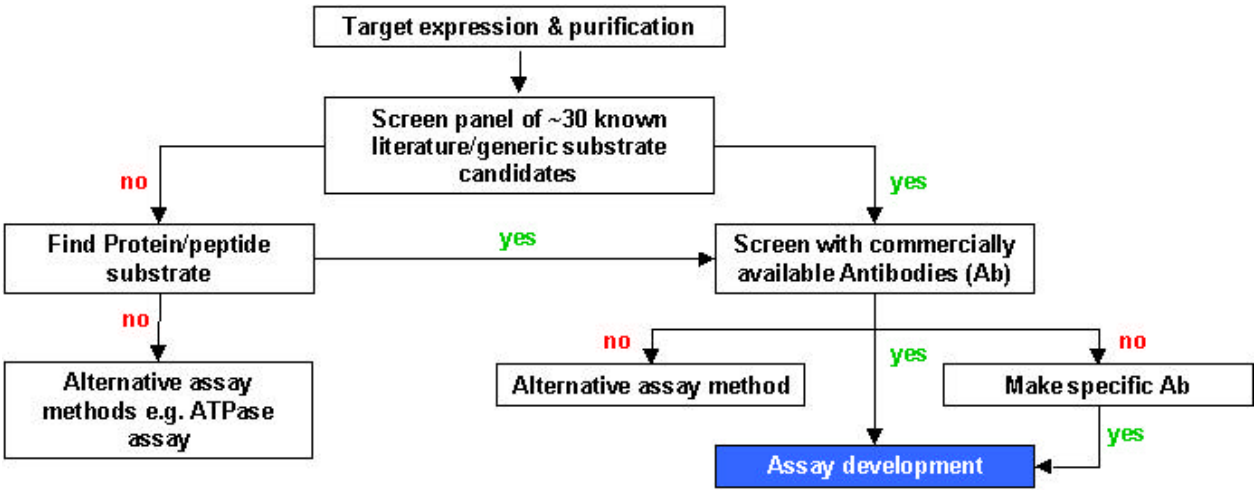

Figure 5: Assay development for a Ser/Thr kinase.(Xu, 2000, modified)

After the target decision is made, the enzyme has to be made available in high enough quantities to enable assay development and, optimally, to support the later HTS. Our MAP KKK is an in-house baculovirus (BV) expressed affinity-tagged fusion protein. Within the baculovirus system, insect cells are transfected with a recombined BV-construct. After absorption of the virus-DNA, the cells start DNA replication as well as BV and foreign protein production. The cDNA encoding the foreign protein is under control of the strong promoter for the polyhedra envelope-protein of the virus. This maximizes the expression level. After cell lysis, the kinase of interest is purified by affinity chromatography.

Within the next steps the tools for assaying the enzyme must be determined. These tools are the substrate and a suitable antibody. Finding a suitable substrate is very important. The actual assay format and, as mentioned, the detection method will be determined by its nature. The detection methods for kinases rely on the phosphorylation of a substrate. Hence, it is necessary to determine the phosphorylation state. There are two methods frequently in use for HTS. One depends on detection by an anti-phospho-antibody, and the other detects phosphorylation by incorporation of radioactive phosphorus into the substrate. The lack of specific antibodies is a major drawback. The cascade format overcomes the lack of antibody. For the last enzyme, in the cascade, MAP K, a direct substrate (myelin basic protein) and a commercially available anti-phospho antibody for this substrate are available. The Target enzyme could be plugged into the cascade in exchange for Raf. Myelin basic protein, MBP, is widely used as a substrate for protein

kinases, including MAP kinases. MAP kinase phosphorylates MBP at several Thr-sites, and the extent of phosphorylation can be monitored by use of phosphorylation state specific antibody. The clone P12 used in our case is specific for Thr-98. The antibody provides the basis for non-radioactive assay. It can be labeled with a variety of fluorescent dyes, used for detection. Fluorescence-based assays play an important role in HTS. When possible, they are the method of choice since radioactive assays have to deal with radioactive waste and the safety risks involved. However, radioactive assays are also of great value in assay development. (Alessi et al., 1995; Upstate Biotech, 2000)

Technology evaluation

After determining the substrate and antibody, the detection method and the assay format must be determined. By looking into various assay formats, one can see that an assay consists of a sequence of independent steps. For example, the steps of an assay might include pipette buffer, add compound, add substrate, incubate, and read. However, if one step fails, the assay fails. HTS systems perform in a very similar fashion. Thus, transferring an assay into HTS results in certain restrictions.

To develop a successful high throughput-screening assay, a scientist has some basic requirements to consider. The assay has to be sensitive, robust, cost-effective, and adaptable for high throughput screening and miniaturization. This makes simple, non-separation based assays favorable for HTS. As mentioned, high throughput detection systems adaptable for kinases are either fluorescence-based or radiolabel-based. Each of the approaches has its advantages and disadvantages. The most common detection systems are summarized in Table 1.

Table 1: Commonly used HTS adaptable kinase assay detection systems.

	Fluorescence based:	Radioactive based:
Homogeneous	Resonance Energy Transfer (RET) Time Resolved Fluorescence (TRF) Fluorescence Polarisation (FP) Fluorescence Correlation Spectroscopy (FCS)	Scintillation Proximity Assay (SPA) Solid Scintillator Counting
Heterogeneous	ELISA	Filter-binding

FLIORESCENCE- AND RADIOACTIVITY BASED ASSAYS

Fluorescence

Every phenomenon where a gas, liquid, or solid emits light after excitation is called luminescence. The excitation can be caused by radiation with light, X-rays, proton- or alpha rays, or electricity. Further, luminescence is divided into phosphorescence and fluorescence.

Fluorescence is the two-phase phenomenon in which a light quantum at a given wavelength, λ_{ex}, and hence with a given energy, equation 1, is taken up in phase one (excitation) by the resonant molecule, a fluorescent molecule.

$$E = h \cdot n \qquad \textbf{[1]}$$

The energy, E, is given through Planck's constant, and the frequency, ν, of the wave. A Jablonski diagram illustrates the process between absorption and emission of (Figure 6).

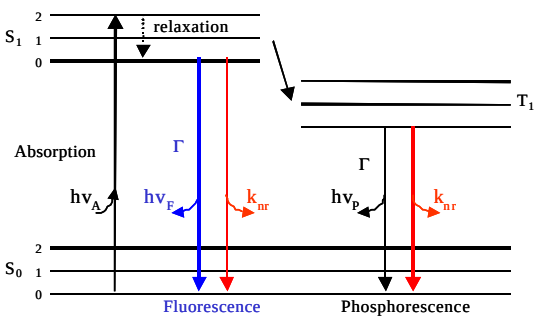

Figure 6: Simplified Jablonski diagram (Lakowicz, 1999, modified).

During excitation, a fluorescent molecule (fluorophor) absorbs light of a particular wavelength. If the energy of the absorbed light (hv_A) is sufficient to boost an electron from the ground-state energy level (S_0) to an excited-state energy level (S_1), excitation occurs. During the brief lifetime of the excited state, the electron generally decays in as second phase toward the lowest vibrational energy level within the electronic excited state (relaxation, hv_F). The return to the resting condition is accompanied by a nonradiative conversion, k_{nr} (e.g. heat), and, more particularly, by a luminescent (radiative) transition, Γ, directly to the ground state (true fluorescence) or through a semi-stable triplet state, T_1 (phosphorescence). The energy of an emitted photon equals the difference between the energy of the electron in the excited state and the

energy level in the ground state to which it falls.The lifetime, τ_F, is defined by the average time the molecule stays in the excited state, and is usually in the range of nanoseconds.

The distribution of wavelength-dependent intensity that causes fluorescence is known as the fluorescence excitation spectrum (Figure7, A), and the distribution as the fluorescence emission spectrum (Figure7, F).

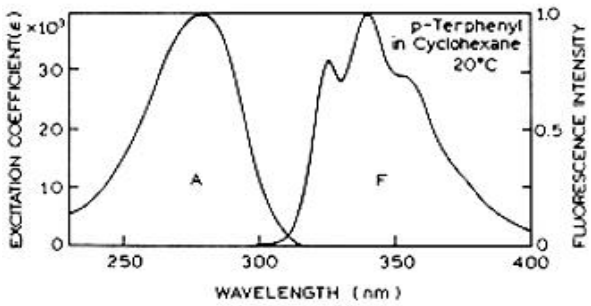

Figure 7: Absorption (A) and emission (F) spectrum of p-Terphenyl. (Lakowicz, 1999)

The emission spectrum is always shifted toward a longer wavelength (lower energy) relative to the excitation spectrum (Stokes' shift). In fluorescence this shift is normally 30 to 50 nm and represents the energy dissipated during the lifetime of the excited state, before the fluorescent light is emitted. A very important feature is that the emission wavelength is independent of the intensity of the excitation wavelength. Hence, the same emission spectrum is obtained regardless of the energy used to excite the fluorophor.

Fluorescence detection has three major advantages over other light-based investigation methods, like for example the ELISA:

- High sensitivity: Fluorescence techniques can accurately measure concentrations in the range of pico- and even femtomolar, once the assay is not susceptible to background effects. In contrast, absorbance measurements can reliably determine concentrations only as low as several tenths of a micromolar,
- High speed: Because the fluorescence lifetime τ_F is usually as short as 10^{-8} to 10^{-9} seconds, this process can be repeated many times before the fluorophore decomposes, for example, by oxidation.
- Safety: Safety refers to the fact that no hazardous products are generated during fluorescence detection in contrast to radioactive measurements.

Fluorophores

Fluorophores can be divided into two classes, intrinsic and extrinsic fluorophores (Figure8). Intrinsic fluorophores are those that occur naturally in the sample, for example, in proteins of the aromatic group from amino acids like, tyrosine, and tryptophan. Extrinsic fluorophores are fluorophores that are added to a sample which does not exhibit the desired properties. Labeling with fluorophores that have longer lifetimes provides features not available with the unlabeled target. An example of a common extrinsic fluorophore is dansyl chloride. An even more widely used example is fluorescein. Fluorescein is widely used because of its high quantum yields and the favorably long absorption maxima, which minimizes quenching from biological samples.

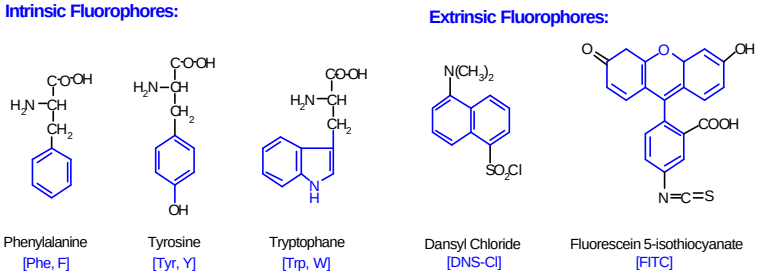

Figure 8: Intrinsic, naturally occurring fluorophores, and extrinsic fluorophores, which are added to a sample.

Choosing the right fluorescence probe for the system is the most important step within the experiment. All the key points in the later experiment, such as the wavelength or the time-resolution required, are highly dependent on the spectral properties of the fluorophore. Therefore the spectral properties of the fluorphore for example must harmonize with the filter and light source used in the detection system. If these properties and the settings of the system filters which do not fit, the data will not be reliable.

Detection

A fluorometer consists of several essential parts. Figure 9 shows the schematic design of a fluorometer. The instrument has a light source, with certain characteristics, for example, a xenon arc lamp or a laser. The excitation monochromator, selects the excitation wavelength, which is directed to the sample. The emitted light is selected by the emission monochromator and detected with a photomultiplier. The output is stored and evaluated in a personal computer.

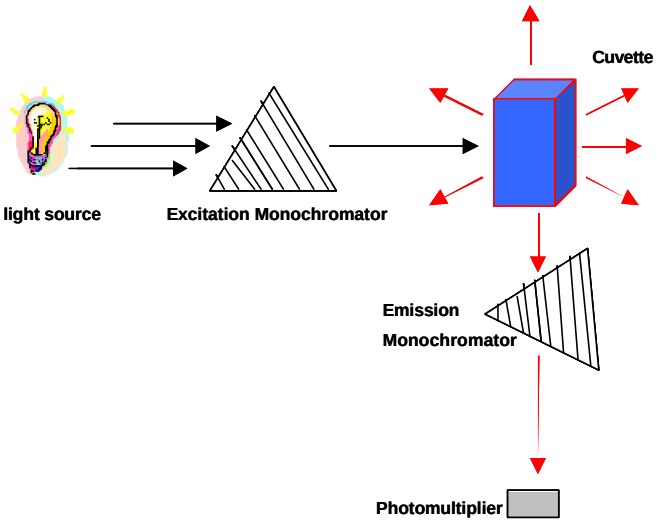

Figure 9: Schematic drawing of a fluorometer (Lottspeich et al., 1998, modified).

There are several characteristics the system has to provide besides the characteristics of the fluorophor:

- The light source must yield a constant photon output at all wavelengths important to the assay.
- The monochromator must pass photons of all wavelengths with equal efficiency.
- The monochromator efficiency must be independent of polarization.
- The detector (photomultiplier tube, PMT) must detect photons of all wavelengths with equal efficiency.

Since PMT are discussed here and later on, a short description of their function will be given.

Photomultiplier Tubes

PMT's can be regarded as a current source, where the current is proportional to the intensity to be detected. A common PMT vacuum tube consists of a photocathode, and a series of dynodes that amplifies the signal. Once a photon passes the PMT window, it will come in contact with the photocathode, a thin layer of metal film on the inside of the window. That each ach photon will cause one electron from this metal film to be ejected in the direction of the dynode cascade, the PMT window should not act as a filter. The electron causes additional electrons to be ejected. These electrons, following the dynode cascade, are even more amplified and hence, can be measured as a change in the current. The size of this pulse can be influenced through the overall

current applied to the PMT. A higher current results in a higher amplification and thus in a higher signal.

Quenching

During a quenching process the energy of an excited fluorophore is transferred to the quenching molecule (quencher) and is not given off as light. Using prompt fluorescence intensity (FLINT) methods, in HTS does not provide sufficient results even though they are sensitive techniques. In theory a single fluorescent molecule can produce thousands of photons, which leads to technologies on the single-molecule level like FCS. The greatest disadvantage of prompt fluorescence, however, is susceptibility to background effects due to light scattering or fluorescence active compounds in the sample. The intensity of fluorescence can be decreased by a variety of processes called quenching. Quenching can be divided into two major effects, referring to the source of interference:

- Chemical quenching: Chemical quenching agents compete with the fluor molecules during the process of energy transfer, causing a reduction in emitted energy. The energy is ultimately dissipated as heat from the quenching agent.
- Color quenching: Color quenching occurs when the emitted energy is absorbed or scattered by colored solutions.

Light scattering caused by the presence of certain substances in the sample, or the biological milieu itself physically causes reduced intensity. Many biomolecules, such as the previously discussed amino acids, are fluorescence quenchers. Under ideal conditions, for example, in a sample with no interfering proteins in the solution, fluorophores can be detected down to 10^{-12} M. In solutions like 1/10 serum dilution, the sensitivity drops to 10^{-8} M. Following pulse excitation of a population of fluorochrome molecules, fluorescent emissions begin instantaneously and decay rapidly. For the fluorochromes commonly used in fluorescence imaging, the lifetime and half-life (decay rate) of the excited state (and, therefore, of the emissions) is usually a few nanoseconds, but can last using metal-chelates until microseconds. Background fluorescence from components in an assay systems (i.e. serum proteins, plastics, assay buffers, etc.) is also relatively short lived, of nanosecond duration and can cause quenching.

Fluorescence Measurements

Different techniques are used to measure fluorescence. Lifetime, brightness, anisotropy (polarization), and energy transfer, are different aspects of the phenomenon fluorescence, The goal in HTS is to simplify assays as much as possible, in order to get a homogeneous and robust test. This guarantees reliability of the HTS system and the assay. At the same time, it is desirable to get as much information out of the assay as possible in order to validate the assay and to correct for interference (multiplexing).

The most common approaches to measuring fluorescence in kinase-based assays, which are resonance energy transfer, time resolved fluorescence are described following. Fluorescence anisotropy (polarization), and fluorescence correlation spectroscopy are discussed in Appendix 3.1 and 3.2. In Appendix 3.3 the ELISA is shortly described.

Resonance Energy Transfer (RET)

RET occurs whenever the emission spectra of a fluorophore donor/acceptor pair overlaps, which is shown in Figure 10.

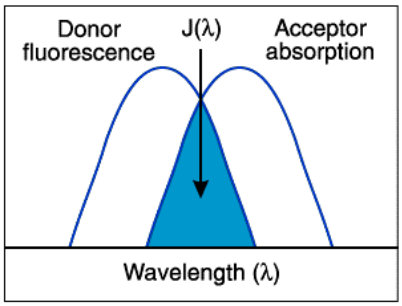

Figure 10: Spectral overlap in RET (Molecular Probes, 1999).

The acceptor does not need to be fluorescent, and the donor does not necessarily emit light during the process. There is no intermediate photon in RET and thus the common nomenclature of RET with fluorescence resonance energy transfer (FRET) is not entirely correct and RET is preferred for this work.

The rate of energy transfer, k_T, is dependent on the distance between the donor and acceptor (r), and can be described through Equation 2.

$$k_T(r) = \frac{1}{t_D} \cdot \left(\frac{R_0}{r} \right)^6 \qquad \textbf{[2]}$$

Here, τ_D is the lifetime of the donor in the absence of energy transfer and R_0 is the Förster distance, at which 50% efficiency occurs. It is clear that the rate is a function of the distance and that the transfer drops very rapidly with the factor $1/r^6$. Förster distances range from 20-60 Å[1] and can be compared with the diameter of proteins, the thickness of biological membranes, and the distances between sites of protein residues. This gives the possibility of using RET as a "ruler". At the same time, this feature limits RET to systems which are in close proximity. Another advantage is that the results are produced in the form of a ratio between excitation and emission wavelength. This reduces artifacts such as variation in volume, probe concentration, and scattering.

Time Resolved Fluorescence (TRF)

During time resolved fluorescence, the fluorophor is typically excited by a light pulse whose lifetime is much smaller than the decay time of the fluorophor. The decay is measured by a high-speed detection system in the range of nanoseconds.

Long-lived emissions, for example, from elements of the lanthanide series, are measured at a fixed time after the pulse. Short lifed decay from proteins, plastics or other quenchers, is almost totally extracted, which lowers the background signals and results in higher sensitivity (Figure 11).

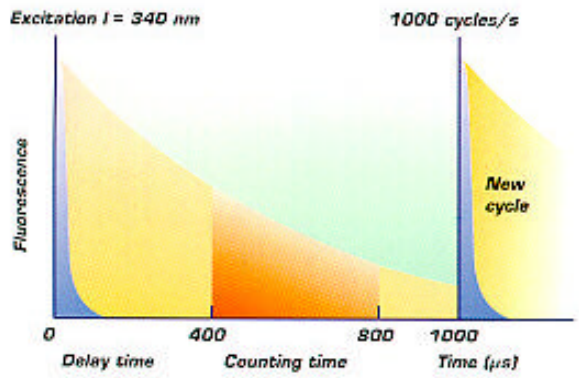

Figure 11: Principles of the measurement for TRF**.** The time resolved counting time between 400-800µs extract the short-lived background fluorescence. (Barnard, 1997).

[1] 1Å = 0.1nm = 10^{-10}m

Lanthanides

Besides the advantage of long-lifetime, it is possible by choosing lanthanides to increase R_0 (Pope et al., 1999). The lanthanide series consists of metals with a ($4f^n$) conformation. The empty 4f orbitals are shielded from the environment and are not involved in bonding. This gives these metals their unique features, such as sharp electron transitions, the ability to accept excitation energy, and their narrow-banded emission with a long-lived signal. Members of this family are terbium (Tb), samarium (Sa), dysprosium (Db), and most frequently used europium (Eu). Their lifetimes range from a few microseconds to 2 milliseconds. These features, along with their hydrophilicity and their small size give lanthanide chelates ideal features as labeling reagents. However, in order to create stabilized chelates, careful attention must be paid to the design of the labels. This means the chelates have to be fit into a surrounding cage that secures the chelate and functions as an antenna (Hemilä et al., 1997).

Radioactivity

Radioactivity is the property of certain nuclides to undergo spontaneous radioactive decay, emitting radiation in the process. All the isotopes of an element have the same number of protons but a varying number of neutrons in their nuclei. Nuclides decay over an unpredictable length of time to a more stable form. The stability depends highly on the relative proportion of the protons and neutrons in the nuclei. During the decay process, these nuclides are emitting electrically charged particles (a-, ß-particles), uncharged particles (neutrons), and electromagnetic beams (X- and ?-rays).

a**-particles:** These particles are structurally equivalent to the nucleus of a helium atom, ^4_2He and have a velocity in the range of 10^7m/s (Equation 3):

$$^{226}_{88}Ra \rightarrow {}^{222}_{86}Rn + {}^4_2He^{2+} + 4.7\ MeV \qquad \textbf{[3]}$$

b**-particles:** A β-particle is an electron emitted from the nucleus of a decaying radionuclide that possesses a neutron/proton imbalance, and can be distinguished by β^- and β^+. They consist of electrons with 99.9% of the speed of light (~10^8m/s):

b⁻-decay: The surplus of neutrons is the reason for the conversion of a neutron to a proton. The released electron (negatron) cannot stay in the nucleus and is hence emitted as a β^--particle (Equation 4).

$$^{33}_{15}P \rightarrow {}^{33}_{16}S + e^- + \bar{\nu} + 0.249\ MeV \qquad n \rightarrow p^+ + e^- + \bar{\nu} + energy \qquad \textbf{[4]}$$

b⁺-decay: With a surplus of protons, positively charged electrons (positrons, β^+-particles) are emitted (Equation 5):

$$^{22}_{11}Na \rightarrow {}^{22}_{10}Ne + e^+ + \nu + 1.83\ MeV \qquad p^+ \rightarrow n + e^+ + \nu + energy \qquad \textbf{[5]}$$

g- and x-rays: These are electromagnetic beams with very short, highly energetic wavelengths. The only difference between these rays is their place of origin. γ-rays arise directly in the nucleus, whereas x-rays are generated within electron orbitals.

