# Studying Sigma-Core Binding of Bacterial RNA Polymerase by LRET:

# Biochemistry and Drug Discovery

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(Dr. rer. nat.)

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der Philipps-Universität Marburg
vorgelegt von

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presented to the
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at the Philipps-Universität Marburg
by

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from Gelsenkirchen-Buer

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**Studying Sigma-Core Binding of Bacterial RNA Polymerase by LRET: Biochemistry and Drug Discovery**

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The focus of this work was to develop a homogeneous assay for sigma-binding to *E. coli* core RNA polymerase and a fragment of the β'-subunit of the core based on luminescence resonance energy transfer (LRET). The technical advances offered by LRET through the use of long-lived lanthanide fluorophores resulted in a very robust assay suitable for biochemical characterization and high-throughput screening. With this assay I was able to obtain binding constants for the sigma-core interaction. It was also used to screen a small crude natural product library consisting of extracts from marine sponges. One of these samples was found active in this screen and was validated to inhibit transcription by *in vitro* transcription assays.

Furthermore a mouse hybridoma cell line was created that produces a monoclonal antibody (8RB13) that binds selectively to core RNA polymerase from many different bacteria. Since this antibody was also selected for polyol sensitivity, i.e. it releases its antigen under very gentle conditions (40% 1,2-propanediol and 700 mM ammonium sulfate), it allows a rapid and efficient purification of RNA polymerase by immunoaffinity chromatography. Active core enzyme was purified from *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. coelicolor*.

A significant part of this work was to optimize techniques for fluorescence labeling of proteins. A fast and convenient way for fluorescence labeling of recombinant proteins was developed combining purification and on-column derivatization. As a further result of the fluorescence labeling studies, a fast protocol for qualitative and quantitative Western blots using fluorescence labeled primary antibodies was established. By the use of IC5-labeled primary Mabs, a secondary antibody and an enzyme-catalyzed signal producing reaction is no longer needed, which reduces the procedure time, starting from a blocked membrane, to less than 75 minutes for quantitative measurements. Depending on the size of the antigen, I obtained a linear working range over more than two orders of magnitude of measured protein within the range from 1 to 1000 ng.

## Zusammenfassung

Das Ziel dieser Arbeit war es einen homogenen Assay basierend auf Lumineszens Resonanz Energie Transfer (LRET) für das Binden des Sigmafaktors an *core* RNA Polymerase von *E. coli* und an ein Fragment der β'-Untereinheit des *core* Enzyms zu entwickeln. Dabei resultierten die technischen Vorteile von LRET, hervorgehend aus einer langlebigen Lanthanid-Lumineszens, in einem äußerst robusten Assay, der sich zur biochemischen Charakterisierung der untersuchten Proteine und zu einem Hochdurchsatz *Screening* von Wirkstoffbibliotheken nach potentiellen Inhibitoren dieser Protein-Protein Wechselwirkung eignet. Mit Hilfe dieses Assays war es möglich, Bindekonstanten für die Interaktion zwischen $\sigma^{70}$ und $\sigma^{32}$ und *core* RNA Polymerase zu messen. Daraufhin wurde eine kleine Naturstoffbibliothek bestehend aus Extrakten von marinen Schwämmen auf Inhibitoren untersucht, und es konnte eine positive Probe gefunden und *in vitro* dessen inhibitiorische Wirkung auf die transkriptionelle Aktivität der RNA Polymerase bestätigt werden.

Zudem konnte eine Hybridoma Zellinie hergestellt und isoliert werden, die einen monoklonalen Antikörper (8RB13) sekretiert, welcher selektiv an *core* RNA Polymerase verschiedenen bakteriellen Ursprungs bindet. Da dieser Antikörper auf Polyolsensitivität hin selektiert wurde, welches ein Ablösen des Antigens unter sehr schonenden Bedingungen (40% 1,2-Propandiol und 700 mM Ammoniumsufat) ermöglicht, kann er zur raschen und effizienten Aufreinigung von *core* RNA Polymerase mittels Immunoaffinitätschromatographie eingesetzt werden. Auf diesem Wege wurden aktive RNA Polymerasen aus *E. coli*, *B. subtilis*, *P. aeruginosa* und *S. coelicolor* aufgereinigt.

Ein Großteil dieser Arbeit konzentrierte sich auf die Optimierung von Techniken zur effektiven Fluoreszensmarkierung von Proteinen. Daraus resultierte die Entwicklung eines

schnellen und leicht durchführbaren Verfahrens zur Fluoreszensmarkierung von rekombinanten Proteinen in Form einer Derivatisierung des Proteins in säulengebundener Form. Ebenso wurde ein verbessertes Protokoll für eine schnelle qualitative und quantitative Auswertung von Western Blots entwickelt. Durch die Verwendung von fluoreszensmarkierten primären Antikörpern kann auf die Verwendung eines sekundären Antikörpers und die daran gekoppelte Enzym katalysierte Farbreaktion verzichtet werden, welche in den gängigen Methoden ein verstärktes Signal erzeugt. Das hierzu benutzte Protokoll verringert die Gesamtzeit, die für einen Western Blot benötigt wird, auf weniger als 75 Minuten für quantitative Messungen, nach Transfer auf eine Nitrocellulose Membran. Es wurde ein linearer Anwendungsbereich von mehr als zwei Größenordnungen erreicht, der sich abhängig von der Größe des untersuchten Proteins von 1 bis 1000 ng erstreckte.

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Without the support and encouragement of several people, my efforts wouldn't have resulted in the presented thesis and rightly so, my gratitude to them precedes my thesis.

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Above all, I would like to thank my family and Karen Abel for their love and support. Especially my parents, who provided me with the education that ultimately counts and who build the foundation that allowed me to pursue this scientific degree. My Dad always tried to keep my aspiration high, but reality in sight. Although he sadly missed the final months and the rewarding finish of this ambitious endeavor, his spirit together with my mom’s exceptional good faith carried me all the way.