The particles and rays discussed are classified as ionizing radiation because of their ability to knock electrons from the orbital shells resulting in the formation of ions. Due to their ionizing ability, these types of radiation have the potential to cause changes in living systems such as cells and biological molecules such as DNA. Strict safety standards have been established to enable scientists to utilize these valuable tools while minimizing exposure and thus potential harm.

Activity and Half-life

The activity of a radioactive source is defined as the number of nuclei decaying per unit of time. According to the SI-system, activity is measured in disintegration per second (dps) in the unit becquerel (Bq), where

$$1\ dps = 1\ Bq \qquad \textbf{[6]}$$

Another pre-SI unit still in use is the Curie (Ci), where

$$1\ Ci = 3.70 \cdot 10^{10}\ Bq \qquad \textbf{[7]}$$

Some of the more common conversions are summarized in Appendix 1.1. In order to get good accuracy, most of the counting devices measure over a period of time of 1 minute.

All the nuclides of a radionuclide decay neither at the same nor after a certain determined time. It is rather the case that the overall number of atoms, dN,

disintegrates in an exponential way during a given time, dt, proportional to the number, N, of radioactive atoms present. This rate is called half-life and can be expressed in Equation 8:

$$-\frac{dN}{dt} = l * N \qquad \textbf{[8]}$$

? is a proportional constant and includes the half-life time, $t_{1/2}$. $t_{1/2}$ is a typical constant for every radionuclide and gives the time after the activity of an sample is reduced by half (Equation 9).

$$l = \ln\frac{2}{t_{1/2}} \qquad \textbf{[9]}$$

Integrating equation 8 from 0 to t results in the disintegration law (Equation 10).

$$N = N_0 \cdot e^{-lt} = N_0 \cdot e^{-0.693\frac{t}{t_{1/2}}} \qquad \textbf{[10]}$$

For convenience, there are decay tables developed for isotopes in which the exponential part of the equation is already solved and one only has to multiply it by the activity of the sample (Appendix 1. 2). The only nucleotide used for kinase assays described in this work is ^{33}P. ^{33}P has a half-life of 25.4 days. It decays by emitting a ß$^-$-particle. The energy emitted is 0.249 MeV, which has a maximum range of 0.6mm in aqueous solutions. This energy is important to know, because it gives a reference for the ability of ionizing the environment.

Detection

Alpha, beta, and gamma rays can be detected because of their above mentioned ability to ionize matter. By applying an electric field, the positive ions start to migrate to the cathode, and the electrons are attracted from the anode. If the field strength is high enough to prevent recombination, all of them arrive at the electrodes. This charge can be detected by using a suitable electric circuit, and hence the indication of ionizing radiation is given. Commonly used detection devices on the basis of beam-dependent ionization of gas, are ion chambers, proportional counters or Geiger-Mueller counters. They differ mainly in the strength of the electric field applied.

However, if a radioactive atom decays in aqueous solution, the distance these particles will travel through the medium is limited. The distance is dependent upon the energy of the particle. When a ^{33}P-isotope decays, the particle will collide with water molecules leading to the dissipation of its energy. This highlights the problem of bringing the detector in close proximity to the sample. Considering the geometry of a microplate, in which most of the screens are performed, the particles within the liquid will be absorbed either by the liquid itself, or will be shielded by the plastic. The signal has to be magnified and/or translated in order to get a measurable result. Detectors based on liquid scintillation counting (LSC) solve this problem. They are based on the discovery that organic chemicals emit fluorescent light when bombarded by nuclear radiation. This signal is converted to photoelectrons and multiplied in a photomultiplier tube (PMT).

Quenching

This phenomenon is similar to the quenching discussed in the fluorescence chapter. Here, the quenching occurs in the same way either by the presence of one or more quenching chemicals in the cocktail (chemical quenching), or by a colored substance that comes with the sample (color quenching). The points of interference of chemical and color quenching are illustrated in Figure 12.

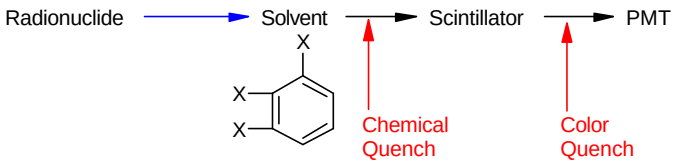

Figure 12: Liquid scintillation process (L'Annunciata, 1998).

Two other phenomena can interfere with the counting: crosstalk between different wells and natural background. In order to avoid crosstalk between different wells, white, reflective, opaque plates should be used. Here, the light generated by the sample is reflected to the PMTs and no optical crosstalk occurs. Natural background is the phenomenon of natural radioactivity. Various methods, such as internal standards, sample spectrum, external standards, and the direct DPM method can correct background. Each has its distinct advantages and disadvantages, and a researcher should choose the method according to the problem (L'Annunciata, 1998).

Liquid Scintillation Counting (LSC)

During a liquid scintillation counting process a radioactive sample is placed in the scintillation cocktail. This cocktail consists of an organic solvent and an organic scintillant. The solvent molecules are in a high concentration and can easily accept the energy released from the nuclear decay. The activated solvent transfers its energy to the fluor. Thus, energy at a certain wavelength is emitted. The intensity of the light flashes is proportional to the number of decays. A photomultiplier tube detects the photons emitted by the scintillant.

The counting efficiency of an LSC is dependent on the original energy that is generated through the decay as well as on the degree of quench in the sample. The higher the energy is during the decay, the brighter the flash. For example, ^{33}P as mentioned is a nuclide with a medium high energy, 0.249 MeV, whereas ^{32}P decays with 1.71 MeV. Thus the ^{32}P-β-particle would generate a light flash with approximately seven times higher intensity than the ^{33}P-particle. As a result, the light intensity reflects the original decay energy, and the number of light flashes is proportional to the number of nuclear decays in the sample. Approximately 10 photons are generated per keV of β-particle decay energy. A liquid scintillation counter must be able to detect light flashes in the sample and determine their number and intensity. It must be able to process the flashes per minute into counts per minute (cpm). Further, the software should be able to handle quench corrections.

The heart of a liquid scintillation counter are its detection and quantification components. Here are the three basic parts: the detector(s), the counting circuit, and the sorting circuit (Figure 13). As discussed, the PMTs convert the light photons to electrons which are amplified and generate the electronic signal. The two PMTs in Figure 13 permit a coincidence light detection, which is necessary to extract background from true nuclear events. During a nuclear decay event, light is emitted equally in all directions, which results in a multiphoton event in the scintillant.

Signals that arrive simultaneously on both PMTs are counted. Background events are usually of a single photon event nature, and only one PMT can detect it in the resolving time. Such signals are not counted. The summation circuit must reassemble the two coincidence signals into an individual signal. Subsequently, the signal is amplified and

sent to an analog-to-digital converter. The digital pulses are finally sorted and plotted in the spectrum analyzer.

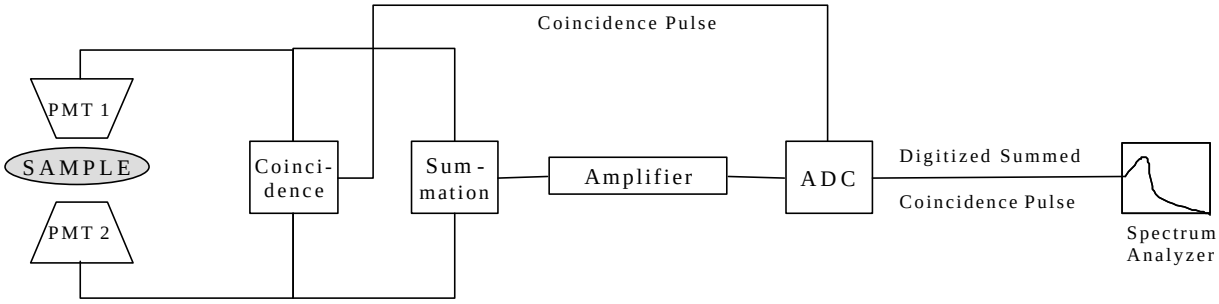

Figure 13: Schematic diagram of the detector components of a liquid scintillation analyzer (L'Annunziata, 1998, modified)

A liquid scintillation counter can also be used to measure fluorescence when radioactive nuclides are absorbed onto or in close proximity to the surface of a plastic or glass scintillator (solid scintillator). Solid scintillation counting uses a solid inorganic scintillator (e.g. yttrium silicate) to produce the light flashes. The different approaches need distinct sample preparation procedures.

Summary

The overall goal of HTS is the efficient discovery of high quality lead structures by reducing time and cost and increasing information gained. Thus the detection methods were evaluated in regard to their key features for HTS, and summarized in table 2.

RET, TRF, and FP all use methods to decrease quenching within the assay. The ratio of the two wavelengths in RET and TRF and the ratio of the excitation parallel and perpendicular to the polarized light in FP, are performed. This reduces the background due to variations in the well volume or concentration of the fluorophore.

Table 2: Summary of fluorescence-based assays.

	RET	TRF	FP	FCS	ELISA	LSC	Filter-binding	SPA	Solid Phase
Homogeneity	+	+	+	+	-	-	-	+	+
Volume Dependence	+	+	+	-	+	+	+	+	+
Single molecule sensitivity	-	-	-	+	-	-	-	-	-
Multiplexing	-	-	-	+	-	-	-	-	-
Components to be labeled	2	2	1	1	1-2 antibody 1 enzyme	0	0	bead	plate
Miniaturization	1536	384	1536	1536	96	96	96	384	384

FP and FCS both need only one fluorophore label. FP functions best if the differences between labeled and unlabeled substrate are big. Small differences in size cannot be

efficiently detected. FCS has the distinct advantage that its sensitivity goes down to the single molecule level. In addition, the readouts can be multiplexed, meaning different readout methods can be applied simultaneously. However, FCS can only be operated within a narrow range of concentrations. Saturation in the observation field becomes limiting at higher-than-optimal concentrations, while slow data accumulation becomes limiting at lower-than-optimal concentrations. Thus, the concentration has to be carefully optimized.

The ELISA, in contrast, is a heterogeneous method with its necessary washing steps, which increases the complexity of the HTS system and, therefore, the reliability of the HTS system decreases. Moreover, depending on the ELISA type, 1-2 antibodies and an enzyme for labeling are needed. The major drawbacks of the ELISA against the fluorescence-based methods are the heterogeneous setup, lower linear range, and lower sensitivity. Another issue is the size of the substrate. For the sandwich ELISA, the substrate has to be big enough for the binding of two antibodies, which limits the use of peptides as substrates. Overall, the ELISA is not the method of choice for high-throughput screening.

LSC and filter-binding assays are, due to their setup, highly volume dependent and are therefore bound to the 96-well format. Within both assays unbound isotope must be removed through cumbersome separation steps, which increases the complexity of the HTS setup.

The similar SPA and FlashPlate approaches remove the separation steps by converting the assay format into a proximity based one. Here, only radionuclides in proximity to the scintillant generate the signal. This also enhances the possibility for miniaturization.

Miniaturization plays an important role, since it reduces the volume and therefore the use of reagents, which saves money and time. This is the major drawback of radioactive-based assays over fluorescence-based ones. Scaling down an LSC from 100 µl by a factor of 100 (1 µl), it would take 100^2 (10,000) times longer to count the plate with the same accuracy. This means a 96-well plate read with one second counting time per well will take 96 second to read. In contrast, 1 µl in the 1536-well plate, would take 1,536x10,000 seconds (six months) to read (Burbaum, 1998).

Fluorescence-based assays are far more scalable, far beyond the 1536-well format, and are thus preferred. Further advantages of fluorescence-based assays are that all methods are very sensitive, nanomolar concentrations, or single molecules in the case of FCS can be measured. Each fluorophore can be excited a thousand times, which amplifies the signal. Assays can provide multiple readouts (FCS), are homogeneous, and robust. Avoiding the handling of radioactive substances, decreasing radioactive waste and contribution to laboratory safety are other key features. However, the major disadvantage is their susceptibility to background effects. Radioactive assays in contrast are independent of the use of antibodies and labeling steps, which makes them an ideal instrument for characterization and assay development of novel and poorly characterized enzymes. In addition, enzymes with higher pH optimum can be assayed. This makes them a valuable assay format for characterization and assay.

MATERIAL AND REAGENTS

All the assays used during this work can be divided in two distinct steps, the kinase reaction, and the subsequent detection of the phosphorylation stage of the substrate after kinase activity. Therefore this section is divided in three topics. First all material and reagents are summarized necessary for the phosphorylation reaction within the MAP kinase assays, second the material and reagents needed for the detection of radioactivity based signals, and third the fluorescence based ones are described. All supplier are noted beside the materials or reagents, and in Appendix 4 a list of all mentioned suppliers is attached.

Raf-1 and Target Reactions

Raf-1 Kinase Cascade Assay Kit, Upstate Biotech

MAP KKK, Raf-1 activated, Upstate Biotech

MAP KKK, Target, inhouse

MAP KK, MEK1 inactive, Upstate Biotech

MAPK, ERK2, inactive, Upstate Biotech

Biotinylated MBP peptide, SynPep

Biotinylated MBP full length, Upstate Biotech

Reaction Buffer: $MgCl_2$, Sigma-Aldrich

EGTA, Sigma-Aldrich

DTT, Roche Diagnostics

Brij35, Sigma-Aldrich

β-phosphoglycerol, Sigma-Aldrich

P105, three different constructs, provided from our collaborator

IκBα, Boston Biologicals

ATP, Amersham

96-well microplates, black, Costar

384-well microplates, black, Costar

Dimethyl Sulfoxide, DMSO, Sigma-Aldrich

Radioactive Assays

White opaque multiscreen filter plates, Millipore

96-well microplates, round bottom wells, flexible used with cassette, Wallac

Isoplate 96-well, white opaque, clear flat bottom plate, Wallac

Vacuum filter unit, Millipore Vacuum control

[γ^{33}P]-ATP, (3000Ci/mmol, 10mCi/ml), NEN

H_3PO_4, Sigma

Scintillation cocktail, Opti Phase 'Super Mix', Wallac

Beta-Counter, MicroBeta 1450, Wallac

Plate seals, Wallac Millipore Multiscreen liner, Wallac

Serine/Threonine kinase SPA assay kit for use with [^{33}P], Amersham Pharmacia Biotech

Dulbecco's Phosphate Buffered Saline, GibcoBRL (Life Technologies)

Homogeneous Time Resolved Fluorescence

EDTA, Sigma-Aldrich

RY01-Eu-labled, Antibody: Upstate Biotech / Label: CisBio

Revelation Buffer: HEPES, Sigma-Aldrich

KF, Sigma-Aldrich

Tween 20, Bio-Rad

BSA, Amersham Pharmacia Biotech

SAXL, Prozyme

Time-resolved fluorescence reader, Discovery, Packard Instruments

96-well, black, opaque microplates, Costar

384-well, black, opaque microplates, Costar

Note: General laboratory equipment like pipettes, pipette tips, centrifuges, tubes, etc. are anticipated and therefore not mentioned. All container and tubes used need to be labeled clearly to avoid mix ups. Enzymes have to be thawed and handled on ice. Stroage of enzymes is @ -80°C.

Enzymes are thaw and temperature sensitive and need to be thawed, and kept on ice as long as possible to avoid loss of activity. Larger amounts need to be aliquoted while keeping the tubes on ice and stored @ -80°C in a freezer.

METHODS

Enzyme Assays

Two distinct methods were used, the MAP kinase cascade using the activated Target with inactive MAP KK, and inactive MAP K. And a direct assay, using only the Target with substrate.

MAP Kinase Cascade

The cascade module was designed to give maximum flexibility during assay development, and the later setup on the robot in the HTS group in Germany. All reagents were added separately to make changes in concentrations or exchange of reagents easier, and to provide possible variability within the assay. This was especially true for the enzymes of the cascade, which could then be independently titrated. The basic module was performed as follows:

- 10 µl reaction buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EGTA; 2 mM DTT; 0.01% Brij35; 5 mM β-phosphoglycerol) were mixed, in a well of a 96-well plate, with
- 10 µl enzyme mix (MAP KKK and/or MAP KK and/or MAP K) and
- 10 µl substrate (MBP full length or MBP peptide)
- 10 µl ATP to start the reaction (mixed with [γ^{33}P]-ATP in the radioactive assay)
- Incubation
- Stop (reagent depending on the assay type)
- Signal development (either radioactivity or fluorescence based)

The reaction buffer, Tris-HCl pH 7.5, was mainly designed to resist a change in hydrogen ion concentration. However additional reagents were added: 50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EGTA; 2 mM DTT; 0.01% Brij35; 5 mM β-phosphoglycerol. Magnesium is necessary for the building of the active Magnesium/ATP complex (see Page 7). EGTA is added to control heavy metal ion impurities. These impurities are due to the purification methods used to obtain the enzymes. EGTA was chosen because it has a low affinity for magnesium and therefore affects the Mg-concentration on a low base. DTT prevents the

uncontrolled oxidation of thiol groups in proteins. The non-ionic detergent Brij35 is used to solubilize proteins and to prevent non-specific binding. Because of its non-ionic nature, Brij will not denature proteins. Finally β-phosphoglycerol is added as an inhibitor of phosphatases in order to avoid dphosphorylation of proteins.

The cascade consists of three enzymes MAP KKK(the target respectively Raf-1), MAP KK (MEK 1), and MAP K (ERK 2). The enzyme mix was freshly prepared before each experiment*. For this reason aliquots of MAP K and/or MAP KK and/or MAP K were thawed on ice and mixed in the needed concentrations and combinations. Depending on the setup of the assay. The cascade reactions were carried out in black 96-well microplates. 10 µl of the enzyme mix and 10 µl substrate dissolved in reaction buffer were added to each other in one well, with 10 µl reaction buffer. If necessary, the cascade could be divided into different fractions. For example, 10 µl titration of MAP KKK could be mixed with 10 µl of the mix from MAP KK / MAP K. The 10 µl buffer could be exchanged either for titrations within the cascade, or for the later addition of compound in the screening. The reaction was started by addition of ATP, and stopped after the appropriate incubation time. The stop solution varied within the different assay types. The signal was developed and recorded according to the assay type, and analyzed with Microsoft Excel. Therefore the data was transfered to a PC using the internal computer network or by using diskettes, uploaded into Excel and depending on the type of assay processed by using the chart wizard and the paste function command.

Direct Kinase Assay

The direct kinase assays were only used within the ^{33}P filter-binding assay. The only difference between the direct kinase assay and the cascade assay was that the direct assay uses the substrate directly, without the need of the two enzymes, MAP KK and MAP K described above. The setup of the assay was therefore similar to the cascade setup. The reagents besides the enzymes, like buffer, substrate etc., in the cascade were used the same as described above.

* For concentration of these and other reagents, see legends of the figures or the text.

Radioactivity Based Assays

Two radioactivity-based assays were used in this work, a filter-binding assay and the scintillation proximity assay (SPA). Both are using ^{33}P as isotope, which is incorporated into the substrate during the phosphorylation reaction catalyzed by the kinase.

^{33}P Filter-binding Assay

Filter counting, or solid phase counting is, in contrast to liquid scintillation counting, heterogeneous counting. The main difference between heterogeneous and homogeneous counting is the sphere in which the particles can travel after a decay event. In homogeneous counting, the particle is completely surrounded by the solvent and can travel in any direction it wants. This sphere can be described as a globe with surface area $4\pi r^2$ whereas in heterogeneous counting, the particle is absorbed into the surface of a filter or membrane. Here, the particles are no longer free to radiate in any direction. Therefore, the emitted light can only occupy the surface area of a hemisphere, $2\pi r^2$ (Figure 14).

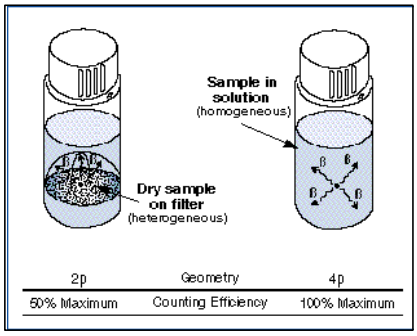

Figure 14: Sample counting geometries encountered in liquid scintillation analysis. (Amersham, 2000)

Filter counting can be a relatively simple technique where the sample is isolated or collected on a filter-microplate and usually dried. The filter binds the protein but not the ATP. Scintillation cocktail is added and, after ensuring that the filter is completely wet, the microplate is counted.

The difficulty in counting on filters or other solid supports is that when the sample is immersed from the solid phase into the cocktail, three situations may develop:

- The sample may remain bound to the filter or solid support.