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# Chapter 1

# Introduction

## 1.1 Characterization of protein-protein interactions

Great interest lies in the identification and characterization of protein-protein interactions. While amino acid sequence, structure and catalytic activity can provide valuable information about the function of a protein, the knowledge about its binding partners and the nature of their interactions is necessary for a more detailed understanding of its role within an organism. It will also be crucial for identifying the function of many hypothetical proteins found by a growing number of genomic sequencing projects. Many processes like cell signaling, metabolism, enzymatic activity and specificity are controlled by modulation of their structure and function upon binding of two or more proteins. Only if we can combine this information, can we begin to comprehend the organization and regulation of cellular processes, and eventually manipulate and harness these processes for their use in biotechnology, pharmacology, agriculture and research.

## 1.2 Transcription and its regulation

Transcriptional regulation is an example of a process that is mediated significantly by protein-protein interactions since binding of transcription factors to RNA polymerase confer its binding specificity for promoters of transcribed genes (1, 2). After DNA-directed RNA polymerase is directed to a specific site on the DNA, it is able to melt the DNA and start transcription by synthesizing RNA in 5' to 3' direction using one strand of the DNA as a template. Transcription factors are involved in melting the DNA, initiation, activation or repression, elongation and termination.

### *1.2.1 E. coli RNA polymerase*

A model system for bacterial RNA polymerases is the *E. coli* enzyme. The core enzyme consists of two α subunits (RpoA, 36.5 kDa), one β subunit (RpoB, 151 kDa), one β' subunit (RpoC, 155 kDa) and one ω subunit (RpoZ, 10 kDa) (3). It can bind DNA non-specifically and initiate transcription from single-stranded DNA and free DNA ends. The holo form of RNA polymerase contains core enzyme plus a sigma factor. It specifically binds DNA-sequences at recognized promoters with significantly higher affinity and is then able to initiate transcription from double-stranded DNA. Sigma factors belong to the family of prokaryotic transcription factors that globally regulate gene expression under different conditions and for large sets of genes. Recognized promoter sites share consensus sequences usually centered around bases -10 and -35 relative to the transcription start site at base +1 and are characteristic for each individual sigma factor. That way a sigma factor can regulate sets of genes controlled by promoters that fall within the corresponding consensus sequence. The affinity for the holoenzyme and therefore the strength of a promoter is governed by its equivalency to the consensus sequence. *E. coli* possesses seven sigma factors which are summarized in Table 1.1 (4-8).

**Table 1.1: Summary of *E. coli* sigma factors. The gene, size, recognized consensus sequence and the classes of genes that are regulated are indicated for each of the seven *E. coli* sigma factors.**

| Factor | Gene | Size (kDa) | Consensus Binding Site | Genes Regulated |
|---|---|---|---|---|
| $\sigma^{70}$ ($\sigma^{D}$) | *rpoD* | **70** | TTGACA-$N_{17}$-TATAAT | **Housekeeping** |
| $\sigma^{54}$ ($\sigma^{N}$) | *rpoN (ntrA)* | **54** | CTGGCAC-$N_5$-TTGCA | **Nitrogen metabolism** |
| $\sigma^{S}$ | *rpoS (katF)* | **38** | TTGACA-$N_{12}$-TGTGCTATACT | **Stationary phase** |
| $\sigma^{32}$ ($\sigma^{H}$) | *rpoH (htpR)* | **32** | CTTGAA-$N_{14}$-CCCCATNT | **Heat shock** |
| $\sigma^{F}$ ($\sigma^{28}$) | *fliA* | **28** | TAAA-$N_{15}$-GCCGATAA | **Flagellar proteins** |
| $\sigma^{E}$ | *rpoE* | **24** | GAACTT-$N_{16}$-TCTGA | **Extreme heat shock** |
| $\sigma^{fecI}$ | *fecI* | **19** | GGAAAT-$N_{17}$-TC | **Iron transport** |

The protein levels of sigma factors in the cell are tightly regulated and vary with the physiological growth conditions and the environment (9). The major sigma factor, $\sigma^{70}$, is present at all times and recognizes the majority of promoters in the cell. It is responsible for the transcription of the genes that are required for the general survival of the cell and is therefore termed the housekeeping sigma. $\sigma^{S}$ is responsible for transcription of the genes in stationary phase (10, 11) and is therefore most abundant during this period of the growth cycle whereas its expression level is otherwise very low. Unlike other bacteria, *E. coli* does not sporulate when it encounters low levels of nutrients. Instead it turns into a quiescent form mainly regulated by $\sigma^{S}$ that reduces the expression of essential genes to a minimum until nutrient conditions have improved. $\sigma^{F}$ regulates the transcription of flagellar biosynthesis genes. $\sigma^{32}$ and $\sigma^{E}$ are both involved in heat shock response (7, 12). $\sigma^{FecI}$ regulates genes involved in the citrate dependent iron transport into the cell (13) and together with $\sigma^{E}$ belongs to the family of extracytoplasmic function (ECF) sigma factors. This means that both are localized at the inner membrane of the cell and respond to extracytoplasmic signals. $\sigma^{54}$ regulates genes in response to nitrogen starvation and is the most different among the sigma factors (14, 15). For example it recognizes promoters at position -12 and -24 and unlike the other sigma factors, is capable of binding DNA on its own. The other sigma factors have to be bound to core RNA polymerase in order to bind their promoter recognition sequence. Thus $\sigma^{54}$ does not belong to the $\sigma^{70}$ family unlike all the other sigmas.

### *1.2.2 Structural characteristics of sigma factors and core RNA polymerase from E. coli*

Although the size of the sigma factors varies significantly, they share four regions highly conserved within the σ70-family (Figure 1.1).

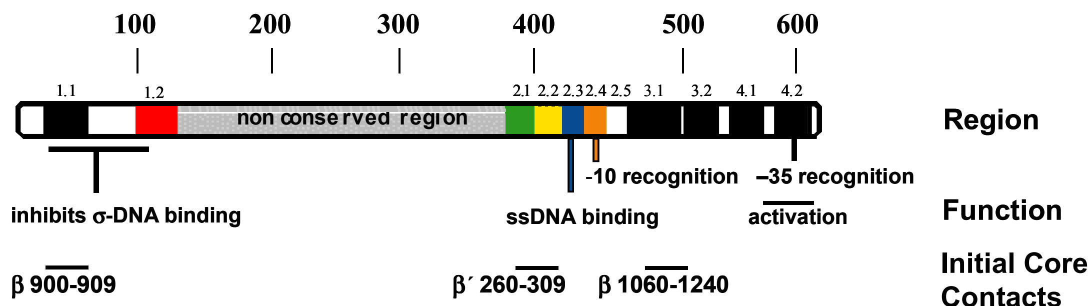


**Figure 1.1: Schematic illustration of the sub-domain structures in $\sigma^{70}$ and their assigned functions (2). Regions of initial contacts to core are indicated at the bottom. The size and position of the regions are indicated by the amino acid residues.**

Region 1 is known to interact with core. The N-terminal region 1.1 is only present in the primary sigma factors $\sigma^{70}$ and $\sigma^{S}$ and it has been shown to mask DNA binding sites of sigma by interacting with region 4 (16, 17). Region 2 contains the main site responsible for sigma binding to core RNA polymerase and is also the most conserved among the $\sigma^{70}$-family (2). It also binds specifically to single-stranded DNA in the -10 binding region of the promoter consensus region. Mutations in region 3 show defects in initiation of transcription. Region 4 is responsible for the contact to the -35 promoter site on the DNA. Although contacts of all four regions of $\sigma^{70}$ to core RNA polymerase have been found, there is evidence for the fact that region 2 contains the most important binding site, that establishes incorporation of sigma into holo RNA polymerase. Single residue mutations in the region 2 have been shown to knock out binding to core and were lethal to the cell. Electrophoretic mobility shift assays have supported these results in vitro (18).

The crystal structure for RNA polymerase $\sigma^{70}$ subunit from *E. coli* is available only for a protease-resistant fragment of $\sigma^{70}$ lacking region 3, 4 and region 1.1 (19). However the crystal structures of core and holoenzyme were solved recently, but only for the evolutionary-

related *Thermus aquaticus* (20) and *Thermus thermophilus* (21). Among many insights into structural and functional features of the enzyme that have been studied for decades with low resolution methods, these structures confirmed the interaction sites for sigma to core RNA polymerase previously suggested by Arthur et al. (22) and Sharp et al. (18) (Figure 1.2).

**Figure 1.2: Structure of holo RNA polymerase from *T. thermophilus*(21). The subunits are colored σ white, β' red, β blue, α green and light yellow. The interaction partner of region 2 of σ contacts the coiled-coil in β' (shown in yellow).**

The key interaction site consists of a coiled-coil corresponding to residues 260-309 within the *E. coli* β' subunit (22, 23). This region was identified by the use of Far-Western blot analysis and the use of an ordered fragment ladder of the β' subunit. Far-Western blot analysis is performed similar to regular Western blot procedures with the exception that a radioactively labeled protein (in this case $\sigma^{70}$) is used instead of an antibody to probe the blot. The SDS-PAGE gel for the blot was prepared with purified $His_6$-tagged fragments of the β'

subunit that set up a ladder on the gel which allowed assessment of the regions necessary for binding $\sigma^{70}$. Additionally these fragments were immobilized on a Ni-NTA resin and tested for their ability to bind $\sigma^{70}$. These investigations and some of the material from the work of T. Arthur were the basis of the binding studies of sigma to the β' subunit of core RNA polymerase described in this thesis.

In the β' subunit, the coiled coil sticks out like a landing pad for sigma. Region 2 corresponding to residues 360-421 in $\sigma^{70}$, interacts mainly through ionic interactions with that region which were independently found through computer docking analysis by Burgess and Anthony (2). This could explain the negative effect of salt on the binding at this interface that has been known since the first identification of sigma in 1969 by R.R. Burgess (3, 24).

### *1.2.3 RNA polymerases from other bacteria*

As mentioned before, there is homology among bacterial RNA polymerases, allowing one to draw conclusions from X-ray crystal structures to homologues from other bacteria. The five subunits of core RNA polymerases are highly conserved among all known eubacterial species although they can vary slightly in size. The sigma factors are also highly conserved in Eubacteria. The level of conservation in region 2 of the $\sigma^{70}$ family, responsible for binding core RNA polymerase, is remarkably high and many sigma factors have been identified by their sequence due to this homology. The number of sigmas factors varies significantly. While *E. coli* manages to get along with 7 sigma factors, other species like *Mycoplasma genitalium* have to live with only one sigma factor (25) whereas *Streptomyces coelicolor* so far holds the record of all sequenced bacterial genomes with 65 different sigma factors (26). Table 1.2 shows a list of bacteria and their repertoire of sigma factors (27).

**Table 1.2: Number of sigma factors from a variety of bacterial species (27)**

| Organism | free living | Total number of sigma factors | # of sigma factors in the σ $^{70}$ famiy | # of sigma factors in the σ $^{54}$ famiy | # of ECF sigma factors |
|---|---|---|---|---|---|
| *B. subtilis* | yes | 18 | 10 | 1 | 7 |
| *E. coli* | yes | 7 | 4 | 1 | 2 |
| *Heamophilus influenza* | no | 4 | 2 | 0 | 2 |
| *Mycobacterium tuberculosis* | yes | 13 | 3 | 0 | 10 |
| *Mycoplasma genitalium* | no | 1 | 1 | 0 | 0 |
| *Pseudomonas aeruginosa* | yes | 24 | 24 | 1 | 19 |
| *S. aureus* | yes | 3 | 3 | 0 | 0 |
| *S. coelicolor* | yes | 65 | 6 | 14 | 45 |

As a common pattern it was found that free-living species generally have a higher number of sigma factors both from the ECF and the $\sigma^{70}$ family due to their need for a more complex transcriptional regulatory pattern, that allows them to adjust to marginal changes in their environment. Many of these are involved in the regulation of pathogen and antibiotic biosynthesis and are therefore especially of pharmacological interest.