- The sample may be partially eluted by the cocktail, which should be avoided. Partially eluted samples are counted in a 4π sphere geometry, whereas the bound ones are counted in a 2π. Hence the measurements are not reproducible.
- The sample may be completely dissolved in the cocktail.

Repeat counting of the sample, over several hours, will determine if an equilibrium situation has been reached. This is evidenced by a constant count rate over time. If the sample is insoluble (no elution), the efficiency and reproducibility of counting will depend on the magnitude of the ß-energy, the nature of the filter or solid support, its orientation in the vial, and the size of the sample molecule. If the sample is completely dissolved or eluted into the cocktail (complete elution), counting considerations will be similar to those of solubilized samples, where a true homogeneous state is obtained. Other factors which affect counting are the presence and composition of the sample precipitate and the amount of sample that becomes soluble in the cocktail.

In practice there are a number of filter types that can be used to isolate or collect various sample types for LSC analysis. The choice of filter type will depend upon both the nature and particle size of the sample. The filters and membranes are made of materials that range from glass fibers or cellulose nitrate to Teflon or normal paper.

Within the kinase approach γ-^{33}P-ATP is used as a tracer and a protein or synthetic peptide as substrate. Kinase activity will incorporate the γ-^{33}P in the substrate. After the phosphorylation reaction is stopped, free and bound radionuclide are separated by a filtration step, and radiolabeled protein or peptide bound to the filter is then analyzed in a counting device.

The filter-binding assay is independent of the use of antibodies and reagents to be labeled, which makes it ideal to determine direct substrates, and for use with targets where no antibodies are commercially available. This makes this assay type a valuable tool in assay development. In this work the filter-binding assay was used to determine if there might be a direct substrate and for the implementation of the cascade. The basic assay was performed as follows:

- 10 µl reaction buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EGTA; 2 mM DTT; 0.01% Brij35; 5 mM β-phosphoglycerol) were mixed, in a well of a 96-well plate, with
- 10 µl enzyme mix (MAP KKK and/or MAP KK and/or MAP K) and
- 10 µl substrate (MBP full length or MBP peptide)
- 10 µl ATP / [γ^{33}P]-ATP - mix (1.25 µCi/well)
- Incubation 2hrs
- Stop with 100 µl stop mix (5 mM ATP / 75 mM H_3PO_4)
- Transfer 120 µl to Millipore 96-well filter plate
- Incubate 20 minutes
- Wash 10 times with 75 mM H_3PO_4
- Dry filter
- Remove underdrain
- Attach adapter and put into plate holder
- Add 25 µl scintillation cocktail
- Seal plate
- Incubate 1 hour
- Count in Wallac β-counter

The phosphorylation reactions were performed in 96-well round bottom flexible plates used with a cassette to shield the individual wells from each other and avoids crosstalk. 10 µl cascade mix* and 10 µl substrate were added to 10 µl reaction buffer in the plate. To start the reaction, 10 µl hot ATP mix (50 µM cold / 40 nM γ-^{33}P; 1.25 µCi/well) was added. The reaction was allowed to proceed for two hours, and after that stopped with 100 µl 5 mM ATP / 75 mM H_3PO_4. 120 µl of this solution was transferred in prewetted, white opaque filter plates on the vacuum control unit and was incubated there for 20 minutes. After the vacuum was opened and the sample filtered, the sample was subsequently washed 6 times with 150 µl, 75 mM, H_3PO_4. The filter with the sample was dried and the underdrain of the plate was removed. The plate was placed into adapters, 25 µl of scintillation cocktail

* For concentration of these and other reagents, see legends of the figures or the text.

were added, and sealed. For equilibration, the sample / scintillation mix was incubated for 1 hour, and read in a Wallac 1450 MicroBeta liquid scintillation counter. The data were analyzed in Microsoft Excel in the same fashion as described above.

Scintillation Proximity Assay© (SPA)

SPA, Amersham Pharmacia Biotech, is designed to avoid the separation step between bound and unbound radionuclide. Glass or plastic solid scintillator microspheres coated with streptavidine are used as acceptor, and isotope bound to a biotinylated substrate is used as energy donor. The scintillant is incorporated into small fluomicrospheres. These microspheres or 'beads' are derived in such a way as to bind specific molecules.

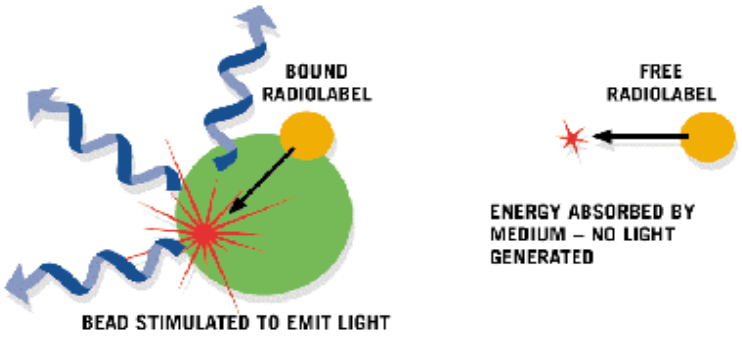

Figure 15: Principle of SPA. Radiolabel bound to the bead is close enough to excite the incorporated scintillant. Free radiolabel cannot excite the scintillant (Amersham, 2000).

If a radioactive molecule, like a ^{33}P-phosphorylated substrate is bound to the bead, it is brought in close enough proximity that it can stimulate the scintillant to emit light (Figure 15). Unbound radioactivity is too distant from the scintillant to emit light and the energy released is dissipated before reaching the bead, and is therefore not detected.SPA was chosen as the method to perform the follow-up assays in order to determine the specificity and potency of possible hits. SPA should provide a homogeneous and direct assay format, as well as detection without the need for an antibody. The basic experiment, based on the Amersham Pharmacia Biotech kit, catalog # RPNQ 0200, was performed as follows:

- 10 µl reaction buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EGTA; 2 mM DTT; 0.01% Brij35; 5 mM β-phosphoglycerol) were mixed, in a well of a 96-well plate, with
- 10 µl enzyme mix (MAP KKK and/or MAP KK and/or MAP K) and

- 10 µl substrate (Bio-MBP-peptide)
- 10 µl hot ATP mix (0.5 µCi/well)
- Incubation
- Stop with 200 µl stop solution (50 µM ATP, 5 mM EDTA, 0.1% Triton X-100, 0.5 mg PVT beads in phosphate buffered saline)

The assay was carried out in 96-well, white opaque, clear, flat bottom plates, in a final volume of 40 µl of reaction buffer. 10 µl enzyme mix* diluted in reaction buffer, and 10 µl biotinylated MBP-peptide were added to 10 µl buffer in the plate. To start the reaction, 10 µl of hot-ATP were added and run the defined incubation time. The hot-ATP was a mixture from non-radioactive and a radioactive marked [γ-^{33}P]ATP. The activity of the isotope had to be 0.5 µCi/well, according to the assay protocol from Amersham. Thus the specific activity had to be calculated on the basis of the calibration date of the isotope batch. The mixture of hot / cold ATP was necessary to obtain a certain specific activity. The plates were sealed with a lid and incubated at room temperature for 2 hours. A volume of 200 µl stop buffer, containing 50 µM ATP, 5 mM EDTA, 0.1% Triton X-100, and 0.5 mg polyvinyl-toluene (PVT) beads in phosphate buffered saline (without Mg^{2+} and Ca^{2+}) were added. The addition of the ATP and Triton helped to minimize unspecific binding of hot ATP to the beads, whereas EDTA chelated all metal ions and subsequently stopped the reaction. The beads were allowed to settle for 6 hours to overnight. The plate was counted in a Wallac 1450 MicroBeta liquid scintillation counter. The data were analyzed in Microsoft Excel.

Time Resolved Fluorescence

HTRF®-Homogenous Time-Resolved Fluorescence, Packard Instruments, uses europium as a donor and a phycobilliprotein from red algae or cyanobacteria as an acceptor fluorophore. Phycobiliproteins are disc-shaped light harvesting protein-pigment complexes. They contain mainly phycocyanin, phycocerythrin, and the here used allophycocyanin. They function like an antenna and absorb light between 550-650 nm and transfer the absorbed energy in the reaction center of the photosynthesis complex (Schlegel, 1992). Europium is surrounded by a protecting cryptate cage, which also functions like an antenna, and the signal-amplifying

fluorescent allophycocyanin. A kinase assay performed with this technology can be divided into two distinct steps, the initial enzyme assay, and the signal development. In the initial step, the kinase of interest, ATP, and a biotinylated substrate are assayed. After stopping the reaction, the revelation mix consisting of an anti-phospho-antibody, labeled with europium, and the streptavidin-labeled allophycocyanine (SAXL) are added. Kinase activity can be monitored according to the amount of phosphorylation detected by the antibody, and the allophycocyanine bound to the biotin label of the substrate.

When two biomolecules labeled with the fluorophores are in proximity (or bound), the Eu^{3+}, surrounded by the protecting cryptate cage, absorbs the energy of the exciting N_2-laser at 337 nm. It then transfers its energy by nonradioactive, direct energy transfer (no light is emitted) to the SAXL, which emits light at 665 nm with a slow decay time (Figure16). The signal is measured over a time period of 50 to 400 µs. Prompt decay background fluorescence is eliminated because the XL665 is measured after 50 µs time delay (time resolved).

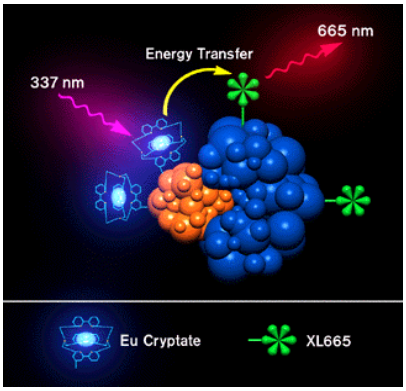

Figure 16: Measuring biomolecular interaction with fluorescence resonance energy transfer (FRET). Eu^{3+}, surrounded by the protecting cryptate cage, absorbs the energy of the exciting laser at 337nm, and transfers its energy directly (no light is emitted) to the XL665, which emits light at 665nm with a slow decay time (Packard Instruments, 1997).

As HTRF requires no separation steps, free (unbound) XL665-labeled biomolecules and free EuK-labeled biomolecules are also present during the measurement. Free SAXL, which also decays at 665nm, can be disregarded because it has a prompt decay and is extracted by the time delay. Free EuK is an important part of the HTRF measurement. It exhibits a slow decay at 620 nm and is measured after a time delay. The EuK signal is used as an internal reference to calculate the ratio of the emission signals. This ratio is built out of the actual long decay signal at 665 nm, and the unbound EuK signal at 620 nm. The ratio is used to compensate for color quenching, turbidity and variations in volume and excitation energy. The

energy transfer process between EuK and XL665 occurs with 50% efficiency at a distance of 90 Å. The efficiency is inversely proportional to the distance ($1/r^6$). Both EuK and proximal bound XL665 are measured simultaneously in black, low fluorescent plates, however the ratio (665/620) is the primary readout. It is multiplied by an arbitrary number, 10,000, simply to get numbers that are easier to use.

The homogeneous and robust setup along with the ability to signal amplification made HTRF to the detection format chosen for the primary screen in the HTS unit in Germany. The basic assay was performed as follows:

- 10 µl reaction buffer (50 mM Tris-HCl pH 7.5; 10 mM $MgCl_2$; 1 mM EGTA; 2 mM DTT; 0.01% Brij35; 5 mM β-phosphoglycerol) were mixed, in a well of a 96-well plate, with
- 10 µl enzyme mix (MAP KKK and/or MAP KK and/or MAP K) and
- 10 µl substrate (MBP full length or MBP peptide)
- 10 µl ATP
- Incubation
- Stop with 10 µl 0.5M EDTA, pH 7
- Add 150 µl revelation buffer with RY01 and SAXL
- Incubate several hours to overnight
- Read plate in discovery

The phosphorylation reactions were performed in 96-well black opaque plates in a final volume of 40 µl. 10 µl cascade mix* and 10 µl substrate, MBP full length or MBP peptide depending on the setup, were added to 10 µl reaction buffer in the plate. To start the reaction 10 µl ATP was added. The addition of 10 µl 0.5 M EDTA, pH 7, stopped the reaction after appropriate incubation. 150 µl of HTRF reagent mixture (8 µl /ml RY01, 26 µl /ml SAXL) diluted in revelation buffer were added. The revelation buffer contained 50 mM HEPES, 400 mM KF, 0,01% Tween 20, and 0.1% BSA at a pH of 7. HEPES adjusted the pH for the antibody/Eu chelate, Kaliumfloride stabilized the chelate, Tween 20 solubilized proteins, and

* For concentration of these and other reagents, see legends of the figures or the text.

BSA avoided unspecific binding. The solution was incubated overnight and read in the time-resolved fluorescence reader (Discovery; Packard Instruments). The data were analyzed in Microsoft Excel as discussed above.

RESULTS AND DISCUSSION

ASSAY DEVELOPMENT

Substrate

As discussed on Page 11 the first step in the assay development is the determination of the substrate. The optimal situation would be to have a direct substrate, however in the case of the MAP KKK discussed direct in vivo or in vitro substrates are not known. The first experiment should clarify if the believed substrate cited in the literature is working. Two substrates were tested using a ^{33}P-filter-binding assay. The first substrate was provided from our collaborator in three different constructs. This is the believed in vivo substrate to our target. All three constructs named S1 to S3, and another substrate IκBα were assayed using a ^{33}P filter-binding assay. The concentrations of all four substrates were adjusted to 1.5 mg/ml and titrated against three different Target constructs named T1 to T3. The titration of the substrates started with 15 ng/well and was titrated by 1/3 two times. The last well contained zero substrate. Three Target constructs were assayed each at a concentration of 50 ng/well. The assay should answer two questions. First can one of the four substrates be used as direct substrate in the mass screening and second which one of the Target constructs shows the most promising activity. 10 µl of the Target were mixed with 10 µl substrate. The reaction was started by addition of 10 µl 50 µM cold and 1.25 µCi/well γ-^{33}P.

Figure 17 shows that all four substrates showed a relatively low signal, around 900 dpm after background subtraction. The Target construct # 1 showed the highest activity and was chosen for subsequent investigations. None of the substrates gave a satisfactory result. Consequent the MAP kinase cascade described on Page 8 was investigated if it could be adapted to the Target.

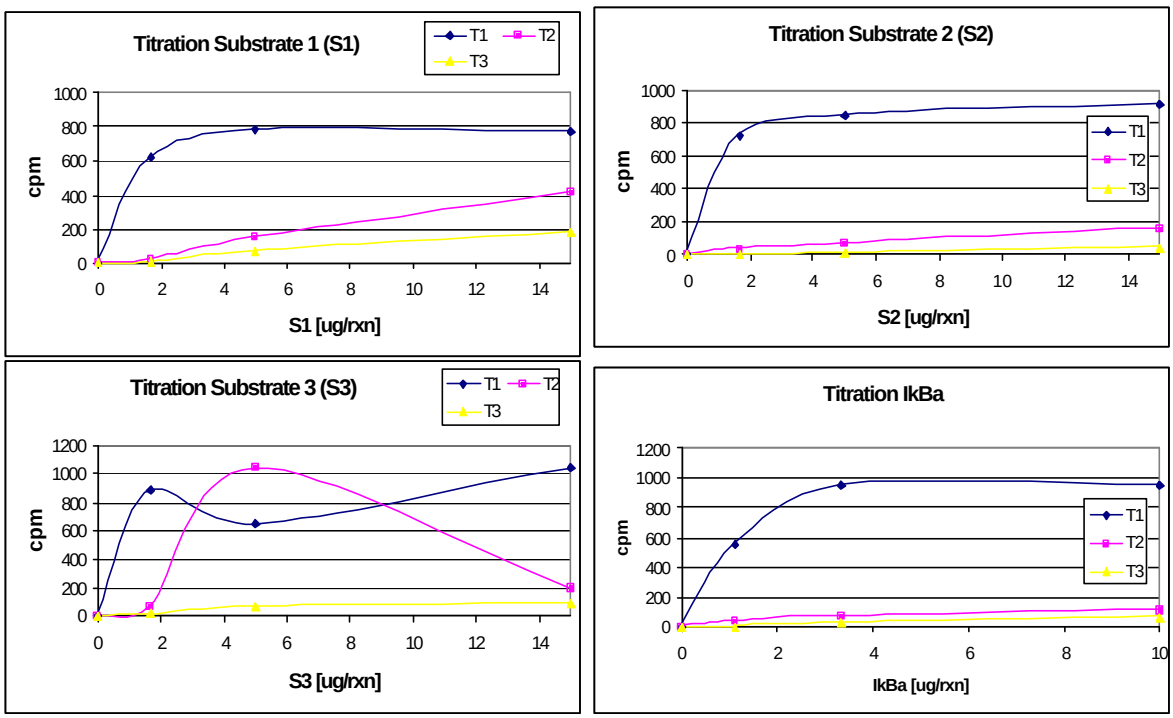

Figure 17: Substrate identification: Three constructs of the same substrate S1 to S3 and I?Ba were assayed with three different Target constructs, T1 to T3. The substrates were titrated against a fixed concentration of the Target constructs.

Implementation of the Cascade

As discussed the blueprint for our target is Raf-1. Raf-1 is well described in the literature, for example, Alessi et al, and cascade assay kits are commercially available. As a platform for assay development, the ^{33}P Raf-1 Kinase Cascade Assay, Upstate Biotechnology, catalog # 17-172, was chosen.

To begin the Raf-1 cascade protocol from Upstatebiotech was used to see if the cascade would work with our target. It was tested on both Raf-1 and our target by using a ^{33}P filter-binding assay. Inactive MAP KK and MAP K were each assayed at a starting concentration of 250 ng/well, the target at 50 ng/well and Raf at 0.2 U/well. As a substrate, full length MBP was used at a concentration of 1 µg/well. ATP was used with 50 µM cold and 1.25 µCi/well γ-^{33}P. The assay was performed with (+) and without (-) substrate in the reaction, and the cascade was broken down into its single steps. Active MAP KKKs (Target respectively Raf) were assayed with a mix of the inactive MAP KK / MAP K, with inactive MAP K, and only active MAP KKK with substrate in order to identify unspecific activity within the cascade components. The reaction was stopped after 30 minutes by adding 100 µl

stop mix (5 mM ATP / 75 mM H_3PO_4). The filter binding was performed as described above, and duplicates were performed.

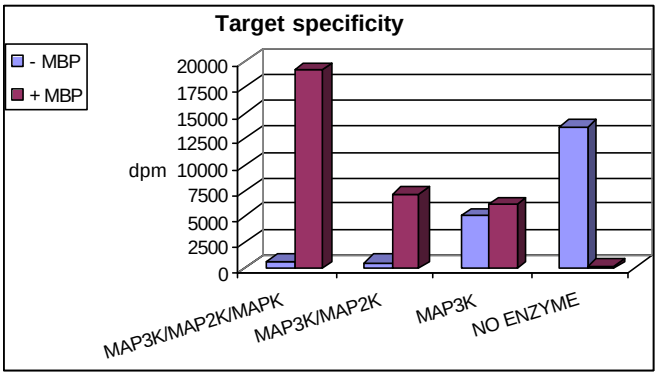

Figure 18: a) Test on Target specificity. All enzymes involved in the cascade were incubated with the target to see whether or not the Target is active on them or not.

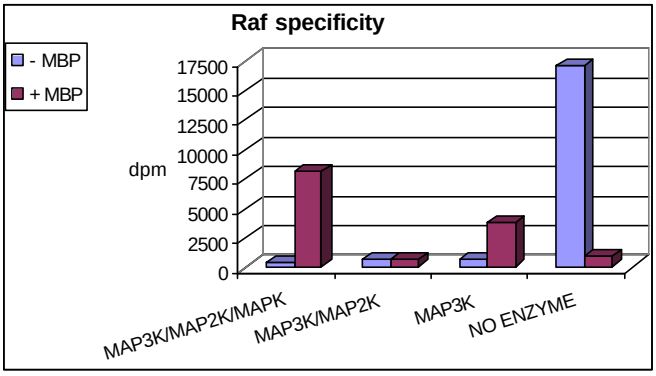

Figure 18: b) Test on Raf specificity. All enzymes involved in the cascade were incubated with Raf to whether is active on them or not.

The obtained data was rather noisy (Figure 18 a and b). This was due to the unsuccessful washing step. Unbound radioisotope in several wells was not successfully removed. The background wells without MAP KKK were especially heterogeneous. Thus, this test is not 100% reliable. However, it was clear that the cascades were working. Both full cascades showed a relatively good signal. In the target cascade a high signal in the wells with MAP KKK / MAP KK and only MAP KKK was noted. This is due to the known low activity of the target to MBP (Alessi et al., 1995).

After determining that the cascade format was working, the next step was to transfer the cascade into the actual HTS assay format, HTRF. The first HTRF cascade experiment was performed in a manner similar to the previous filter-binding assay. The Raf-1 and Target cascades were incubated with (+) and without (-) substrate. At this point, the biotinylated MBP-peptide (biotin-ahx-KNIVT^{P}PRTPPPSQGK) was introduced into the cascade format. Therefore, the

activated MAP KKKs were incubated directly with and without peptide to make sure that they would not use it directly as a substrate.

For the first shot, the concentrations of the enzymes were doubled. The MAP KK and MAP K concentration were each 500 ng/well, Target was 100 ng/well and Raf 0.4 U/well. ATP was used at 250 µM and the peptide at 3 µM. This reflects the concentration of SAXL used in the assay. The assay was performed by running duplicates. The reaction was stopped after 30 minutes by addition of 10 µl 0.5 M EDTA, pH 7. 150 µl revelation mix, 8 µl/ml RY01, 26 µl SAXL/ml diluted in revelation buffer, was added. RY01 detects the phosphorylation within the MBP substrate. The streptavidine labeled allophycocyanin, SAXL, binds to biotynilated MBP. Once there is a antibody substrate complex bound to SAXL the europium and the allophycocyanin are in close proximity and RET can occur. The plate was read after 15 minutes.

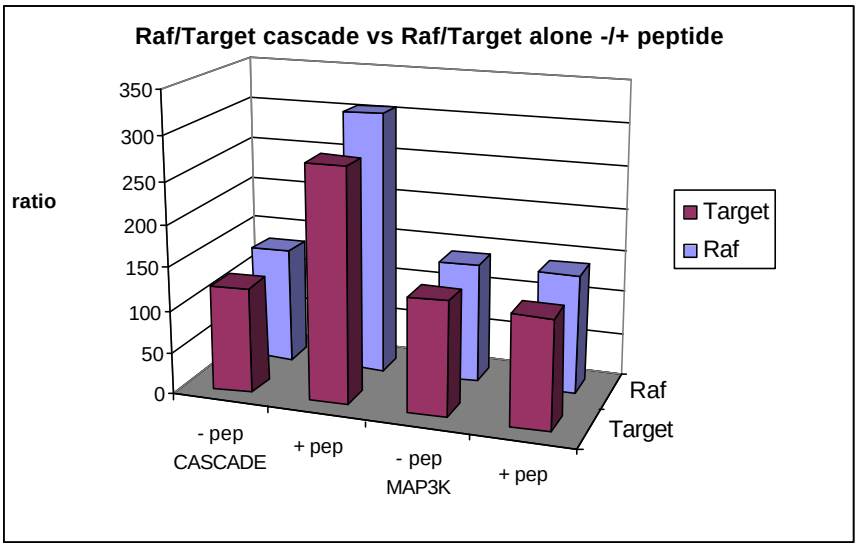

Figure 19: Raf vs Target cascades, assayed with and without substrate; MAP KKKs assayed directly with the substrate.

The signals obtained for the duplicates were tight. Figure 19 shows the average of the values. Both cascades gave similar results: signal in the complete cascade, no signal in the cascades without peptide. The MAP KKKs alone with and without substrate showed exactly the same result, and confirmed the peptide as a poor direct substrate for MAP KKKs. However, the signal was very low. This was due to the short incubation time after addition of the revelation mix; longer incubation enhanced the signal (data not shown). Therefore, 2 hours incubation time were subsequently used. The background ratio was around 120. This background is not

a true background; it is more of a zero line. The background ratio is calculated from the ratio, which is obtained by dividing the signal of the total amount of europium in the sample through the real signal from europium and SAXL in close proximity (see HTRF Page 40). Overall, it was obvious that the cascade would also work in this format. The next step was to optimize the concentration of each enzyme in the cascade.

Effect of enzyme concentration

The biggest advantage of the cascade format, besides the already mentioned known substrate and the availability of the antibody, is its ability to amplify the signal, initiated by the MAP KKK. This saves precious enzyme, and reduces the capacity for its purification. In order to get the best possible signal, the enzymes in the cascade have to be optimized relative to each other such that the signal produced by the target has a linear output at the end. MAP KK and MAP K could therefore be used at saturation levels, which would achieve the highest possible signal. To determine the optimal concentration of each enzyme in the cascade, several titrations of each enzyme were performed. This allowed determination of where the linear range of the target would end. The concentration shortly before the end of the linear range should be chosen for MAP KK and MAP K. It was necessary to find the lowest possible concentration with the best possible signal for efficient use of enzyme.

To start the optimization, the Target was titrated against the MAP KK / MAP K mix. 100 ng/well Target was diluted, six times by 1/3. The eighth measurement point contained 0 ng/well target. In contrast to the experiment above, the amounts of MAP KK and MAP K were cut. MAP KK was used with 12.5 ng/well and MAP K with 250 ng/well. Using less enzyme would result in lower costs for the screening. ATP was used with 100 µM and Bio-MBP-peptide with 3 µM per well. The reaction was stopped after 2 hours with 0.5 M EDTA, pH 7. The revelation mix was used at the same concentrations as before. Besides the titration, the time dependence of the signal development was investigated This time dependence strongly depends on the affinity of the anti-phospho-antibody to the substrate. The plate was

therefore read after 30 minutes incubation and after overnight incubation in the refrigerator.

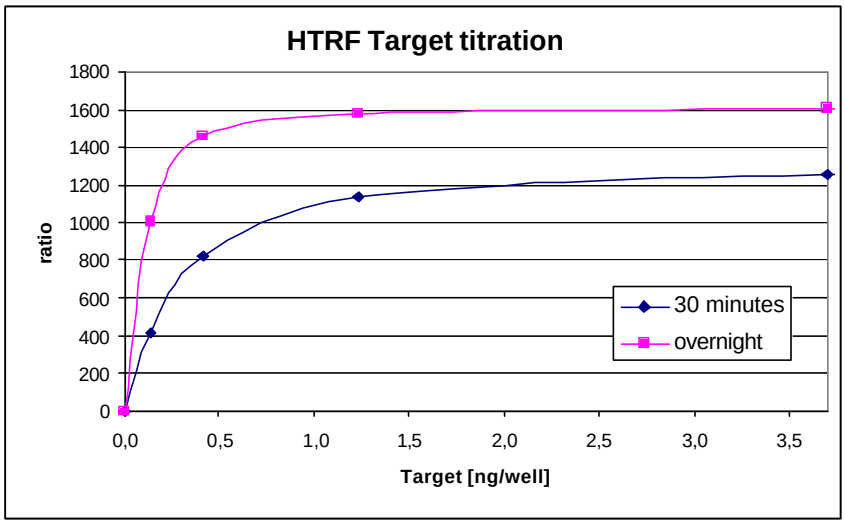

Figure 19: Target titration 100 ng/well – 0 ng/well by 1/3. Since the titration saturated between 1-1.5 ng / well (overnight incubation), the graph is limited to only 2 ng / well. Overnight incubation increased the signal by 25%.

The difference between the 30 minutes and overnight incubation reflects the low affinity of the antibody to the substrate (Figure 20). Overnight incubation increased the signal by 25%. This phenomenon will be discussed in more detail later. The highest signal, ratio = 1600, was reached with an enzyme concentration of 1.4 ng/well. This reflects the predicted potential of amplification of the cascade. For further titrations, the amount of 2.5 ng/well was chosen. This ensures the highest possible signal-output, while cutting down the concentrations of MAP KK and MAP K at the same time in order to save reagents.

The next step was to titrate MAP KK against the Target / MAP K mix. MAP KK was titrated in the same fashion as the target. Starting with the previously used concentration of 12.5 ng/well, it was titrated in six steps by using a one to three dilution. The eighth well contained 0 ng/well MAP KK. Target was used in the previously described concentration of 2.5 ng/well, and MAP K was assayed by using 125 ng/well. ATP and bio-MBP-substrate were kept the same, with 100 µM and 3 µM, respectively. After 2 hours, the reaction was stopped with 0.5 M EDTA, pH 7, and revelation mix was also added at the same concentrations as before. The plate was read after 1 hour.

Figure 20 shows linearity over the whole range. The one-hour incubation reached a ratio of 460. By applying the previous observation that the signal increases by

about 25% overnight, it leads to an overall signal of about 560. The linear response indicates that the MAP K concentration is too high to be saturated from the Target / MAP KK combination. Although the signal dropped significantly from around 1600 to around 460, these settings were used as a standard for the following investigations.#

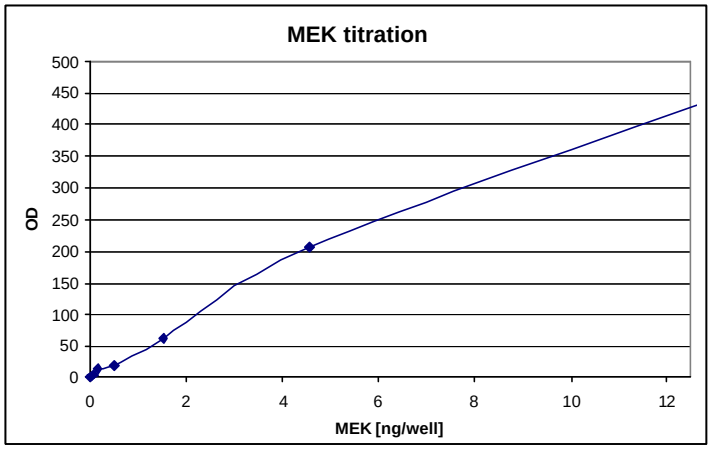

Figure 20: MAP KK titration 12.5ng/well-0ng/well by 1/3. The chart shows linearity over the whole range.

Signal Development Time-Course

The primary goal was to determine the optimal incubation time for obtaining the best possible signal. It should not take too long to perform the later HTS assay. Another aspect of the experiment was to control the previously discussed concentrations of the cascade enzymes.

The Target was titrated starting with a concentration of 25 ng/well. MAP KK and MAP K were added at 275 ng/well and225 ng/well, respectively. ATP and bio-MBP-peptide were kept the same, at 100 µM and 3 µM, respectively. Duplicates were performed. The reaction was stopped after 2 hours with 0.5 M EDTA, pH 7. After the last stop was performed, the revelation mix was added (same concentrations as above). The plate was read after 5 minutes, 30 minutes, 1 hour, 1.5 hours, and 2.5 hours. In addition an overnight read was performed. After reading, the duplicates were averaged and the results summarized after subtraction of the background.

Figure 21 shows the results of the two dimensional experiment: the Target titration, left to right, and the signal development time course bottom to top. The Target titration saturated at a concentration of about 2.5ng/well. This was confirmed by

comparing that with the previously obtained Target titration results. The obtained ratios for the signal development results after an incubation time of 5 minutes, ratio 800, and overnight, ratio 1600, show an increase in the signal of about 50%. This factor was subsequently used to predict signal increase.

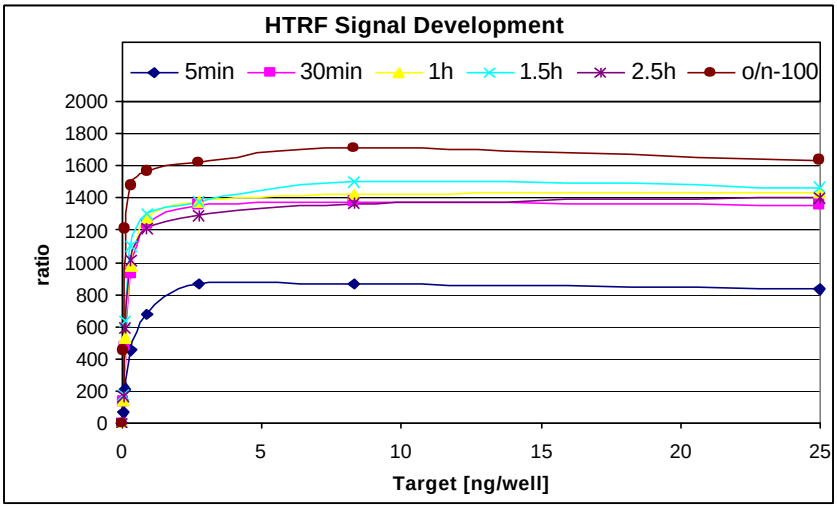

Figure 21: HTRF signal development. The plate was read after 5 and 30 min, and 1, 1.5, and 2.5 hours. In addition, an overnight read was performed.

ATP dependence

As discussed phosphate transfer is the basic reaction catalyzed by kinases. To avoid substrate inhibition, the ATP concentration has to be optimized. Therefore, the next series of experiments was performed, where ATP was titrated against the previously optimized cascade, to determine the optimal concentration.

The titration started with 500 µM ATP and was diluted in the same fashion as the other titrations, with dilution steps of 1/3. MAP KK and MAP K were assayed at the above optimized concentrations, 12.5 ng/well and 125 ng/well, respectively. The Target was used at 3.75ng/well. The peptide was also used as above at 3 µM. After two hours the reaction was stopped by addition of 0.5 M EDTA, pH 7. The revelation mix remained the same as above.

After averaging the signals and background subtraction, concentrations below 6 µM ATP/well resulted in zero signal (Appendix 1.9). The signal started to develop between 6 µM and 19 µM (Figure 22). The half signal is around 150 µM ATP, and the graph makes a turn around the same concentration. At this point it is not clear if this behavior is due to the performance of the assay or to the increasing ATP

concentration. Two standard concentrations, 10 µM and 100 µM were chosen for further investigations.

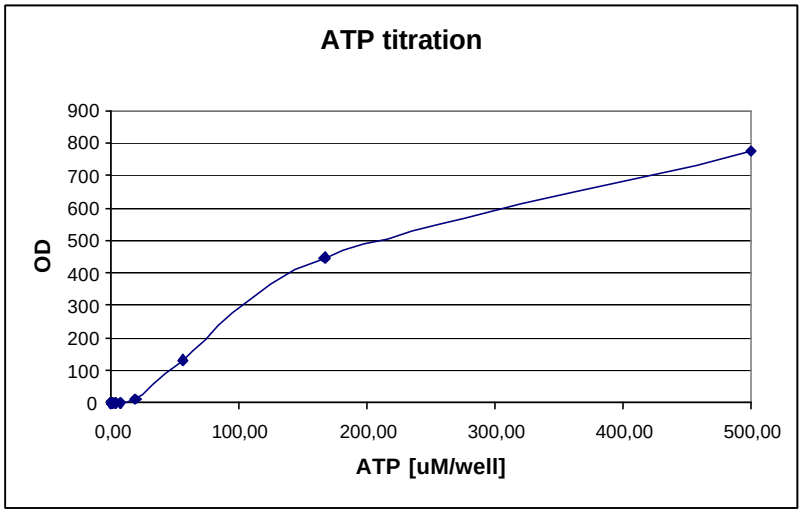

Figure 22: ATP titration starting with 500 µM/well.

Time Dependence

At this point, the optimal incubation time should be determined. It is important to obtain a linear output over the whole incubation time. If the assay were to be performed at saturation level the results would not be of any use. The best assay form to address this question is the time-course.

To see the progress of the cascade over time with the determined conditions, a time-course experiment was performed. Target was used at 3.75 ng/well, MAP KK at 12.5 ng/well, MAP Kat 125 ng/well, and the peptide at 3 µM. ATP was tested at the two above-mentioned concentrations, 10 µM and 100 µM, respectively. This should give an idea of how low the ATP concentration could be without affecting the signal output. The reaction was stopped after different time periods between 0 minutes and two hours, with 0.5 M EDTA, pH 7. The revelation mix was added at the same concentrations as before.

A long lag phase of about one hour could be observed at the beginning of the time course (Figure 23) McDonald, et al, 1999, also reported this lag phase. They explain this phenomenon as a reflection of the time required for the Target to phosphorylate and activate MAP KK, and for the DSK MAP KK to phosphorylate and activate MAP K. After the lag phase, the signal increased in a linear fashion over 2 hours until the end of the time-course. An HTS experiment with these

settings and 2 hours reaction time would gain an over all signal to noise ratio of about 1:10. This, compared to a desired signal to noise ratio of about 1:4 - 1:6 is very good, however, it requires a long reaction time of 2 hours. The next experiment should answer the question of whether or not an increase in Target concentration could significantly decrease the lag phase.

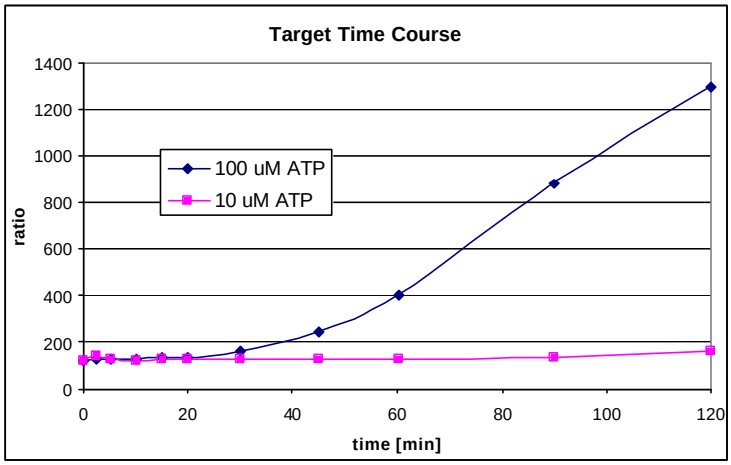

Figure 23: Target time course. Two different ATP concentrations were assayed, 10 µM and 100 µM.

An additional experiment was performed due to the above discussed long lag phase of 60 minutes. The Target concentration was increased threefold to 12.5 ng/well. The concentration of MAP KK was increased to 125 ng/well, and the concentration of MAP K was decreased to 62 ng/well. The peptide concentration was used as always at 3 µM, and ATP at 100 µM. After two hours, the reaction was stopped by addition of 0.5 M EDTA, pH 7. The revelation mix was added at the same concentrations as before.

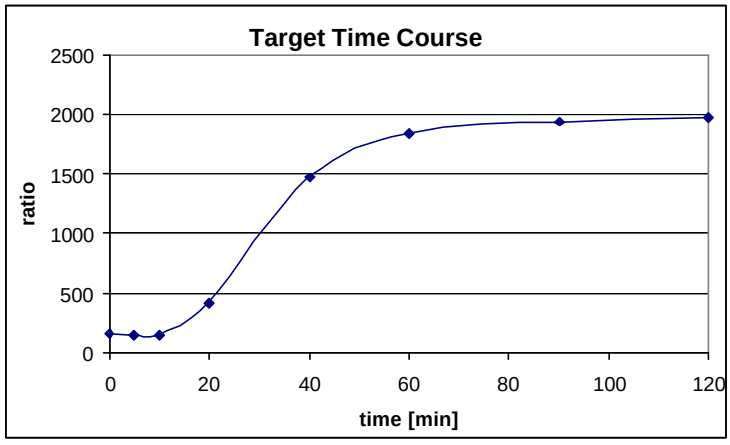

Figure 24: Target time course.

The lag phase could be decreased to 20 minutes (Figure 24). However, the amount of target used to achieve this is not affordable for HTS, since not enough enzyme is available using this conditions. The Target amount of 2 ng/well was chosen for the screening. The long lag phase and, subsequently, the reaction time of 2 hours had to be accepted in order to preserve the scarce enzyme source.

Validation

As discussed, an assay has to be validated before use. Investigating day-to-day variations and testing the system against known inhibitors ensures stability. The in-house purification group immediately implemented the above-determined settings to test the activity of the purified Target. Thus, the cumbersome radioactive filter-binding assay was avoided. Their experience and results using the cascade were used for the evaluation of day-to-day variations and performance of the assay through different investigators (data not shown). The cascade proved to be stable and reliable. Duplicates were usually very tight, and day-to-day variations were not observed. Next, the active target construct was compared with a mutant inactive construct.

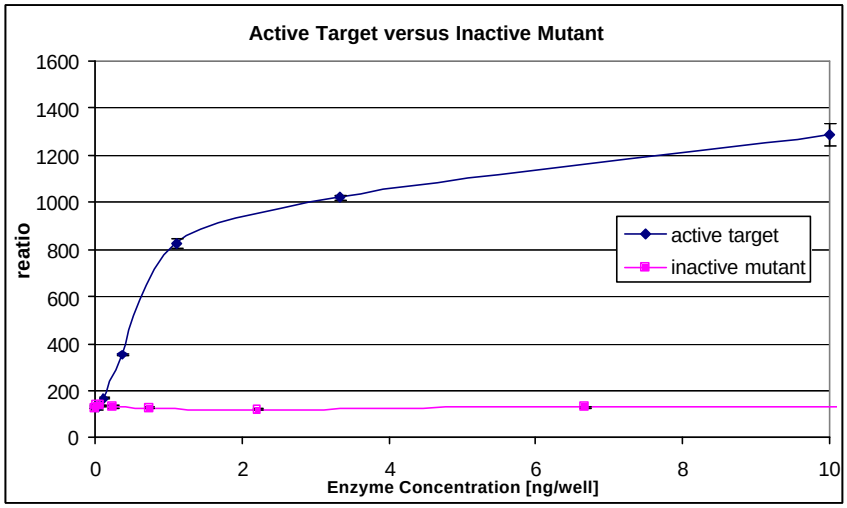

Figure 25: Comparison of the active Target versus an inactive Target mutant.

The activated Target construct was assayed in comparison to an inactive kinase mutant. Two titrations were performed. The active Target titration started at a concentration of 10ng/well and was diluted by 1/3. The inactive Target mutant was assayed at the same concentration and in the same fashion. MAP KK and MAP K were used with the now standardized concentrations of 12.5 ng/well and

125 ng/well, respectively. The same was done with Bio-MBP-peptide peptide and ATP. Their now standardized concentrations are 3 μM and 100 μM, respectively. Duplicates were performed. The reaction was stopped after 2 hours with 0.5 M EDTA, pH 7. The revelation mix was used at the same concentrations as above.

The result was as expected. The active Target titration showed a good titration curve, whereas the inactive mutant showed no activity (Figure 25). This result proved the specificity of the active target enzyme.