### *1.2.4 Eukaryotic RNA polymerase II - the bacterial counterpart*

The mRNA in eukaryotic cells is synthesized by eukaryotic RNA polymerase II (Pol II). Mechanisms in eukaryotic transcriptions are of much greater complexity and far less thoroughly understood and much can be learned from studying their bacterial counterpart. However there is enough similarity between these two enzymes, that the corresponding subunits of bacterial RNA polymerase can be assigned as homologues to some of the known subunits in PolII. Partially these homologies are based on structure rather than sequence similarities. An overview of the core subunits in Pol II and its corresponding homologues in *E. coli* RNA polymerase is shown in Figure 1.3.

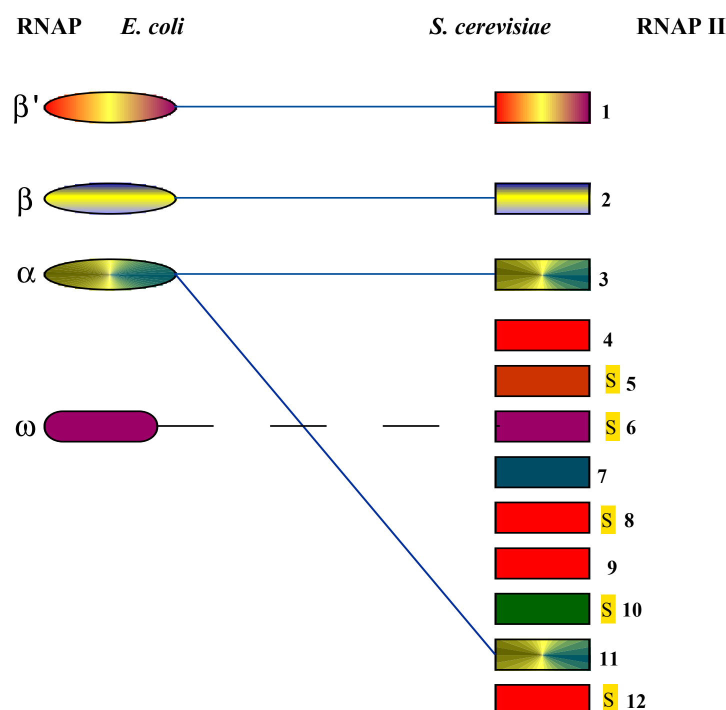


**Figure 1.3: Homologous subunits in RNA polymerases from *E. coli* and *S. cerevisiae*. The four subunits of *E. coli* core RNA polymerase are shown on the left and their homologues in *S. cerevisiae* RNAP II to the right. The lines indicate homologous subunits. Note that there are no known sigma homologues in eukaryotic cells. S indicates subunits that are shared between RNAP I (synthesis of rRNA), RNAP II (synthesis of mRNA) and RNAP III (synthesis of tRNA and other small RNAs).**

### *1.2.5 Bacterial RNA polymerases as a target for drug discovery*

The demand for new antimicrobial drugs is becoming increasingly urgent, since resistance mechanisms against whole classes of antibiotics are currently developing faster than research generates new structurally-different agents. Proteins involved in the central processes of the life cycle of a cell like replication, transcription, translation and cell wall biosynthesis have been traditionally exploited for drug discovery. As the key enzymes involved in these essential processes, DNA polymerase, the ribosome and the transcription machinery offer attractive targets for inhibitor screening. An important issue for the use of such an inhibitor as a drug in clinical applications is that the target site of the drug be absent in human cells.

The interface between sigma and core RNA polymerase offers such an attractive target, since no sigma homologue has been found in archaea and mammalian cells except for sigma factors in mitochondria (28) and chloroplasts (29). However, they do not show a significant homology to bacterial sigma factors. This fact implies that there is very little chance of a potential new antibiotic interfering with eukaryotic RNAP assembly, which could otherwise lead to serious side effects if it were used as a drug. Any inhibitor of sigma-core interaction should inhibit the initiation of transcription and should therefore act as a bacteriostatic agent. This is supported by the fact that induction of expression of the β'-coiled-coil fragment (residues 260-309) stops cell growth in *E. coli* (30). Although numerous antibiotics are known to inhibit transcription, none is known to interact either with region 2 of sigma or with the coiled-coil of the β' subunit of core. This offers the potential for a new class of antibiotics with a low initial occurrence of resistance. On the same note one could expect, that it would challenge the organism massively to develop resistance-conferring mutations in this site, since it is expected to interact with most of the sigma factors, which cannot be compromised in their affinity to that site by such a mutation. The homologies among different bacterial sigma factors and the coiled-coil in the β'-subunit suggest that any inhibitor of this interaction might lead to a broad-spectrum antibiotic. A sequence alignment of the coiled-coil in the β' subunit from several bacterial species as well as its human homologue is shown in Figure 1.4 (2).