Further validation had to be performed in the HTS group in Germany under real screening campaign conditions on the robotic system.

Optimized Protocol

All of the above-determined settings were summarized in a protocol, which is attached in Appendix 2.1. The design of the protocol should allow any investigator to conduct the experiment without difficulties, which was important for later assay transfer in the HTS group. There the protocol would be used for performing the screening.

FOLLOW UP ASSAYS

Follow up assays have the task to remove false positive and false negative compounds from the 'hit-list'. They are designed to prove authenticity and confirm the ability of a given compound to inhibit the Target. Due to the cascade format, the follow up assay should also have the ability to determine at which point the compound inhibits. There are three possibilities for a compound to inhibit within the cascade, the MAP K, MAP KK, or MAP KKK. In addition, in cases of unspecificity the compound can inhibit more than one of the kinases in the cascade.

Two different approaches are possible for the setup of a follow up assay based on the HTRF technology. First, testing of the hits, achieved the primary screening in the Raf cascade. Only compounds that had an effect in the Target cascade (primary screen) and no effect in the Raf cascade would be considered specific hits. Second, the cascade could be broken down into its different steps. This format would use active Target, inactive MAP KK and inactive MAP K for the whole

cascade. The intermediate step would use active MAP KK and inactive MAP K, and the last step would be performed using only active MAP K. This approach would give the highest level of information since the inhibition of all three enzymes could be separately detected and further investigated. Both approaches, the Raf cascade and the splitted cascade, were developed, for counter screens and for further investigations. Scintillation proximity assay (SPA) was investigated as a third possibility. This technology would give an advantage compared to HTRF with a radioactive-based assay. Hits in both formats could be considered as real.

Split Cascade

The split cascade combined three different experiments in one setup, the complete Target cascade, activated MAP KK with inactive MAP K, and activated MAP K. The MAP KK / MAP K and MAP K approaches were developed in the same fashion as the target cascade described above. After investigating the effect of the enzyme concentration within the setups, ATP and time dependence were determined.

Based on the results and the experience made by the optimization of the full cascade the titration for the active MAP KK / inactive MAP K cascade was started. 125 ng/well inactive MAP K were used. Active MAP KK was titrated down outgoing from 0.1 U/well by 1/3 six times. The last well contained MAP K but no MAP KK. As substrate Bio-MBP-full length at a concentration of 2.5 µg/well was used. The ATP concentration was the same as in the optimized protocol, 100 µM. 10 µl of the MAP KK titration were mixed with 10 µl MAP K and 10 µl Bio-MBP-full length. Adding 10 µl ATP started the reaction.

The reaction procedded very fast (Figure 26). For the next experiment 0,01 U/well MAP KK were chosen. Inactive MAP K was titrated to determine the lowest concentration of MAP K in connection with active MAP KK needed for running an assay (Figure 27). The MAP K titration was started at an concentration of 125 ng/well. ATP and Bio-MBP-full length were kept the same as in the previous experiment. Figure 27 shows that the reaction started to saturate at a MAP K concentration of 50 ng/well. As a result the 0.01 U/well of active MAP KK and 50 ng/well inactive MAP K were chosen for the optimized protocol.

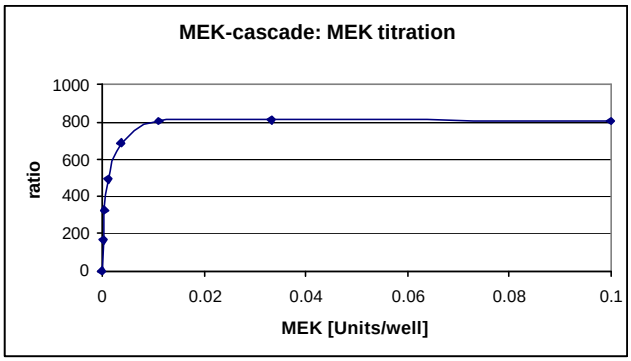

Figure 26: MAP KK-Cascade; Titration of activated MAP KK against the inactive MAP K to determine the optimal concentrations of both.

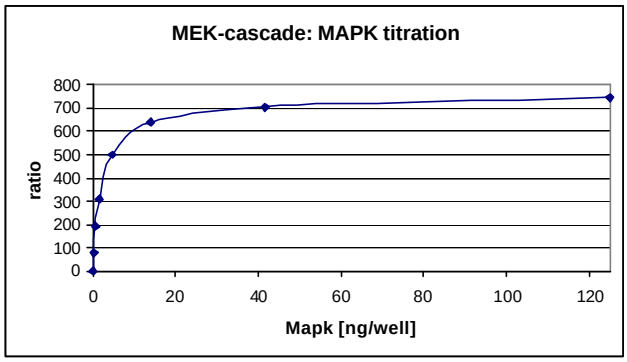

Figure 27: MAP KK-Cascade; Titration of inactive MAP K against MAP KK to determine the optimal concentrations of both.

Following the MAP K assay was optimized. Active MAP K was titrated starting with 100 ng/well 1/3 six times. The eight's well contained 0 ng/well MAP K. ATP and Bio-MBP full length were kept the same. The optimal for active MAP K was determined at 1ng/well (Figure 28).

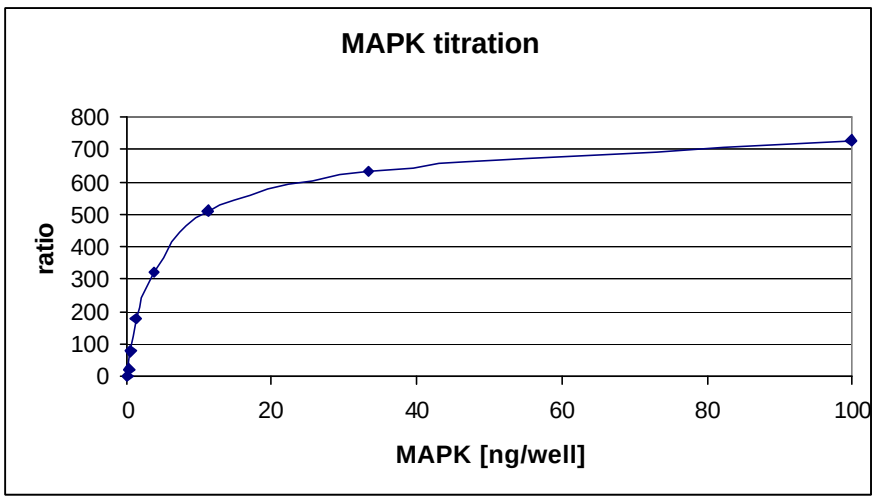

Figure 28: Activated MAPK-Assay; Titration of the enzyme to determine the optimal concentration.

Finally a time course was performed using the above determined settings. This experiment should show how good and how long the reaction was linear. The above determined settings were used and the reaction was terminated after defined time intervals between 0 to 70 minutes.

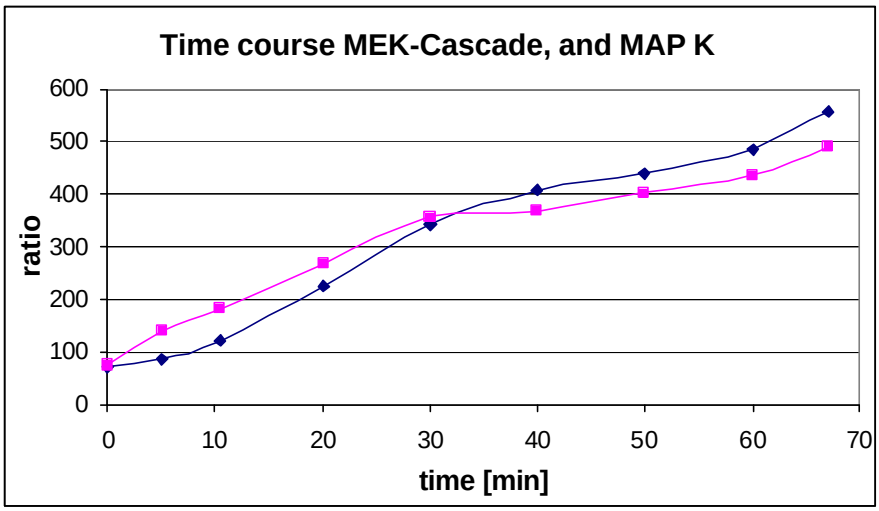

Figure 29: Time course of the optimized MAP KK-cascade and MAP K assay.

The time course showed linearity of the assay during 70 minutes (Figure 29). Surprisingly the assay showed no lag phase, which suggests that the lag phase discussed earlier is due to the Target and not as thought to the MAP KK. However it confirmed the settings, and the final protocols were written. Both protocols, for the active MAP KK with the inactive MAP K cascade, and the active MAP K alone are attached in Appendix 2.2 and 2.3. Due to time shortage the assay could not be validated in the same fashion as the above described cascade assay. This assay should be used later on in the project for more detailed inhibition studies.

Raf Cascade

The Raf cascade is favorable for counter screen because it is one single experiment and not three experiments in one, like in the split cascade. The Raf cascade was optimized from another group (data not shown). The performance was similar to the Target cascade, and the assay is ready to be used.

Scintillation Proximity Assay (SPA)

Scintillation proximity assay was chosen as a possible alternative to perform the follow up assays to determine the specificity and potency of the hits. The assay development was based on the Serine / Threonine kinase assay kit for use with ^{33}P, Amersham Pharmacia Biotech, catalog # RPNQ 0200. SPA, in contrast to HTRF, is a radioactive-based assay without the need for an antibody or labeling steps. Hits showing up in the same fashion in both approaches could therefore be considered as real, and not as a technology-induced artifact. The coupling technology of SPA is

similar to the HTRF approach. The streptavidine labeled beads capture the biotinylated substrate, and bring bound radiolabel in close proximity to the scintillant incorporated in the beads (Page 38). The cascade with the biotinylated peptide can be applied to the SPA approach without modifications to the substrate. However since ^{33}P has a relatively high maximum decay energy, 0.249MeV, its ß-particle has therefore a path length of 0.6 mm in aqueous solution. Thus a high background due to unspecific proximity effects were expected.

To address the background issue and to determine the best plate to use with the Wallac MicroBeta 1450 reader, an experiment was designed. Two plates were tested a white opaque, clear flat bottom plate, and a clear flexible poly propylene plate used with a cassette to avoid crosstalk. The in the Amersham protocol suggested ^{33}P concentration of 0.5 µCi/well was added to water, water with 0.5 mg SPA beads/well, and kinase reaction buffer with 0.5 mg beads/well. Duplicates were performed.

Table 3: Background check

flexible plate	water	water+33P	water+33P+beads	buffer+33P+beads
Sample 1	39	5691	23041	20187
Sample 2	36	5206	21986	16656

white plate	water	water+33P	water+33P+beads	buffer+33P+beads
Sample 1	47	4320	23360	23609
Sample 2	56	4511	23048	21152

The white plate showed slightly lower signals in the water / ^{33}P samples as the flexible plate. The signal water / ^{33}P / beads, and buffer / ^{33}P / beads showed no significant differences (Table 3). The background from 0.5 µCi ^{33}P in combination with 0.5 mg beads could be determined around 23,000 dpm.

Within the first SPA experiment, according to the Amersham protocol, the Target was titrated outgoing from a high concentration of 250 ng/well 1/3 two times, the fourth sample contained 0 ng Target per well. The substrate Bio-MBP-peptide was used with 3 µM per well. After 10 µl of the Target was mixed with 10 µl substrate in 10 µl reaction buffer the hot ATP mix, 40 µM cold ATP containing 0,5 µCi/well hot γ-^{33}P[ATP]. The reaction was stopped after 30 min using the on Page 38 described stop buffer. Duplicates were performed.

Table 4: First SPA experiment. Target titration assayed with Bio-MBP-peptide.

Target [ng/well]	1 [dpm]	2 [dpm]
250	42153	43140
83	36195	36631
28	32622	29227
0	27854	26937

The background signal with 27,000 dpm was very high as expected (Table 4). The duplicates came out close, but the signal to noise ratio of 1,6 is not acceptable. To decrease the high background and to increase the very low signal to noise ratio, the samples obtained in this experiment were used and more cold ATP, respectively Tween 20 were added. The additional ATP (20 µM/well) should compete with the hot-ATP mix in the sample for unspecific binding places, the Tween 20 should solubilize all unspecific bound ^{33}P, to avoid unspecific signals. Instead of decreasing the signal increased (Table 5). One reason for this might be that the time the beads were allowed to settle was to short. In this case unspecific proximity effect occur and hence increase the signal.

Table 5: Experiment above after addition of 20uM cold ATP, and 0.04% Tween 20.

	Additional ATP [20uM]		Addition of 0.04% Tween 20	
Target [ng/well]	before add.	after 6 hrs	before add.	after 6 hrs
250	28621	35781	27441	34961
83	23558	27984	25061	30359
28	22917	27278	20814	26574
0	20316	26999	19483	26493

Following the Target was assayed in the same fashion as above. As Target concentration was chosen 10 ng/well, three setups were performed. The Target alone, Target with 20 µM of a known inhibitor, and one setup without Target. The substrate and the other reagents were used at the same concentrations as above. Since the calibration date of the ^{33}P was seven days over the actual activity was calculated with 0.4 µCi/well instead of 0.5 µCi/well.

The assay showed specific activity (Figure 30). Background signal of about 14,000 cpm, signal of inhibited cascade slightly higher, and a signal at 16,500 cpm in the cascade setup without inhibitor. The assay seemed to work, however the signal to noise ratio of 1.2 is still much too low. Subsequently the reaction time was increased to one hour. All the other parameter were held constant. The target was titrated outgoing from 50 ng/well 1/3 down to a concentration of 0.07 ng/well.

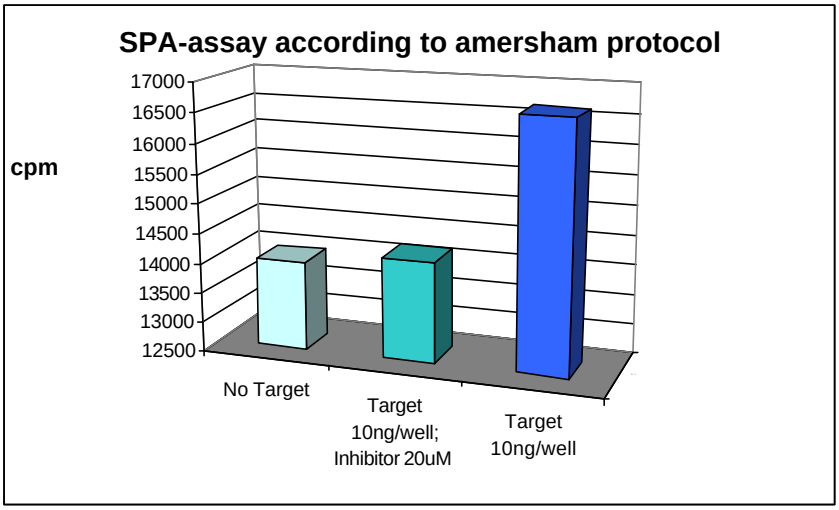

Figure 30: SPA assay with the Target cascade and with the addition of an unspecific MAP kinase inhibitor.

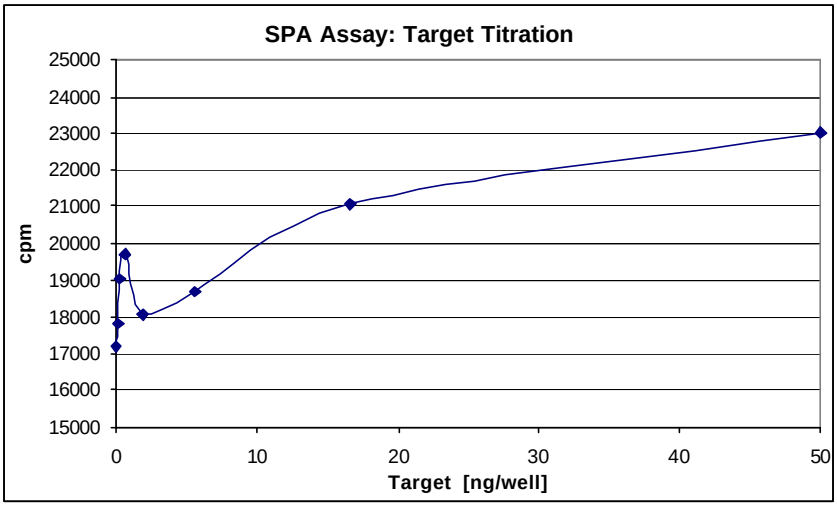

Figure 31: SPA assay Target titration.

The extended incubation time increased the signal in the 10 ng/well range of about 2,000 cpm (Figure 31). But since the titration curve peaks the first time at 0.6 ng/well with 20,000 cpm and drops to 18,000 cpm for increasing again steadily with concentration the experiment is not very reliable.

After performing this series of experiments, it turned out that optimization could only be accomplished by expending a great deal of effort and resources. The low signal to noise ratios, about 1:1.5 to 1:2, in addition to the short time-frame available to transfer the assay, were the reason for sidelining this approach until resources will be available. Therefore the splitted cascade and the Raf cascade were developed as alternatives.

MASS SCREENING PREPARATION

The central HTS unit of BASF Pharma is based at Knoll AG, Ludwigshafen, Germany. The task of the HTS unit is to automate the assay as much as possible and to optimize the assay on the robot. The primary screen should be performed and the hits validated in the follow up assay. The evaluation of the data (hit; no hit; %-inhibition, IC50's) would be sent back to BBC and evaluated by the medicinal chemists. They would then propose possible potent lead structures for further investigations.

To transfer an assay to the HTS unit, the amount of necessary reagents must be calculated, and their supply ensured. Running one campaign with the same batch of reagents makes the process simpler, because time consuming tests to determine whether a new batch has the same quality as the old one are unnecessary. Optimal is to run the whole screening, including the follow up assays, possible failed runs, optimizing the HTS system, and dead volume in the pipette system of the robot. New batches of reagents has to be evaluated, which cost time. The follow up-assay has to be in place, and the protocols had to be written in detail. Therefore, after the design of the protocols these numbers were calculated on the basis of the amount of data points which should be acquired during the screening campaign. This number is the amount of compounds, which should be tested against the cascade. The actual amounts are summarized in Table 6.

Table 6: Amount of reagents needed for the HTS.

Reagent	Amount needed	Vendor
Target	2mg	In house
MEK1	6.25mg	Upstate Biotech
ERK2	62.5mg	Upstate Biotech
RY01	1.115mg	Upstate Biotech / CisBio
SAXL	80mg	Prozyme
Bio-MBP-peptide	130mg	Syn Pep

After the amounts were determined, and 50% of the total was added for security, all vendors were contacted and the bulk rates negotiated. Within these negotiations the amount for the order at Upstate Biotech could reduced by 2/3. The antibody had to be sent to Cis-Bio, France, in order to get the Europium label in place. After labeling the antibody had to be shipped directly to Germany.

Both BBC and Knoll AG dedicated staff to the assay transfer project. After preparations at both sites, four days were determined for the transfer of the assay to Knoll AG, Germany. The protocol and reagents for optimization and setup of the robotic system were sent to Germany in advance. Since Upstate Biotech could not hold the promised deadline for the delivery of the reagents. Germany could not be supplied with the actual batches of MAP KK and MAP K ordered for the screening and its optimization. To speed up the process and older batch was sent to Germany, even this meant additional validation experiments with the new batches later on. However the setup of the robot could already be performed. Compared to this an additional titration makes far less work.

ASSAY TRANSFER

After the assay development and the organization of the mass screening, the assay had to be transferred to Knoll AG in Germany where the HTS activities of BASF Pharma are centralized. One biology-lab-technician was assigned to the assay. The assay had to be passed over within four days. To introduce the staff, responsible for running the assay on the robot, to the project and the assay format, I held a seminar on the third day. The seminar provided basic information about the detection method, HTRF, and introduced the special kinase cascade format and the overall conception of the assay.

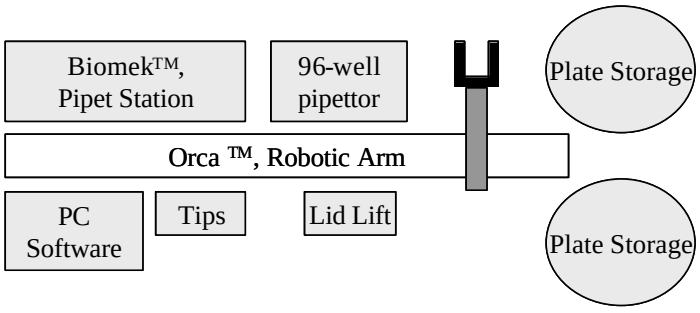

Figure 32: HTS setup for the Target campaign (Eckard et al., 1996, modified).

For transferring the assay, a series of experiments were designed, primarily to make the technician familiar with the cascade and the format of the assay. The later task of the technician was to optimize the assay on the robot. These experiments were designed to help the technician to adopt the assay as quickly as possible. In addition, the HTS setup protocol was reviewed and details of the setup,

the cascade, and HTRF were discussed. The HTS system consists of a robotic arm, a 96-well pipettor, a Biomek™ pipette station, and storage for plates and consumption materials (Figure 32). Due to the setup of the system, the amount of reagents to pipette had to be limited. This would simplify the programming of the robot and speed up the assay time.

Before optimizing the robot setup, assay elements had to be tested under the different conditions in the laboratory in Germany. These elements include the detection device, Discovery, new reagents, and the handling (pipettes, plates etc.).

One part of the first experiment was performed according to the protocol without modifications in the setup. All buffers were prepared with reagents purchased in Germany. The target was titrated starting with a concentration of 10 ng/well. MAP KK and MAP K were used with 12.5 ng/well and 125 ng/well, respectively. The Target was diluted two times by 1/3, and the fourth well contained 0 ng/well. ATP and Bio-MBP-peptide were used at 100 µM and 3 µM, respectively. 10 µl of the target titration was mixed with 10 µl of the MAP KK / MAP K mix and10 µl of Bio-MBP-peptide. The reaction was started with 10 µl ATP, and stopped after two hours by addition of 10 µl 0.5 M EDTA, pH 7. 150 µl revelation mix containing 8 µl/ml RY01 and 26 µl/ml SAXL was added. The plate was incubated overnight in the refrigerator and read the next morning.

Within the second part of the experiment, the Bio-MBP-peptide was mixed with the ATP. 10 µl of this mix were mixed with 10 µl of the Target titration above, 10 µl MAP KK/MAP K mix, and 10 µl reaction buffer. The reaction buffer was added to achieve the same 40 µl volume as above. The combination was performed to see if it would have any impact on the cascade.

Both cascades got the same result with an enormous signal ratio of 13,000 (Figure 33), in contrast to the expected ratio of 1000. Also, the background increased about sevenfold, from the expected ratio of 120 to a ratio of about 880 in both cascades. This increase shows a higher sensitivity of the systems in Germany, compared to the results achieved in America. Since the assay worked in the same fashion, this result could be explained by differences in the setting of the detection device, Discovery. The voltage applied to the detector within the machine in the

HTS unit seemed to vary from the settings of the machine on which the assay was developed. This made it possible to reduce the amount of reagents used to develop the HTRF signal.

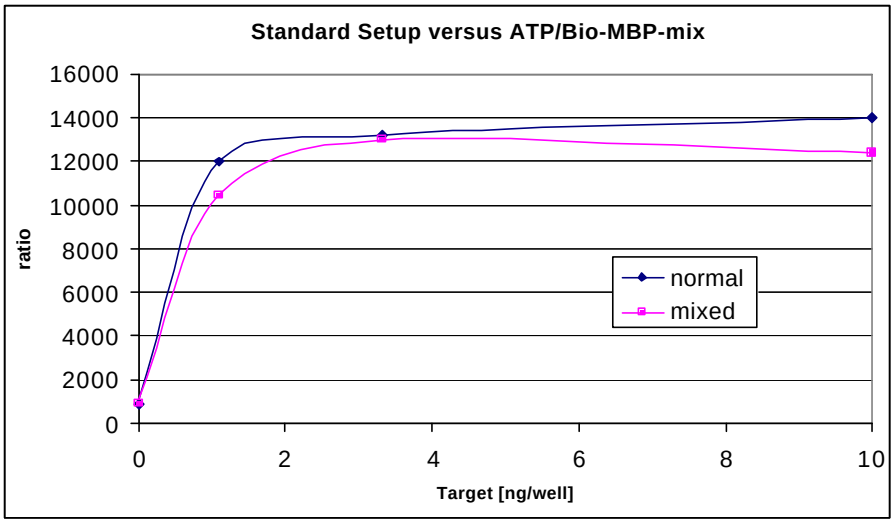

Figure 33: First experiment to see if the assay would work in Germany. In addition, ATP and substrate were mixed to decrease the number of reagents to be pipetted.

The next experiment was designed to validate the assay and find out optimum conditions for RY01 and SAXL . The cascade was basically performed in the same fashion as above. The target titration was diluted six times by $^1/_3$. The eighth well contained 0 ng/well. MAP KK and MAP K were applied in a mix with 12.5 ng/well and 125 ng/well, respectively. ATP and Bio-MBP-peptide were added at 100 µM and 3 µM, respectively. After a 2 hour incubation, the reaction was stopped by addition of 0.5 M EDTA. The amount of RY01 in the revelation mix was reduced by $^1/_3$ (2.5 µl/ml), and that of SAXL by ½ (2.5 µl/ml).

After 50 minutes the plate was read. The ratio in the highest concentration was around 3,000 (Figure 34). To see if the amount of SAXL was cut too much, the other half of the SAXL, 2.5 µl/ml, was added. Since the coupling reaction between the streptavidine of the alophycocyanine and the biotin of the peptide is very fast, the plate could be read after 5 minutes. The signal increased to a ratio of about 4,000. To see if the amount of RY01 would be saturated by the amount of SAXL, another 1/3 of RY01 was added to the wells and incubated for 50 minutes. The signal increased to a ratio of around 5,000. This, plus the knowledge that longer incubation would increase the signal further, indicated no more reagents were necessary. The signal to background ratio was about 1:5, and therefore sufficient for HTS. The plate was incubated for another two hours and read again. This

further incubation increased the signal by a ratio of around 500 to 5,800. This rough experiment resulted in a cut-down of the antibody by one third, with further optimization possibilities in later steps.

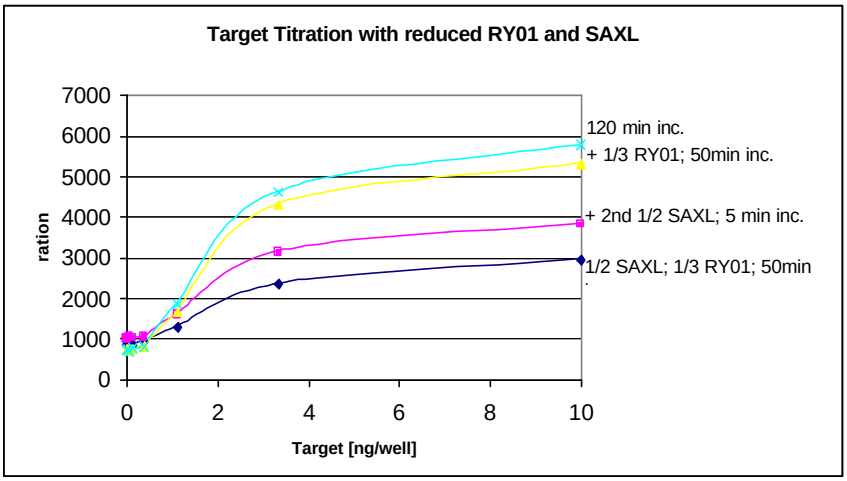

Figure 34: Target Titration to determine the amount of SAXL and RY01 necessary for signal development.

The next experiment addressed the question of variance between the different plate formats, 96- and 384-well plates, and their different maximum volumes. The laser of the detection device focuses on a certain height of the well. This experiment should give an idea of whether different plate formats produce different results. The lower volume of the 384-well plate made it necessary to reduce the volume used for revelation of the signal. The reaction volume should be held constant.

Table 7: Signal dependence of the signal to the volume.

96 well (200ul/well)		384well		
Background	**Signal**	**ul/well**	**Background**	**Signal**
748	7189	30	956	7531
		40	852	7544
		50	814	7561
		60	811	7940
		70	733	7700
		80	765	7779

To save Target and other reagents, the reaction mixture of the plate used in the experiment before was pooled. Background wells were pooled with background wells and titration wells with titrations wells. 200 µl of the pooled mix were pipetted into a 96-well plate. This is the overall volume used in the experiments before and the signal should act as a standard for the evaluation. 30, 40, 50, 60, 70, and 80 µl of the pooled mix were pipetted into a 384-well plate.

The signal in the 96-well format reached a ratio of 7,189 and a background ratio of 748 (Table 7). The signal in the 384-well plate showed small variations, whereas the background ratio varied from 733 to 956. The rough setup of the experiment did not allow differentiated evaluation, but it seems to be necessary to use at least 60 µl. Since the reaction volume had 40 µl, the revelation mix should have at least 20 µl. In total a 384-well plate can hold 100 µl liquid. However, this volume should not be completely filled in order to avoid run over of the liquid and cross contamination. 40 µl was chosen as the volume for the revelation mix. The actual concentrations of RY01 and SAXL had to be determined within further studies.

The next experiment was set up to compare the performance of the new settings in the 96- and 384-well format. Another feature was the mixture of the stop solution with the revelation mix. This should further reduce the exertion of pipetting and help to simplify the HTS programming.

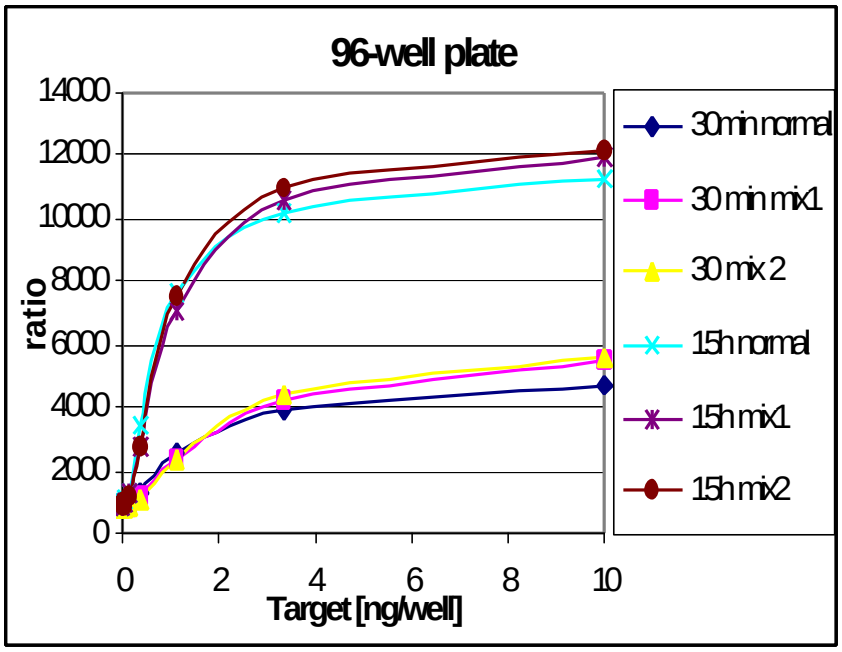

Figure 35: Three setups for reducing pipette steps in a 96-well: First, normal cascade; second one-step reduced cascade with a mix of MAP KK, ERK, and substrate (mix1); third, a two-step reduced cascade with the addition of the EDTA mixed into the revelation buffer (mix2).

The Target was titrated starting from 10 ng/well by 1/3 six times, the eighth well contained 0ng/well. MAP KK, MAP K, Bio-MBP-peptide, and ATP were used in the same concentrations as before. Three setups were made: First, the normal cascade as previously described; second, the one-step reduced cascade with a mix of MAP KK, MAP K, and Bio-MBP-peptide (mix1); and third, a two-step reduced cascade with the addition of the EDTA mixed into the revelation buffer (mix2). The 96-well plate was set up with all three, whereas the 384-well plate, due

to Target shortage, was set up with only two reduced setups. The overall volumes after revelation were 190 µl for the 96-well plate and 80 µl for the 384-well plate.

In the 96-well plate, the signal developed to a ratio of 12,000 regardless of the setup used (Figure 35). This showed that both mixtures were adaptable for the HTS system, and could reduce the steps necessary for pipetting. The zero line within the plate was around a ratio of 900. The 384-well plate showed a three times lower signal compared to the 96-well plate (Figure 36). The maximum ratio here was 4,500. The background signal was also lower than the signal obtained in the 96-well plate, and differed by a factor of 1.2. The signal in the 384-well plate started to saturate at a concentration of 3 ng/well, close to the concentration of the 96-well plate.

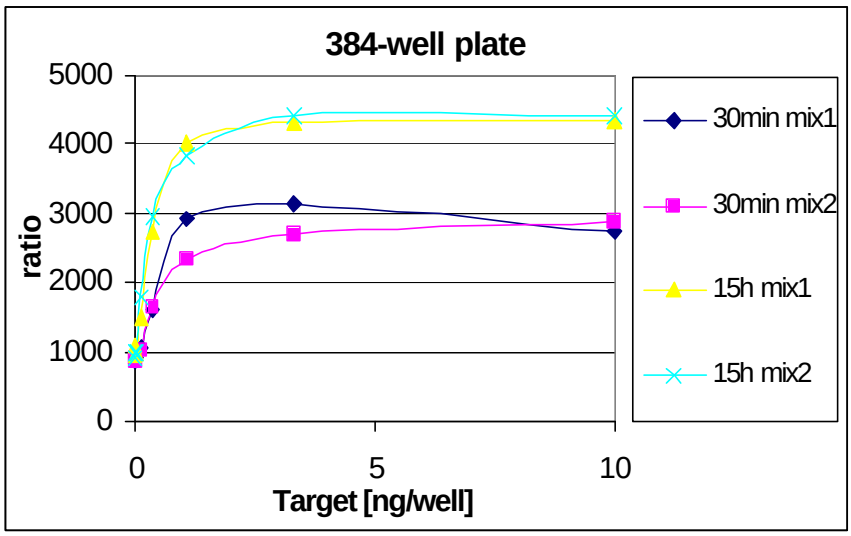

Figure 36: Two setups for reducing pipette steps in a 384-well plate: one-step reduced cascade with a mix of MAP KK, ERK, and substrate (mix1); and a two-step reduced cascade with the addition of the EDTA mixed into the revelation buffer (mix2).

MBP-PEPTIDE VERSUS MBP-FULL LENGTH

To the beginning of the project biotinylated full length MBP was not available. For this reason the biotinylated peptide was used for the assay optimization. After the assay transfer to Germany Upstate Biotech offered customized biotinylation for an affordable prize. A sample batch was purchased and investigated which of the two above mentioned substrates was preferable. The full length MBP was derived from bovine brain and customized conjugated with one biotin molecule per MBP molecule.

Within the first experiment the Target was titrated starting with 10 ng/well 1/3 two times, the fourth well contained 0 ng/well Target. The substrates were assayed at concentrations of 3 µM for the Bio-MBP-peptide respectively 1 µM for the full length MBP. MBP full length was believed to have a higher background because of multiple phosphorylation sites within the molecule to gain a higher signal, thus the concentration could be lower as the peptide concentration. With the MBP Upstate Biotech sent the new batches of MAP KK and MAP K meant for the mass-screening, for quality control these were compared with the old batches. The concentrations for these reagents and ATP were as described in the protocol Appendix 2.1.

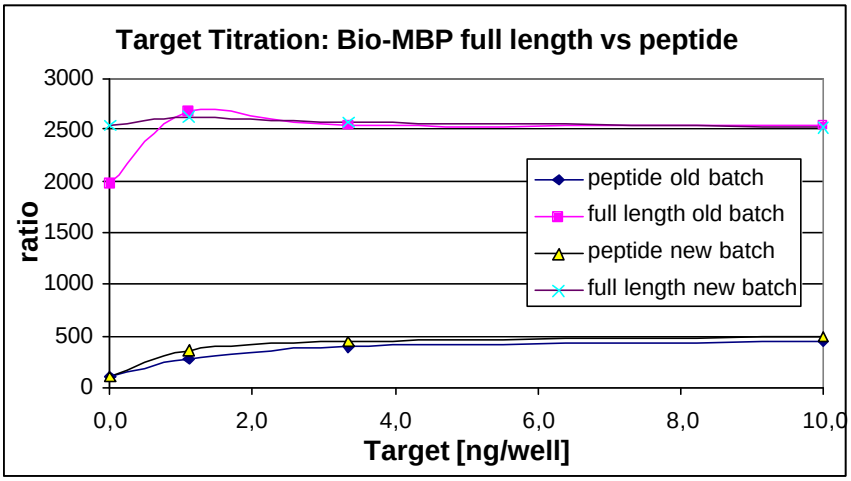

Figure 37: Target titrated using Bio-MBP-peptide and full length, as well as different batches of MAP KK and MAP K.

The MBP-full length signal was as expected about six times higher (Figure 37). Surprisingly the background signal of the cascades using the full length protein were at nearly the same level as the signal in the highest concentration of Target. This suggested two things, first the amount of either SAXL or RY01, or both were at saturation levels, and second since in the background was no Target, either MAP KK or MAP K, or both enzymes in the cascade mix work on the substrate. To address the later suggestion another experiment was designed.

To verify the above obtained data the experiment was repeated, in addition inactive MAP KK, and inactive MAP K on their own and in combination were assayed with the MBP-peptide and the full length MBP. All enzymes were assayed at the same concentrations as described in the optimized protocol. The peptide was again used at 3 µM and the full length MBP with 1 µM.

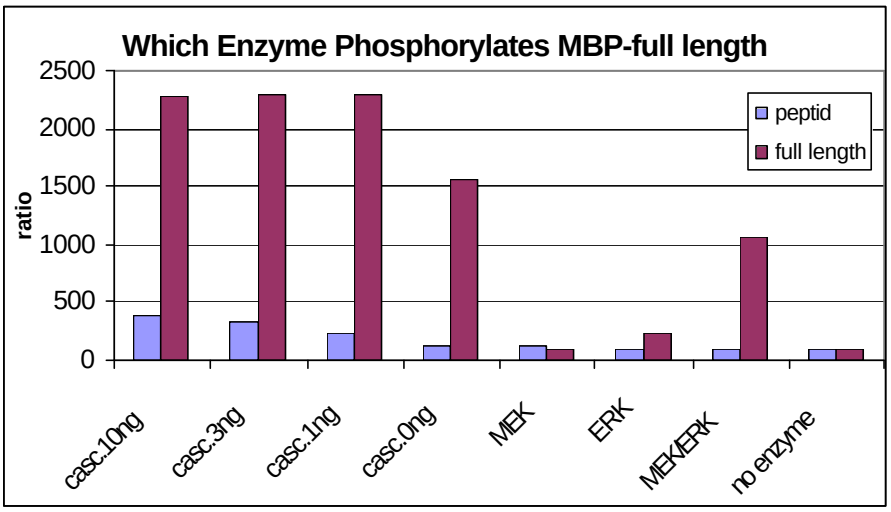

Figure 38: Target cascade, inactive MAP KK, inactive ERK, and inactive MAP KK in combination with inactive MAP Kassayed with the MBP-peptide and the full length MBP

The full target cascade came out in the same fashion as the previous experiment, low background in the peptide setup and high background in the MBP full length setup (Figure 38). MAP KK alone showed no activity in either setup where MAP K showed some activity against the full length MBP. However MAP KK and MAP K combined show a very high background signal, where no signal actually should be observed. This suggests that MAP KK uses MAP K as a substrate and MAP K used MBP as a substrate. Both enzymes should have no activity since they are supposed to be inactive.

In order to confirm the above observed results, and before putting the order of MAP KK and MAP K from Upstate Biotech on hold, the optimized RAF cascade was assayed with the MBP-peptide and the full length.

The assay was performed according the optimized protocol as seen in Appendix 2.4. Raf was titrated outgoing from 250 ng/well 1/3 six times the eighth sample contained no Raf. MAP KK was assayed at a concentration of 12.5 ng/well, and MAPK at 50 ng/well. Bio-MBP-full length was used with 2.5 µg/well and ATP with 100 µM/well. Along with this setup, activated and inactivated MAP KK respectively MAP K were assayed alone and in combination. The amounts of inactive MAP KK and MAP K were used the same as above. The activated enzymes were used with 0.01 U/well for MAP KK respectively 1 ng/well MAPK.

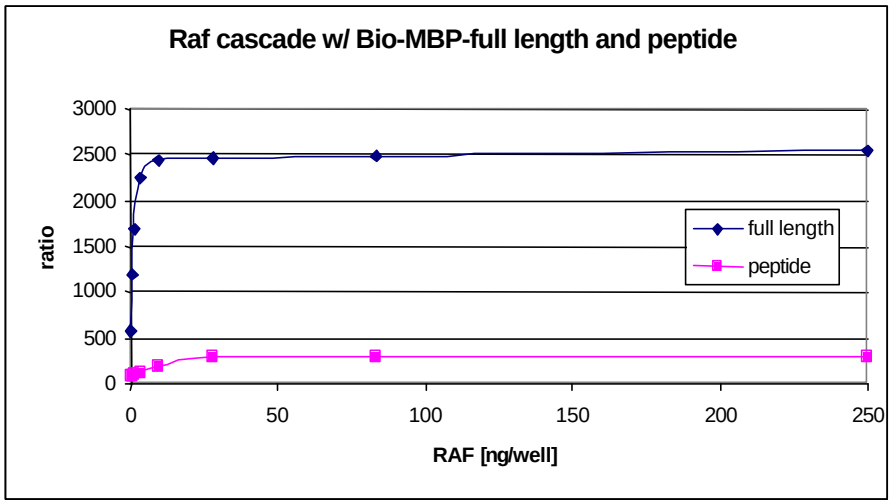

Figure 39: Raf cascade, assayed with the MBP-peptide and the full length MBP.

The Raf cascade assayed with Bio-MBP-full length, performed in the same fashion as the Target cascade, high signal (ratio 2,500) in the range from 25 – 250 ng/well Raf, along with high background (ratio 500) (Figure 39). Whereas the Raf cascade assayed with the Bio-MBP-peptide gave a signal under a ratio of 500 and a lower background.