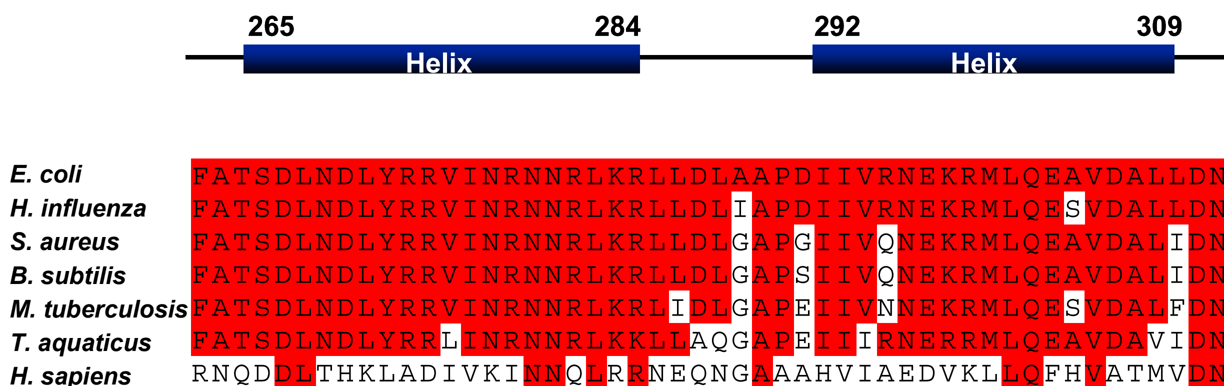


**Figure 1.4: Alignment of sequences from the coiled-coil in the β'-subunit. Sequences from *E. coli*, *Haemophilus influenzae*, *Staphylococcus aureus*, *B. subtilis*, *Borrelia burgdorferi*, *Mycobacterium tuberculosis* and *Thermus aquaticus* comprising the residues from the coiled-coil in the β'-subunit that is crucial for sigma binding. For comparison, the sequence of the structurally conserved coiled-coil from human RpbI is shown at the bottom. The schematic illustration of the α-helices within this region is shown above the sequences.**

## 1.3 Methods to study protein-protein interactions

Several techniques are commonly used in order to study protein-protein interactions. These can be divided into homogeneous, i.e. studying the binding partners while they are free in solution and inhomogeneous if one or more of the binding components are bound to a matrix or must be separated before they are analyzed. Crucial to inhomogeneous methods is that they often result in a change of the equilibrium conditions at which initial binding occurs. Procedures that involve immobilization often perturb the structure of the immobilized protein or the chemical environment during the binding event. Separating the complex from the unbound binding partner (either by size, charge, hydrophobicity or other physical properties) perturb the equilibrium and thus weak interactions (i.e., complexes that have a half life shorter than the time scale of the separation step are underrepresented or cannot be detected since the interaction doesn't persist throughout the procedure). This is true for all non-homogenous techniques like surface plasmon resonance (SPR, used in BIAcore instruments), co-immunoprecipitation or "pull-down" assays, ELISA assays, Far-Western blotting, affinity

chromatography, as well as size exclusion chromatography and sucrose gradient centrifugation.

In homogeneous techniques, all components of the assay are free in solution and no separation steps are used. A key benefit of such an assay is that the binding measurement is done without perturbing the binding equilibrium. This often allows more accurate assessment of the binding forces and mechanisms present. In most cases it is the superior technique, although technically more demanding. Many homogeneous techniques involve spectroscopic methods because the use of light as a probe doesn't change the conditions in most systems. These methods use NMR, light scattering, and very often fluorescent probes like in fluorescence polarization (FP) and fluorescence resonance energy transfer (FRET) assays. Recent developments of FRET also allow analysis of protein-protein interactions *in vivo* (31). Its close relative luminescence resonance energy transfer (LRET) makes use of luminescent lanthanide derivatives. LRET allows a remarkable reduction of the measured background fluorescence due to the enhanced fluorescence halflives of these luminescent probes like europium (1 ms) and terbium (2 ms). Most organic dyes have fluorescence lifetimes of less than 1 ns and decay by the time emission signals are acquired in a typical LRET experiment (50 to 400 μs). The key mechanism in these techniques is a radiationless energy transfer due to dipole-dipole interactions at resonance conditions. These occur if two suitable dyes come within a distance of less than 10 nm and decay due to their nature with the inverse sixths power of their distance. Measuring the emission of the donor and acceptor fluorophores gives information about the extent of the energy transfer and allows monitoring the distance of two fluorescence-labeled proteins within these dimensions and more generally whether two proteins are bound to each other or not. The theory and principles of are described in Chapter 3 "LRET-Based High-Throughput Screening Assay for Inhibitors of Essential Protein-Protein

Interactions in Bacterial RNA Polymerase". Together with two-hybrid screens and functional assays these are currently the only established methods to obtain reliable *in vivo* data.

**1.4 Thesis Plan**

The goal of this thesis was to develop a homogenous assay for sigma-binding to core RNA polymerase from *E. coli* by LRET and to investigate its use as a high-throughput screen for inhibitors of this protein-protein interaction.

Chapter 2 describes a rapid and efficient fluorescence-labeling procedure for proteins that includes TCEP-mediated reduction prior to cysteine-directed labeling and subsequent dye removal while the protein is bound to a resin for purification. This was necessary to achieve a maximum fluorescence-labeling efficiency of proteins for their use in LRET assays. It was crucial to efficiently remove free dye since it can cause diffusion-controlled LRET leading to false signals in the assay.

Chapter 3 outlines the development of a homogeneous LRET-based assay for sigma-binding to its major binding site in the β' subunit of core RNA polymerase from *E. coli.* It contains the IC5-labeled fragment (residues 100 to 309) of the β' subunit and a single cysteine mutant of $\sigma^{70}$ labeled with an Eu-chelate. The use of the assay for high-throughput screening was evaluated and an initial screen of a small natural product library isolated from marine sponges for inhibitors of sigma-binding to core RNA polymerase was performed.

Chapter 4 reports the initial results of a further developed LRET-assay using the full length core RNA polymerase randomly labeled through lysine residues with IC3-succinimide and $\sigma^{70}$ or $\sigma^{32}$ randomly labeled through lysine residues with cs124-Tb-TTHA-NSC. The goal of this Chapter was to use the LRET-assay to derive equilibrium binding constants for sigma binding to core RNA polymerase.