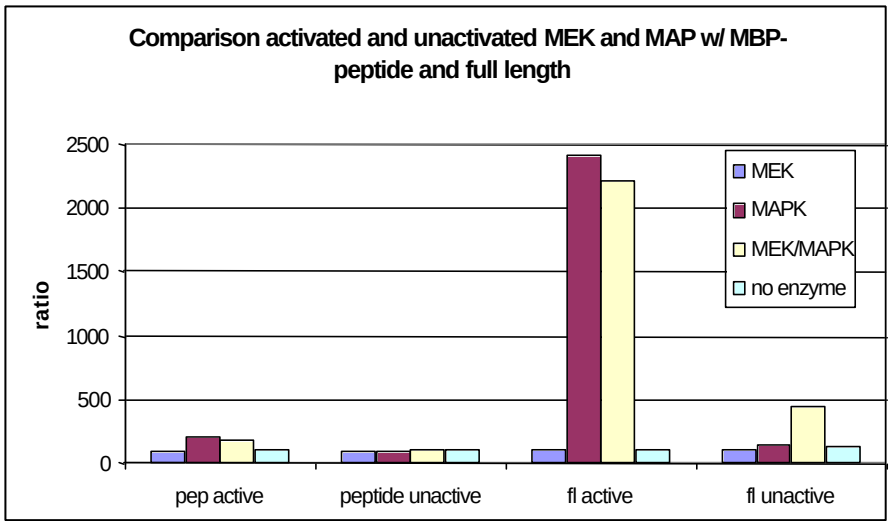

Figure 40: Activated and inactivated MAP KK respectively MAP Kwere assayed alone and in combination.

The above observed results could be confirmed (Figure 40). Using inactive enzymes no signal could be observed while assaying them with MBP peptide. Whereas by using the MBP full length a significant increase by using inactive MAP KK in combination with inactive MAPK could be observed. As expected MAP KK alone showed no activity in either setup.

These results stopped the order with Upstate Biotech. After contacting their technical service people in the purification group further purification steps were negotiated. And the order was set on hold.

Upstate Biotech purified a new batch of MAP KK, now as human MAP KK. This new batch was compared together with two older batches of MAP KK. All three batches were assayed using 12.5ng/well MAP KK, and 50ng/well MAPK in the same fashion as above.

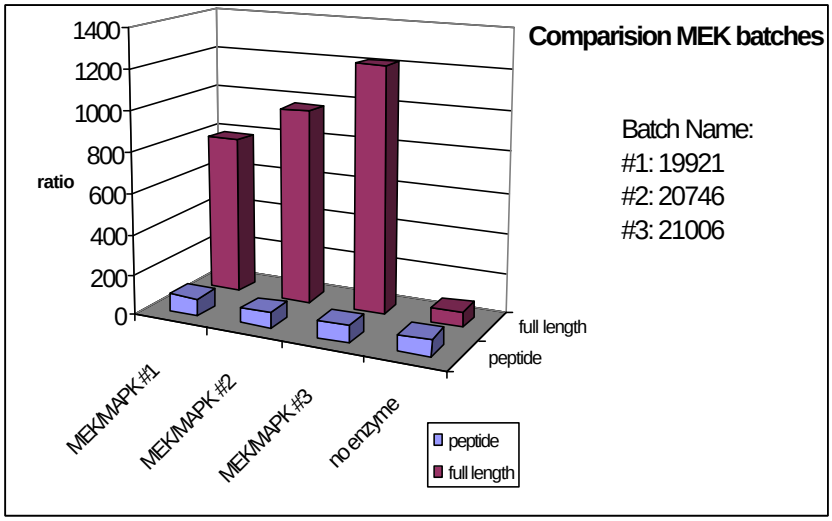

Figure 41: Comparison of three different MAP KK batches.

All batches showed activity (Figure 41). However another group optimized the Raf and the Target cascade using MBP full length and the partly activated MAP KK (data not shown). Although the MAP KK was partly activated this work was done because the MAP KK is of human origin and the setup of the assays will come closer to the actual environment in the body.

CONCLUSION

The goal of developing a HTRF based MAP kinase cascade assay and transferring it into the mass-screening group in Germany was reached in three major steps. First, the assay was developed and optimized; Second, the screening campaign was organized; and third, the assay was transferred to Germany. Where the HTS activities of BASF Pharma are centralized within the facilities of Knoll AG based in Ludwigshafen.

Assay Development and Transfer

The assay development is described in detail on Page 11. The first step was to find a direct substrate. So far no substrates have been found that are phosphorylated at significant rates by the Target. Two substrates which are believed to be in vivo substrates of the Target were assayed using a ^{33}P filter-binding assay. Substrate one was tested using three different constructs provided by our collaborator, substrate two was I?Ba. All substrates gave a relatively low signal. This result lead to seek another strategy, the described MAP kinase cascade module, McDonald et al., 1999. This cascade format has several advantages. It has a high amplification ability, which would decrease the amount of Target used in the cascade to get a decent signal to noise ratio. The assay consists of three enzymes in a row, activated MAP KKK (Raf1), inactive MAP KK (MEK 1), and inactive MAP K (ERK 2). In the modified assay the Target was used instead of Raf 1. The Target and Raf 1 are both MAP KKK and use MAP KK as a substrate, the activated MAP KK phosphorylates in turn MAP K. MAP Ks can be assayed using their ability to phosphorylate myelin basic protein (MBP) at high rate. MBP is commercially available, as full-length protein, or as a truncated peptide version. An anti-phospho antibody, specific for a particular phosphorylation site of MBP, Thr 98, is also commercially available. This allows the use of fluorescence technologies such as HTRF as detection method which is preferred for HTS. It is known that MAP KKK can phosphorylate MBP but only to a low level (Alessi et al., 1995). This low level of phosphorylation can be disregarded since it occurs on a different site from that detected by the antibody, thus yields no signal in the detection.

At the beginning a modified Raf 1 ^{33}P filter-binding assay was used to introduce the Target into the cascade format by comparing it with Raf 1. The obtained results were inhomogeneous since the filter washing step was not 100% successful. However it was obvious that this setup would work for the Target. Furthermore, a signal was observed by directly incubating the Target with, confirming that MBP is a substrate for the Target.

The cascade was transferred from the radioactivity based format into the fluorescence based format HTRF. A truncated MBP peptide was introduced at this point, since biotynilated full length MBP was not available. This peptide with the sequence Biotin-Ahx-KNIVT^{P}PRTPPPSQGKK-Amide is phosphorylated at only one sight, which can be detected by the above mentioned antibody. The results of both, Target and Raf1 cascade, came out similar. All wells containing no peptide and the MAP KKKs alone showed the same result. Whereas the cascade signals were significantly higher. The activated MAP KKK showed both no signal assayed directly with the peptide which eliminated the above mentioned background totally. However a high noise-signal was observed. This noise is due to the HTRF format, and the ratio measurement within it. This ratio is due to the signal caused from the free europium and to europium in proximity with SAXL. The cumbersome filtering step involved in the ^{33}P assay, the safety issues of the handling of the isotope, and low throughput, make the radioactive assay less preferable, thus HTRF was further pursued.

Further optimization of the cascade was carried out. At the beginning the Target was titrated against MAP KK and MAP K. The two later enzymes were used at excess amounts. The over all amount of Target should be kept as low as possible, since the expression and purification of it is very cumbersome due to difficulties in activation and conglomeration. The lowest Target concentration was determined. The signal started to saturate at a Target concentration of 1.2 ng/well. Another important piece of information which could be observed from this experiment is the time needed for the signal development. After the stop of the enzyme reaction, the revelation buffer is added. The binding of the biotinylated substrate to the streptavidine labeled allophycocyanine is very fast due to the high binding affinity. The time limiting factor is the affinity of the antibody epitope to its paratope at the

substrate. It seems that this affinity is low and in order to develop a good signal the mixture has to be incubated several hours.

Subsequently the MAP KK and MAP K concentrations were optimized by tritrating them against each other. The setup should use as less Target as possible, the other enzymes could be purchased to a reasonable price and could use in excess amounts. After a series of titrations MAP KK was standardized with 12.5 ng/well and MAP K with a concentration of 125 ng/well.

After the optimizing the enzyme concentrations, the reaction time was optimized. This reaction time should neither be too long nor to short, both would cause problems later for the robotic system. The reaction time had to be coordinated with the necessary pipetting steps. Very short incubation time would collide with the pipetting steps, and very long incubation time would decrease the throughput. Therefore a time course experiment was performed. This time course showed a very long lag phase of about one hour. McDonald, et al, 1999, also reported this lag phase. They explain this phenomenon as a reflection of the time required for the Target to phosphorylate and activate MAP KK, and for the DSK MAP KK to phosphorylate and activate ERK. MAP KK phosphorylates the MAP K at Thr-183 and Tyr-185 in two steps. A single molecule of MAP KK binds to MAP K and phosphorylates one residue (Thr or Tyr), dissociates, and has to bind again in order to phosphorylate the second residue (Burack et al., 1997). This two step phosphorylation takes time and slows down the reaction. After two hours the signal rose to a ratio of 1,200. Over the whole time course the signal to noise ratio was 1:12, 100 at time point 0 minutes and 1,200 after two hours. Compared to the desired signal to noise ratio of about 1:4-1:6 for HTRF, this ratio is very good, however, it requires a long incubation time of 2 hours. Another experiment was carried out to determine if this lag phase can be decreased. To achieve this the amount of Target and the amount of MAP KK were increased, whereas the amounts of MAP K was decreased. More active Target should phosphorylates more MAP KK. The increased amount of MAP KK could in turn phosphorylates the decrease amount of MAP K faster and hence shorten the lag phase. The time course came out as expected. The lag phase could be shortened from 60 to 20 minutes. After consulting the HTS group it was decided to use the setup with lower

amounts of Target. The two hours incubation time was manageable by the robotic system and Target could be saved.

Once the optimized protocol was in place the mass screening was prepared. All enzymes for the cascade, the substrate, the europium labeled antibody, and the SAXL were ordered, and their prices were negotiated. A screening campaign should optimally run with the same batch of each reagent, which could avoid timely intensive quality control of the samples as well as additional optimization experiments. The amounts were calculated based on the number of data points to be collected, and a total of 50% were added for security. This was necessary for setting up the robotic system, eventually failed runs, and the unavoidable dead volumes in the pipetting system on the robot. Timelines for the screening were agreed with the HTS-group in Germany and the reagents had to be available according to these. Unfortunately Upstate Biotech could not hold their promised time line for providing the reagents. The assay transfer to Germany however could not be delayed. For this reason Suitable amounts of the old batches of enzymes along with the optimized protocol were shipped to Germany in advance for the transfer and initial experiments within the HTS group. The transfer was completed within four days. I presented a seminar in order to introduce the assigned biology-lab-technician and the staff to the detection method, HTRF, the cascade format, and the overall conception of the assay.

Because of the short time window, only a few experiments could be performed, to investigate how the assay would perform under the altered conditions in the laboratory in Germany. These different conditions included the detection device, new reagents, and the handling, like the pipetting etc. For the first experiment all buffer and reagents were prepared fresh with the reagents purchased in Germany while the enzymes, the substrate, the labeled antibody and the SAXL were sent from BBC. The inital experiment was done in the 96-well format and according to the protocol. The setup was modified altered in a way to reduce pipetting steps within the robotic system. The substrate was mixed with ATP, to reduce the pipetting steps on the robot from four to three. The signal came out surprisingly high at an ratio of about 13,000 with a background of about 900. The assay worked in a similar fashion as it was performed at BBC. The maximum signal was reached

with an amount of about 1 ng Target per well. The differences in the intensity of the signal could be explained by differences in the detection device. The signal to noise ratio rose to 1: 14, which gave the possibility to further reduce reagents. Subsequently an experiment to determine the optimal SAXL and RY01 concentrations was performed. As a result the amount of antibody needed for the signal development could be decreased by one third. Since this experiment was rough more reductions could be expected through further optimization. Next it was tried to miniaturize the assay from the 96- in the 384-well format. First the optimal volume for the plates had to be determined. The laser of the reading device focus on a distinct height in the well, a volume too low results in a false reading, a volume too high would waste the reagent. A volume of roughly 60 µl was determined to be necessary for the detection. Since the 384-well plate can hold about 100 µl, a volume of 80 µl was chosen, on one hand to be on the safe side and on the other hand to avoid run over of the liquid which would result in cross contamination. With the above determined settings a final experiment was performed in order to compare the 96-well performance with that of the 384-well. At the same time it should be investigated if the pipetting steps could be reduced further by mixing the revelation-cocktail with the stop solution, 0.5 M EDTA, and the MAP KKK / MAP KK / MAP K cocktail mixed with the substrate. The 96-well format showed a much higher signal, but the assays performed in the same fashion as the 384-well plate. Both setups reached their highest signal at a concentration of about 3 ng Target per well. Due to time and Target shortage no further experiments were performed. Further optimization and teaching of the robot were carried out independently in the HTS group. After the setup of the robot 50 of 384-well plates could be assayed per day, four days a week. The whole screening campaign should take about three months once all reagents are in place.

Follow-Up Assay

Besides the above described assay development and transfer, follow up assays had to be set up. Three different approaches were used to design a suitable follow-up assay to rule out false positive hits and to determine specificity. First scintillation

proximity assay, second a splitted cascade and third the above mentioned Raf-cascade.

The scintillation proximity assay was approached as an alternative technology to detect phosphorylation. Even though this approach is using ^{33}P, it would be a valuable alternative to HTRF, since compound showing up as inhibitors in both distinct different detection methods could easily be recognized as true inhibitors. Due to the high background and time-consuming optimization this approach was sidelined, and will be eventually picked up at a later stage.

The second approach was the breakdown of the cascade into three independent assays: First, the actual cascade with the active Target with inactive MAP KK and Map K; Second, the activated MAP KK with the inactive MAP K, and third, the activated MAP K alone. Within this format it could be determined at which step an inhibitor really inhibits. This development was successful and can be used later for the follow up assay.

However the third approach, a Raf-cascade, will be used as a follow up assay in Germany. This setup was optimized by another group, and therefore the data are not shown. In this assay only one experiment need to be performed besides the primary screen, in contrast to the two experiments needed for the 2nd approach (splitted cascade). Compounds showing up in both cascades could be considered as non-specific. Compounds showing up in only one cascade could be considered as specific for that cascade, either the original Target or Raf, and should be investigated further.

MBP full length vs. peptide

As mentioned at the beginning of the project biotinylated full length MBP was not available. After the offer from Upstate Biotech a sample was purchased and tested in the cascade assay. MBP full length was believed to result in a stronger signal and it should be tested if reagents could therefore be saved. The results were as expected, a higher signal as MBP peptide, however, at the same time a huge background signal could be observed. After further investigations this could be traced to the MAP KK. The results showed that the believed inactive enzyme is not

fully inactivated and was able to phosphorylate MAP K at detectable amounts. The above mentioned order of the enzymes was put on hold until Upstate Bioteck was able to produce a fully inactivated MAP KK. Therefore they switched from a mouse MAP KK to a human MAP KK construct. Even this did not remove the activity. However the human construct had its advantages. The setup would be closer related to the Target environment in vivo. This and the still not available direct substrate for the target were the reason to look if the cascade assay could somehow optimized by using MBP full length in combination with the partly active MAP KK. The optimization of the cascades Raf as well as the Target were done by another part of the group (data not shown).

OUTLOOK

So far the assay is established and validated in the HTS screening group in Germany and the screening campaign will be performed in the near future. The campaign will take about three months to detect eventual hits, which could be further investigated at BBC in the USA.

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APPENDIX 1: TABLES

Appendix 1. 1: Conversion table curie (Ci) in becquerel (Bq) and in disintegrations per minute (dpm) (Maekinnen). (Page 22)

Curie	Becquerel	Disintegrations / minute
1Ci	37GBq	$2.2*10^{12}$ dpm
1mCi	37MBq	$2.2*10^{9}$ dpm
1µCi	37kBq	$2.2*10^{6}$ dpm
1pCi	37mBq	2.2*dpm
1Bq	27pCi	60*dpm
1kBq	27nCi	$60*10^{3}$ dpm
1Mbq	27µCi	$60*10^{6}$ dpm

Appendix 1. 2: Extract of the decay table from ^{33}P. The numbers must be multiplied with the theoretical activity to get the actual activity (Amersham Pharmacia Biotech: Ser/Thr kinase SPA assay handbook). (Page 23)

days	0	1	2	3	4	5	6	7	8	9
-10	1.314	1.287	1.244	1.211	1.178	1.146	1.115	1.085	1.056	1.028
0	1	0.973	0.947	0.921	0.897	0.872	0.849	0.826	0.804	0.782
10	0.761	0.741	0.721	0.701	0.683	0.664	0.646	0.629	0.612	0.595

Appendix 1.3: Figure 17, Page 43: Substrate identification: Three constructs of the same substrate S1 to S3 and IkBa were assayed with three different Target constructs, T1 to T3. The substrates were titrated against a fixed concentration of the Target constructs.

S1 [ug/rxn]	T1	T2	T3
15	773	415	181
5	783	153	70
1.7	617	28	11
0	0	0	0

S2 [ug/rxn]	T1	T2	T3
15	913	153	44
5	848	65	6
1.7	722	33	0
0	0	0	0

S3 [ug/rxn]	T1	T2	T3
15	1043	202	87
5	651	1046	74
1.7	882	73	24
0	0	0	0

IkBa [ug/rxn]	T1	T2	T3
10	952	113	64
3.3	944	69	25
1.1	547	40	0
0	0	0	0

Appendix 1. 4: Comparison of the Target cascade vs. the Raf-1 cascade, by using a ^{33}P filter-binding assay (Figure 18, Page 44).

CASCASDE	RAF		Target	
Substrate	-	+	-	+
MAP3K/MAP2K/MAPK	462	7398	717	21269
	261	8806	58543	17166
MAP3K/MAP2K	1106	515	527	7466
	218	806	602	6646
MAP3K	125	299	784	5880
	1071	7426	9491	6299
NO ENZYME	209	1134	14566	292
	34098	892	12736	140

Appendix 1. 5: Raf vs Target cascades, assayed with and without substrate; MAP KKKs assayed directly with the substrate. (Figure 19, Page 45)

	Full cascade		only inactive MAP3K	
Substrate	-	+	-	+
Raf	135	272	133	132
	135	345	146	149
Target	119	262	136	128
	128	284	133	127

Appendix 1. 6: Target titration 100ng/well-0ng/well by 1/3. Since the titration saturated between 1-1.5ng/ well (overnight incubation), the graph is limited to only 2ng/well. Overnight incubation increased the signal by 25%. (Figure 19, Page 47)

					Average	
Target [ng/well]	Incubation 30 minutes		Incubation overnight		30 minutes	Overnight
100.0	1325	928	1571	1515	1126	1543
33.3	1323	1363	1642	1667	1343	1654
11.1	1317	1364	1636	1624	1341	1630
3.7	1266	1252	1594	1610	1259	1602
1.2	1138	1127	1525	1626	1133	1576
0.4	805	840	1419	1492	822	1455
0.1	413	421	980	1032	417	1006
0.0	126	126	144	131	0	0

Appendix 1. 7: MAP KK titration 12.5ng/well-0ng/well by 1/3. The chart shows linearity over the whole range. (Figure 20, Page 48)

MEK [ng]	Value 1	Value 2	Average	Background
12.5	583	595	589	462
4.2	311	357	334	207
1.4	180	197	189	62
0.5	145	146	145	18
0.2	144	141	142	15
0.1	139	124	132	5
0.02	130	128	129	2
0	127	127	127	0

Appendix 1.8: HTRF signal development. The plate was read after 5 and 30 min, and 1, 1.5, and 2.5 hours. In addition, an overnight read was performed. (Figure 21, Page 49)

Target [ul]	5min	30min	1h	1.5h	2.5h	o/n-100
25	835	1359	1432	1471	1403	1630
8.3	868	1377	1419	1499	1362	1715
2.8	862	1361	1374	1374	1290	1621
0.9	676	1230	1276	1304	1210	1572
0.3	457	930	975	1095	1007	1479
0.1	213	474	534	632	584	1209
0.03	72	138	147	178	162	456
0	0	0	0	0	0	0

Appendix 1.9: ATP titration starting with 500µM/well Experiment 18. (Figure 22, Page 50)

ATP [uM]	Ratio		Average	Background
500	862	964	913	774
166.7	554	619	587	448
55.6	256	281	268	129
18.5	146	149	147	8
6.2	132	114	123	0
2.1	133	116	125	0
0.7	131	128	129	0
0.2	120	127	123	0
0.08	124	126	125	0
0.03	131	123	127	0
0.01	135	131	133	0
0	137	142	139	0

Appendix 1.10: Target time course. Two different ATP concentrations were assayed, 10µM and 100µM. (Figure 23, Page 51)

TIME [min]	0	3	5	10	15	20	30	45	60	90	120
100 uM ATP	123	123	126	124	133	130	163	245	401	882	1291
10 uM ATP	120	137	125	123	125	129	129	129	127	135	157

Appendix 1.11: Target time course. (Figure 24, Page 51)

stop time [min]	0	5	10	20	40	60	90	120
ratio	152	146	145	414	1472	1840	1946	1982

Appendix 1.12: Comparison of the active Target versus an inactive Target mutant. (Figure 25, Page 52)

Enzyme [ng/well]	Active Target	Inactive Mutant
10.00	1288	125
3.33	1020	131
1.11	822	123
0.37	351	127
0.12	168	133
0.04	132	136
0.01	130	139
0.00	133	129

Appendix 1.13: (Figure 26 / 27, Page 55) 27: MAP KK-Cascade; Titration of activated MAP KK against the inactive MAP K to determine the optimal concentrations of both. 27: MAP KK-Cascade; Titration of inactive MAP K against MAP KK to determine the optimal concentrations of both.

MEK [U/well]	1	2	Average	background
0.1	869	908	889	807
0.03	872	911	892	810
0.01	866	909	888	806
0.004	757	774	766	684
0.001	554	591	573	491
0.0004	396	418	407	326
0.0001	252	249	251	169
0	101	100	101	0

MAP K [ug/well]	1	2	Average	background
125	810	837	823	746
41.7	771	790	781	703
13.9	676	756	716	638
4.6	582	576	579	501
1.5	383	398	390	313
0.5	305	241	273	195
0.2	153	162	157	80
0	81	74	78	0

Appendix 1.14: (Figure 28, Page 55) Activated MAPK-Assay; Titration of the enzyme to determine the optimal concentration.

MAP K [ng/well]	1	2	Average	background
300	746	679	713	636
100	905	780	843	766
33.3	909	881	895	818
11.1	862	832	847	770
3.7	751	726	739	662
1.2	556	347	452	375
0.4	371	375	373	296
0	76	80	78	0

MAP K [ug/well]	1	2	Average	background
100	797	808	803	728
33.3	708	708	708	633
11.1	591	585	588	513
3.7	386	411	399	324
1.2	263	246	254	179
0.4	146	164	155	80
0.1	93	99	96	21
0	78	71	75	0

Appendix 1.15: (Figure 29, Page 56) Time course of the optimized MAP KK-cascade and MAP K assay.

time [min]	0	5	10.5	20	30	40	50	60
MEK-cascade	71	87	121	227	344	407	439	486
MAPK	74	140	182	270	357	369	403	436

Appendix 1.16: (Figure 30, Page 59) SPA assay with the Target cascade and with the addition of an unspecific MAP kinase inhibitor.

	1	2	Average
Target 10ng/well	16647	16674	16661
Target 10ng/well; 421386 80uM	14441	13946	14194
no enzyme	13795	14164	13980

Appendix 1.17: (Figure 31, Page 59) SPA assay Target titration.

Target [ng/well]	1	2	Average
50	23093	22934	23014
17	21348	20763	21056
6	18773	18640	18707
2	18122	17977	18050
0,6	19709	19684	19697
0,2	19069	19011	19040
0,1	18152	17488	17820
0	16866	17547	17207

Appendix 1.18: First experiment to see if the assay would work in Germany. In addition, ATP and substrate were mixed to decrease the number of reagents to be pipetted. (Figure 33, Page 64)

Target [ng/wel]	Normal	Mixed
10	13974	12376
3.3	13178	12959
1.1	119525	10422
0	850	922

Appendix 1.19: Three setups for reducing pipette steps in a 96-well: First, normal cascade; second, one-step reduced cascade with a mix of MAP KK, ERK, and substrate (mix1); and third, a two-step reduced cascade with the addition of the EDTA mixed into the revelation buffer (mix2). (Figure 34, Page 65)

Target [ng/well]	1	2	3	4
10	2938	3837	5315	5784
3.3	2353	3139	4302	4621
1.1	1290	1604	1657	1852
0.4	928	1044	819	805
0.1	834	1033	748	766
0.04	877	1038	731	709
0.01	873	1017	729	735
0	861	1009	736	743

Appendix 1.20: Two setups for reducing pipette steps in a 384-well plate: one-step reduced cascade with a mix of MAP KK, ERK, and substrate (mix1); and a two-step reduced cascade with the addition of the EDTA mixed into the revelation buffer (mix2). (Figure 35 / 36, Page 66 / 67)

	96				384	
	30min		15hours		30min	15hours
Target ng/well	Normal	Mix	Normal	Mix	Mix	Mix
10	4731	5524	11307	12055	2813	4378
3.3	3954	4305	10135	10797	2925	4374
1.1	2585	2339	7648	7287	2616	3915
0.4	1257	1127	3473	2752	1639	2857
0.1	858	817	1299	1236	1049	1639
0.04	837	793	1118	939	884	990
0.01	775	804	938	906	874	925
0	802	766	905	888	919	1037

Appendix 1.21: (Figure 37, Page 68) Target titrated using Bio-MBP-peptide and full length, as well as different batches of MAP KK and MAP K.

	MEK/ERK old batch	
Target [ng/well]	peptide	full length
10,0	451	2537
3,3	394	2548
1,1	264	2670
0	109	1980

	MEK/ERK new batch	
Target[ng/well]	peptide	full length
10,0	489	2525
3,3	448	2581
1,1	357	2621
0	108	2539

Appendix 1.22: (Figure 38, Page 69) Target cascade, inactive MAP KK, inactive ERK, and inactive MAP KK in combination with inactive MAP Kassayed with the MBP-peptide and the full length MBP

	casc.10ng	casc.3ng	casc.1ng	casc.0ng	MEK	ERK	MEK/ERK	no enzyme
peptid	383	337	239	131	114	103	109	109
full length	2269	2293	2291	1552	106	245	1059	108

Appendix 1.23: (Figure 39, Page 70) Raf cascade, assayed with the MBP-peptide and the full length MBP.

Raf [ng/well]	full length	peptide
250	2544	309
83,3	2480	295
27,8	2455	294
9,3	2437	201
3,1	2245	136
1,0	1687	108
0,3	1196	96
0	579	95

Appendix 1.24: (Figure 40, Page 70) Activated and inactivated MAP KK respectively MAP Kwere assayed alone and in combination.

	pep active	peptide unactive	fl active	fl unactive
MEK	100	99	117	113
MAPK	201	93	2419	142
MEK/MAPK	168	106	2205	446
no enzyme	107	105	114	132

Appendix 1.25: (Figure 41, Page 71) Comparison of three different MAP KK batches.

	MBP peptide	MBP full length
MEK / MAP # 1	85	788
MEK / MAP # 2	82	945
MEK / MAP # 3	80	1220
no enzyme	80	79

APPENDIX 2: PROTOCOLS

Appendix 2.1: Target Protocol

HTRF Target Cascade Assay: Measure IC50 using HTRF assay format with biotinylated – MBP (myelin basic protein) as substrate.

Ernzyme Reaction:

Reagent:	Stock	Stock [mM]:	Volumes	Conc. / reaction	Enzyme mix
Target	0.05mg/ml		2ul + 1mlRB	1ng/ well	2ul Target + 5ul MAP KK + 50ul MAPK + 945ul RB reagents a [??] enough for 100 data points
MEK	0.25mg/ml		5ul+1mlRB	12.5ng	
MAPK (ERK2)	0.25mg/ml		50ul+1ml RB	125ng	
Bio-MBP-peptide*		5mM	2.4ul+1ml RB	3uM	
ATP		10mM	40ul+1ml RB	100uM	
EDTA		500mM, pH7	1ml		

*Sequenz: Biotin-Ahx-KNIVTPRTPPPSQGKK-Amide

Reaction buffer (RB):	Stock	Stock [mM]:	Conc in 10x buffer [mM]	Dilution of stock for 10x	Needed vol. For 100ml	Final conc [mM]
Tris-HCl pH 7.5		1000	500	1:02	50	50
MgCl2		1000	100	1:10	10	10
EGTA		500	10	1:50	2	1
DTT		1000	20	1:50	2	2
Brij 35	30%		1%	1:300	0.333	0.01%
Beta-phosphoglycerol		2500	50	1:50	2	5
dH2O					33.7	

Signal Development:

Reagents	Stock solution
RY - 01 ab*	0.223mg/ml
SAXL (SA conc)	1.6mg/ml

Revelation Mix	Volumes
RY - 01 ab (diluted)	125ul
SA-XL	15ul
Rev Buffer with 0.1% BSA and 0.01% Tween	15 ml

Revelation Buffer	Final Concentration
2.5 ml of 1 M HEPES	50mM
10 ml of 2M KF	0.4M
bring to pH 7.0 add 49.5 ml H2O	
50ul 10% Tween 20	0.01% Tween**
500ul 10%BSA	0.1% BSA**

* Dilute 10x with Rev Buffer +0.1% BSA for storage

**Added just before use

Procedure:	
Enzyme Reaction	**Volume (ul)**
Buffer or compound	10
Enzyme Mix	10
Bio-MBP	10
ATP	10
2 hrs reaction	
Add 150ul of revelation mix to each well	Allow to sit overnight, then read on the HTRF Machine

Appendix 2.2: MEK/MAP K-protocol

The only changes to the prior Target protocol are in the enzyme reaction. Signal development and procedure are the same.

Reagent:	**Stock**	**Stock [mM]:**	**Volumes**	**Conc. / reaction**	**Enzyme mix**
MEK active	100U/ml		10ul+1mlRB	0.01U/well	10ul MEK active + 6ul MAPK inactive + 9850 ul RB
MAPK (ERK2)	0.25mg/ml		20ul+1ml RB	50ng	
Bio-MBP full length		2.58 mg/ml	1ul+1ml RB	2.5ug	
ATP		10mM	40ul+1ml RB	100uM	
EDTA		500mM, pH7	1ml		

Appendix 2.3: MAPK-protocol

The only changes to the prior Target protocol are in the enzyme reaction. Signal development and procedure are the same.

Reagent:	**Stock**	**Stock [mM]:**	**Volumes**	**Conc. / reaction**	**Enzyme mix**
MAPK (ERK2) active	0.1mg/ml		1ul+1ml RB	1ng	1ul MAPK active + 1ml RB
Bio-MBP full length		2.58 mg/ml	1ul+1ml RB	2.5 ug	
ATP		10mM	40ul+1ml RB	100uM	
EDTA		500mM, pH7	1ml		

Appendix 2.4: Raf-protocol

The only changes to the prior Target protocol are in the enzyme reaction. Signal development and procedure are the same. This assay was optimized by Andrew Biliecky in another part of the group.

Reagent:	Stock	Stock [mM]:	Volumes	Conc. / reaction	Enzyme mix
Raf	0.2U/ul		25ul + 1mlRB	0.05ng/ well	25ul Target + 5ul MEK + 20ul MAPK + 950ul RB
MEK	0.25mg/ml		5ul+1mlRB	12.5ng	
MAPK (ERK2)	0.25mg/ml		20ul+1ml RB	50ng	
Bio-MBP-full length	2.58mg/ml		1ul+1ml RB	2.5ug	
ATP		10mM	40ul+1ml RB	100uM	
EDTA		500mM, pH7	1ml		

Target Purified by N. Bump

Human Mek-1 Inactive: UBI Cat# 14-420

Mapk 2/ Erk2 Inactive: UBI Cat# 14-198

MBP, dephosphorylated: UBI Cat# 13-110

RY01 ab (anti-phosopho MBP clone P12 UBI# 05-429) labeled by cis-bio with Eu-Cryptate

SA-XL Prozyme cat# PJ25S

APPENDIX 3: FLUORESCENCE POLARISATION / FLUORESCENCE CORRELATION SPECTROSCOPY / ELISA

Appendix 3.1: Fluorescence Polarization (FA /FP)

Fluorescence anisotropy, FA, and fluorescence polarization, FP, are two different ways to measure the same process.

After the excitation of fluorophores with polarized light, their emission light is also polarized. The extent of polarization is described as anisotropy (r). Anisotropy highly depends on the orientation of the fluorophores to the excitational polarized light. However, molecules move and tumble in solution (Brownian molecular rotation) and the planes in which light is emitted can vary substantially from the plane used to excite the molecules. One structural characterisitic of fluorophores is the existence of transition moments for absorption and emission, which lie along a specific direction within their fluorophor structure. In solution, fluorophores are normally randomly oriented and those that have their absorption transition moment situated in the direction of the polarized light will be excited, resulting in a partially excited population of fluorophores. Rotational diffusion of a fluorophor (or fluorophor labeled analyte) leads to depolarization of the fluorescence. High polarization values indicate a large molecule that has rotated little during excitation; low values mean the molecule is small and moves very quickly. The time the molecule needs for rotation is related to the viscosity of the solvent, temperature, the gas constant and, most importantly, to the molecular volume. The binding of two molecules to each other, degradation, or conformation changes can cause changes in molecular volume (Chechovich, 1995).

More technically, within an electromagnetic wave, the electrical field is perpendicular to the magnetic field in the z-axis. Unpolarized light has equal amplitudes of the electrical vector normal to the direction of light propagation. In contrast, polarized light has greater amplitude in one of the directions. After excitation of the sample with the polarized light, derived from polarizer 1, the polarized excitation is analyzed by polarizer 2. This is accomplished by orienting the

polarizer parallel (II) to the direction of the polarized light, as well as perpendicular (⊥) (Figure 42).

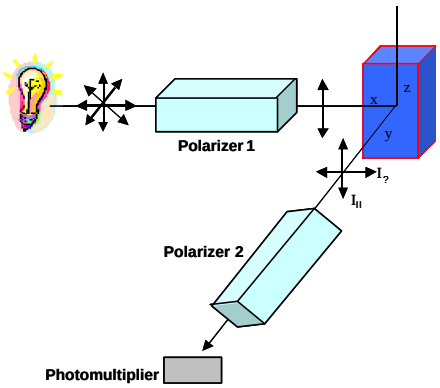

Figure 42: Schematic diagram for the measurement of fluorescence anisotropy. The polychromatic light beam is horizontally polarized by polarizer one, and the emission is analyzed parallel and perpendicular to the direction of the polarized emission (Lakowicz, 1999, modified).

The values derived from polarizer 2 are used to calculate the anisotropy according to equation 6.

$$r = \frac{I_{II} - I_{\perp}}{I_{II} + 2I_{\perp}} \qquad \textbf{[8]}$$

The anisotropy is a dimensionless quantity that is independent of the total intensity of the sample, because the difference (I_{II} - $I_{\perp}$) is normalized by the total intensity (I_{II} + 2$I_{\perp}$). The term polarization is given by equation 7.

$$P = \frac{I_{II} - I_{\perp}}{I_{II} + I_{\perp}} \qquad \textbf{[9]}$$

Equations 8 show the relationship between anisotropy and polarization.

$$P = \frac{3r}{2 + r} \qquad r = \frac{2P}{3 - P} \qquad \textbf{[10]}$$

For the kinase approach, several scenarios are possible. For example, a fluorescence-labeled peptide, which is phosphorylated by tyrosine-kinase activity, is immunocomplexed with an anti-phospho-tyrosine antibody. Unphosphorylated substrate will not bind to the antibody and hence keeps its free rotational mobility, resulting in a low polarization signal. The larger fluorescent peptide-antibody complex will lose its mobility and increasing concentration will cause an increase in polarization.

Another approach is to use the same format as a competition immunoassay. Here the phosphorylated kinase product competes with another peptide, which is labeled with a fluorophor, for binding to the anti-phospho-tyrosine antibody. Phosphorylated

peptide causes less-fluorescent peptide to bind and thus there is a decrease in the FP signal. The signal is inversely proportional to the kinase–product concentration (Seethala et al., 1998).

Appendix 3.2: Fluorescence Correlation Spectroscopy (FCS)

Fluorescence correlation spectroscopy is a statistical-physics-based tool to obtain information of the fluorescence activity of small molecules, down to the single-molecular level. Within this technique a well-defined space of time is observed as a function of time.

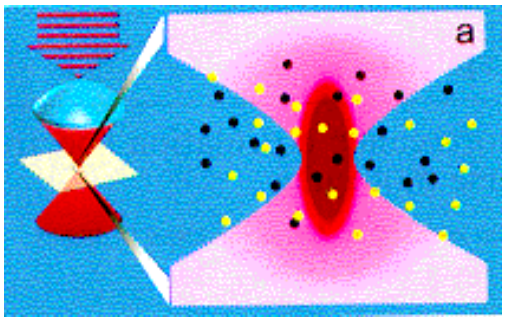

Figure 43: Schematic drawing of a molecules in confocal microscopic optical systems (Maiti et al., 1997).

The most common way to get fluorescence signals is to excite a suitable volume of molecules and to measure the average amount of fluorescence. At the same time, the background fluorescence will be extracted in some way, for example, through the time delay of the measurement during TRF. As described earlier, it is difficult due to background problems to measure concentrations $<10^{-9}$. Multiplying this number by Avogadro's constant[2], we yield an amount of around 10^{15} molecules per ml. The average effect of the signal is a result of all incidents generated by any single molecule in the detection space and time. To get a single signal, the space for the molecule has to be as big (small) as the single molecule itself. As in our example of the 10^{-9} solution, with 10^{15} particles per liter each single molecule would occupy the space of 10^{-15} liter = 1 femto liter. In order to detect a single molecule, the light beam must be focused in a volume of as little as one femtoliter. This can be accomplished through confocal microscopic optical systems (Figure 43).

[2] $N = 6{,}022 \cdot 10^{23}$

Once the detection volume is decoupled from the sample volume, the movement of the molecules can be measured as a function of time in the range of their lifetime τ_F. The signal is analyzed by statistical techniques. These techniques provide autocorrelated information such as concentration, diffusion time, and average brightness per molecule.

Background consists of scattering laser light from the solvent and the optics. Therefore, the success of getting a good signal depends on the ability to detect as large a photon flux as possible. On the other hand, the photostability of the dyes has to be taken into account. Whether a dye is usable for FCS depends on its quantum yields. Whereas photobleaching or loss of fluorescence activity, influences the statistical accuracy of the measurement. Loss in fluorescence activity means loss in signal, which in the case of bleaching does not correlate to the actual activity, for example, of a kinase. (Eggeling, et al., 1998).

In the case of a kinase assay applied to FCS, the difference in translational diffusion rates of large versus small molecules will be measured. As discussed above, kinase activity can be measured by, for example, coupling an anti-phospho-fluorescence labeled antibody to a phosphorylated substrate. Each molecule that diffuses through the illuminated confocal focus gives rise to bursts of fluorescent light quanta during the entire course of its journey, with each individual burst being registered. The length of each photon burst corresponds to the time the molecule spends in the confocal focus. The photons emitted in each burst are recorded in a time-resolved manner by a highly sensitive single-photon detection device. An uncoupled antibody will diffuse faster than an antibody-substrate complex (Eigen et al., 1994). The signal derived diffusion coefficient of the observed molecules (unbound fluorophor labeled antibody / antibody-substrate complex) allows to distinguish between different states of these particles without physical separation. The diffusion coefficient itself can be used to determine such factors as concentration or degree of binding (Trier et al., 1999).

Appendix 3.3: Enzyme Linked Immunosorbent Assay (ELISA)

ELISA is an amplification method and is widely used in immunology and other research fields. Even though ELISA is not a fluorescence-based assay, but rather an absorbance-based one, it will be discussed at this point briefly.

There are two different versions in use, the single and the sandwich ELISA. The single ELISA uses only one antibody. Here the microplate is coated with the substrate, for example, kinase substrate. After adding kinase and removing it by washing, the phosphorylated peptide is labeled with an anti-phospho antibody labeled with horseradish peroxidase (HRP). The surplus antibody in the solutions is also removed by washing and the substrate specific for the HRP is added. HRP activity is measured by absorption (Eckard, et al., 1996). Within the sandwich ELISA, an antibody is bound to the surface of, for example, microplate. Its function is to bind antigens dissolved in the solution. All other molecules in the solution can subsequently be removed by a washing step. Next, a second antibody is introduced into the plate. This detection antibody is labeled with an enzyme, for example, HRP, and has a different epitope than the catching antibody, but for the same antigen. Bound antigens can therefore be labeled with a second antibody (sandwich). Finally, the substrate for the enzyme is added and the reaction can be measured through absorbance.

APPENDIX 4: VENDORS-LIST

Amersham Pharmacia Biotech Inc. 800 Centennial Ave PO Box 1327 Piscataway, NJ 08855, USA www.apbiotech.com	ATP, BSA, Ser/Thr kinase SPA assay
Upstate Biotechnology 10 Old Barn Road Lake Placid, NY 12946, USA www.upstatebiotech.com	MEK1 inactive, MAPK inactive Raf-1 Kinase Assay Kit
Sigma-Aldrich PO Box 14508 St. Louis, MO 63195, USA www.sigma-aldrich.com	EDTA, MgCl, HEPES, KF, Brij 35 β-phosphoglycerol
Bio-Rad Laboratories 2000 Alfred Nobel Drive Hercules, CA 94547 www.bio-rad.com	Tween 20
Roche Diagnostics Corporation 915 Hague Road PO Box 50414 Indianapolis, IN 46250, USA www.IBuyRMB.com	DDT
Cis-Bio B.P. 32 91192 GIF-SUR-YVETTE, France http://www.cisbiointernational.fr	RY01
SynPep Corporation PO Box 2999 Dublin, CA 94568, USA www.synpep.com	Bio-MBP-pep

Prozyme 1933 Davis Street, Suite 207 San Leandro, CA 94577, USA www.prozyme.com	SAXL
Millipore 80 Ashby Road Bedford, MA 01730, USA www.millipore.com	Multiscreen- filter plates
NEN Life Science Productions Inc. 549 Albany Street Boston, MA 02118, USA www.nen.com	Gamma-33P-ATP
Life Technologies 9800 Medical Center Drive PO Box 6482 Rockville, MD www.lifetech.com	Dulbeco's Phosphate Buffered Saline w/o CaCl
EG&G Wallac 9238 Gaither Road Gaithersburg, Maryland 20877 www.wallac.com	β-counter, plate seals, scintillation cocktail
Packard Instrument Company 800 Research Parkway Meriden, CT 06450, USA www.packardinstrument.com	Discovery
Boston Biologicals 49 Thackery Road Wellesley, MA 02481 www.bosonbiologicals.com	IkBa