Chapter 5 describes the making of a monoclonal antibody 8RB13 with a high cross-reactivity for RNA polymerases from different bacterial species and its performance in immunoaffinity chromatography. The purification of active core RNA polymerase from *E.*

*coli*, *B. subtilis*, *S. coelicolor* and *P. aeruginosa* was done to confirm its utility in protein purification. This project was conducted mainly for my own educational purposes, although it turned out to be a powerful tool for getting access to a diversity of RNA polymerases and their future characterization with the LRET-based binding assay.

Chapter 6 summarizes the efforts to use fluorescence-labeled antibodies in quantitative Western blots. The goal was to get a reliable tool to measure protein levels of sigma factors in crude cell extracts from *E. coli* that could later be used for *in vivo* studies of sigma competition for core RNA polymerase.

The Appendix contains a summary of methods and results already outlined in Chapters 2 and 3 in the form of protocols and was added to this thesis only for the convenient use of the described methods in the laboratory.

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# Chapter 2

## On-column TCEP Reduction and IC5-Maleimide Labeling During Purification of a RpoC-Fragment on a Ni-NTA Column

This Chapter has been published as:

Bergendahl, V., Anthony, L. C., Heyduk, T., and Burgess, R. R. (2002) On-column tris(2-carboxyethyl)phosphine reduction and IC5-maleimide labeling during purification of a RpoC fragment on a nickel–nitrilotriacetic acid column. *Anal Biochem* **307,** 368.

My contributions are that I performed all the experiments and wrote the manuscript. Larry C. Anthony introduced me into the protein purification methods for $\sigma^{70}$ and the β'-fragment. Tomasz Heyduk provided the Europium label used in this study.

R. R. Burgess is the principal investigator.

**2.1 Summary**

Fluorescence labeling of proteins has become increasingly important since fluorescent techniques like FRET and fluorescence polarization are now commonly used in protein binding studies, proteomics, and for high-throughput screening in drug discovery. In our efforts to study the binding of the β'-subunit from *E. coli* RNA polymerase (RNAP) to σ70, we synthesized a fluorescent-labeled β'-fragment (residues 100-309) in a very convenient way, that could be used as a general protocol for hexahistidine-tagged proteins. By performing all the following steps, purification, reduction, derivatization with IC5-maleimide, and free dye removal while the protein was bound to the column, we were able to reduce the procedure time significantly and at the same time achieve better labeling efficiency and quality. The β'-fragment with a C-terminal $His_6$-tag was purified from inclusion bodies and could be refolded prior to or after binding to a Ni-NTA affinity column. Reduction prior to labeling was achieved with TCEP that does not interfere with Ni-NTA chemistry. The labeled β'-fragment was tested with σ70 that was labeled with an Europium-based fluorophore for binding in a electrophoretic mobility shift assay. The sigma-to-core protein interaction in bacterial RNA polymerase offers a potentially specific target for drug discovery, since it is highly conserved among the eubacteria, but differs significantly from eukaryotes.

**2.2 Introduction**

Fluorescence-based assays play an important role in biochemistry and molecular biology, due to their sensitivity and versatility in studying the properties of nucleic acids and proteins (1-3). Although the physical properties of fluorophores and chemical tools for derivatization of selective functional groups in the target molecules have been well known for more than 20 years, the integration of this knowledge into established biochemical techniques has just begun. The requirements for the almost exclusively aqueous biochemical or physiological assays like salt, pH, reducing agents and temperature are typically different from the conditions in which these compounds and reactions were originally studied. Nevertheless, it is very often possible to combine common biological and biochemical techniques with the conditions required for coupling fluorescent dyes to functional groups like amines, phosphates, sugars, sulfhydryl and carboxyl groups, which are abundant in proteins and nucleic acids. In this work we present an example of how an efficient labeling procedure can be integrated into the currently very popular IMAC protein purification method (4) using Ni-NTA resin (5). The benefit of this combined approach over performing purification and labeling separately is a clearly shorter and more efficient procedure. Excess dye and byproducts can be washed away from the final product, while the target protein is still bound to the column matrix during purification.

The bacterial transcription machinery appears to offer an attractive target for drug discovery and drug design. In that respect we decided to study the assembly of the major sigma factor σ70 with core RNAP to form the holoenzyme by the use of fluorescent probes. The postulated region mainly responsible for core binding (region 2.1-2.2, Figure 2.1) of bacterial transcription factors (σ70 in *E. coli*) shows remarkable sequence identity (>80%) among the major sigma factors and is also conserved among the minor sigma factors of

bacteria (6, 7). Additionally their binding partner β' (Figure 2.1) in core RNAP exhibits a very high sequence conservation in the previously identified sigma-binding region (between residues 260-309 in the β'-subunit of *E. coli*) (8, 9).

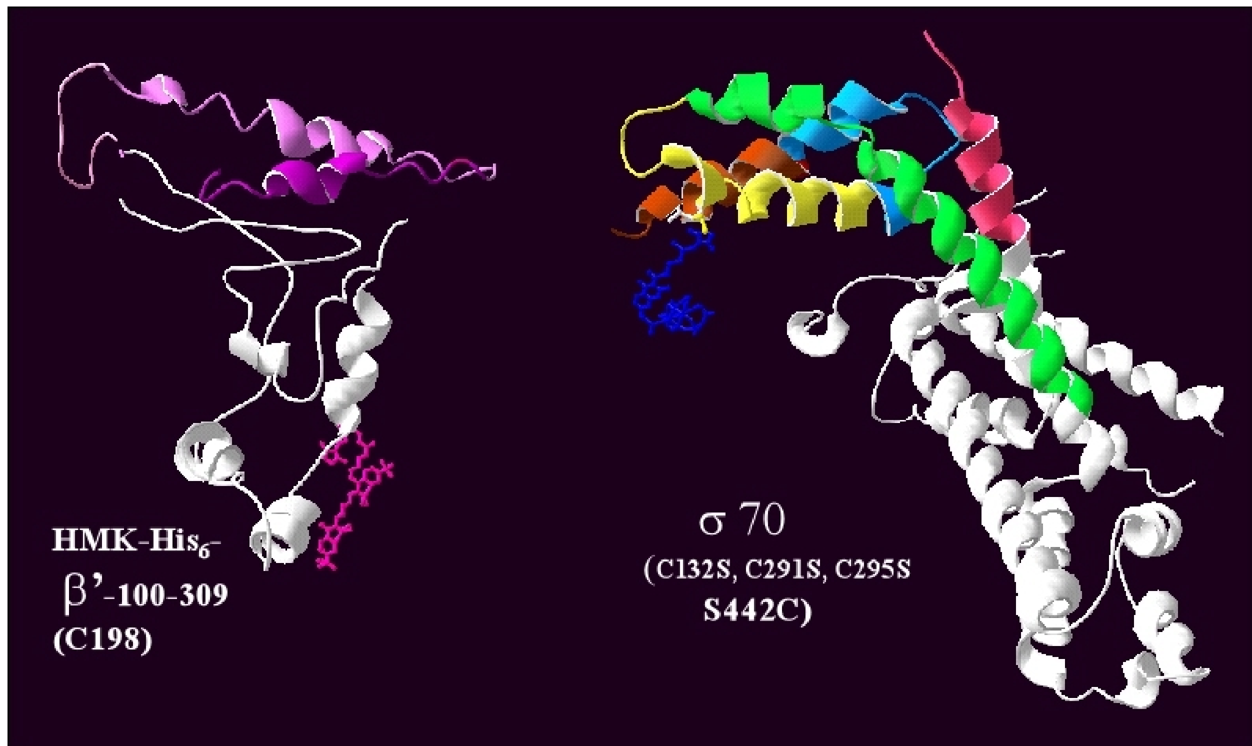


**Figure 2.1: Structures of the β'-fragment (A) and σ70 (B) that are the targets for the protein binding assay. Next to the names are the mutations and the derivatization sites (indicated by white letters). HMK-$His_6$-β'-100-309 indicates that the fragment of β' contains residues 100-309 and is a N-terminal fusion to a heart-muscle kinase (HMK) recognition site and a $His_6$-tag. The structure was taken from (10). The coiled-coil α-helical structure colored black in the β'-fragment is thought to be a primary sigma binding element in the RNAP (9). The regions in σ70 responsible for binding to the β'-contact region of core RNAP are regions 2.1 (green) and 2.2 (yellow). Regions 2.3 and 2.4 are indicated in blue and brown, respectively. The non-conserved region is in white. The structure was taken from (11). The labels IC5 on the β'-fragment and Eu-DTPA-AMCA on σ70 are also indicated in black in their expected positions.**

These homologies suggest a highly conserved structure within the holoenzyme form of RNAP, whose formation is crucial for a correct initiation of transcription. Any inhibitor of this protein-protein interaction can thus be expected to be a broad-spectrum antibiotic candidate. No σ70 homologue has been found in mammalian cells except for sigma factors in

mitochondria (12) and chloroplasts (13). However, these sigma factors do not show strong similarity to their prokaryotic counterparts. This implies that there is very little chance of a potential new antibiotic interfering with eukaryotic RNA polymerase assembly leading to serious side effects if it were used as a drug.

In order to screen for inhibitors of RNAP assembly with sigma, a simple, fast and reliable assay had to be found for the formation of the σ70-β'-complex. We have chosen to use LRET (Luminescence Resonance Energy Transfer) a recent modification of FRET, that can create the desired signal upon complex formation (14-16). The more general term luminescence instead of fluorescence (as in FRET) indicates that lanthanide emission is technically not fluorescence (i.e. arising from a singlet to singlet transition) (17). Differences in the physical processes result in different fluorescent lifetimes of the lanthanide-based donor fluorophore (> microseconds up to several milliseconds) compared to the short lifetime of most organic-based fluorophores in FRET (picoseconds up to a microsecond). The conventional dipole-dipole theory of Förster is still applicable for LRET, so that the difference of the two lies mainly in the technical advantages offered by LRET (15). Heyduk and co-workers have used a Europium-chelate fluorophore to measure DNA binding to σ70 in holoenzyme using the same pair of dyes (18). They have been able to show its applicability within this system. We adapted their procedure to synthesize IC5-labeled β'-fragment (residues 100-309 with a $His_6$-tag followed by a heart-muscle kinase (HMK) recognition site fused to the N-terminus) and integrated it into our current purification protocol. For the desired homogenous assay, we labeled σ70 with a Europium-DTPA-AMCA complex as a donor as described by Heyduk et al (18). The structures of the dyes are shown in figure 2.2. We tested the labeled proteins for their binding activity with native PAGE gels in electrophoretic mobility shift assays.

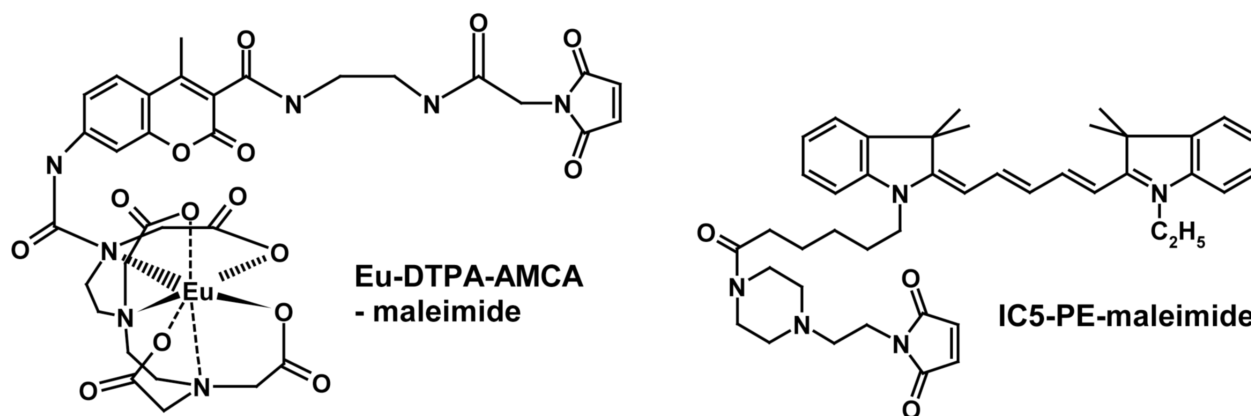


**Figure 2.2: The fluorophores that were used to derivatize the proteins. The Eu-chelate DTPA-AMCA-maleimide was used to label sigma70. IC5-PE-maleimide served as the label for the β'-fragment.**

Controlled labeling of proteins with only one fluorophore can be performed by exploiting the specificity of maleimide-linked dyes with cysteine residues (19). For uniform labeling it is desirable that the protein has only one cysteine residue. The maleimide moiety of the label usually reacts readily in most buffers used in biochemistry and is highly specific to sulfhydryl residues at pH-values below 7.5 (20). However oligomerization and oxidation of the sample can lead to a significant loss of labeling. Getz et al. (21) have found that any reducing agents such as 2-ME and TCEP (22) can inhibit maleimide labeling significantly, so that their presence during the labeling reaction is undesirable. Another challenge when labeling proteins is the separation of labeled product from the excess dye. This is especially important for samples used in FRET and LRET assays, since too much free dye can cause additional signal by diffusion-controlled resonance energy transfer when working at concentrations above 100 nM. For cyanine dyes like IC5 it very difficult to remove excess dye by dialysis and usually requires an extra gel filtration step. In our system, we tried to combine all steps by working on a Ni-NTA column throughout containing a hexahistidine-tagged beta prime (RpoC) fragment (residues 100-309), that has a single naturally-occurring cysteine at residue 198. We circumvented reducing the Ni-resin by using TCEP instead of 2-

ME. After reduction, TCEP was washed away and the on-column derivatization was carried out immediately to avoid oxidation. Excess dye could be washed from the column while the labeled product remained bound to the resin. Since we started with purified inclusion bodies, we tested refolding before IMAC and on-column before and after labeling.

## 2.3 Experimental procedures

### *2.3.1 Materials and chemicals*

IC5-PE-maleimide was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, USA). DTPA-AMCA-maleimide was produced as described (18) by T. Heyduk. (CAS numbers of the used starting materials: DTPA-anhydride: 23911-26-4, Maleimido-propionic acid, NHS ester: 55750-62-4, AMCA: 106562-32-7). The identity of AMCA-DTPA-maleimide was established by its spectroscopic properties (ability to sensitize Europium; blue-shifted emission and absorption of AMCA moiety which is characteristic of reaction of its 7-amino group), its ability to react with thiols and by mass spectrometry. A single peak on reverse phase HPLC was taken as an indication of the purity of AMCA-DTPA-maleimide (data not shown). Ni-NTA resin was purchased at Qiagen. TCEP and TritonX-100 were purchased at Pierce. $EuCl_3$ and all other chemicals were purchased from Sigma unless otherwise indicated in the text.

### *2.3.2 Buffers*

NTG buffer: 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 % glycerol; NTGED buffer: 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 % glycerol, 0.1 mM EDTA, 0.1 mM DTT; TGE buffer: 50 mM Tris-HCl, pH 7.5, 5 % glycerol, 0.1 mM EDTA; NTTwGu buffer: 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1 %Tween 20, 6 M Gu-HCl; NTTw buffer: 500 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1 %Tween 20; Storage buffer: 50 mM, 200 mM Tris-HCl, pH

7.5, 50 % glycerol, 0.5 mM EDTA, 0.1 mM DTT; Native buffer: 200 mM Tris-HCl, pH 7.5, 20 % glycerol, 0.005 % Bromphenol blue

### *2.3.3 Overproduction of HMK-His$_6$-β'(100-309) and σ70 (S442C)*

HMK-His$_6$-β'(100-309) was overproduced from pTA133 (9) and is a chimera (25 kDa) with the amino acid sequence MARRASVHHHHHHM N-terminally fused to β'(100-309). The underlined sequence represents a heart muscle kinase (HMK) recognition site and was used in the original construct to allow $^{32}$P-labeling of the protein (23), but we did not make use of it in this paper. Sigma70(442C) is overproduced from a plasmid (18) derived from the σ70-expression system pGEMD (24, 25) that had a HindIII fragment containing the *rpoD* gene from *E. coli* cloned into a pGEMX-1 (Promega) vector. Both are T7 expression systems allowing controlled induction by IPTG and selection with ampicillin. The plasmids were transformed into BL21(DE3) (Novagen) for expression. The cells were grown in 1-L cultures at 37°C in LB medium with 100 µg/ml ampicillin. The cultures were grown to an $OD_{600}$ between 0.5-0.7 and then induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG, Roche Diagnostics Corporation, Indianapolis, USA). After 2 hours of induction, the cells were harvested by centrifugation at 8,000 x g for 15 min and frozen at -20 °C until use.

### *2.3.4 Purification of inclusion bodies for σ70 and the β'-fragment*

A cell pellet (2 g wet weight) was resuspended in 10 ml NTGED-buffer + 10 mM EDTA and 100 µg/ml lysozyme. The cells were incubated on ice for 30 min and then sonicated at 4°C (1 cm diameter tip, three times 1 min in 1-s pulses, 80% duty cycle, output level position 9, Sonicator W-385 Ultrasonic Inc.). TritonX-100 (1% v/v) was added and

vortexed. The recombinant protein in the form of inclusion bodies was separated from the soluble lysate by centrifugation at 25,000 x g for 15 min. The inclusion body pellet was resuspended by short sonication in 10 ml NTGED-buffer + 1% (v/v) TritonX-100. The mixture was centrifuged at 25,000 x g for 15 min and the supernatant discarded. The washed inclusion bodies were resuspended in 10 ml NTGED-buffer + 0.1% (v/v) TritonX-100 and centrifuged at 25,000 x g for 15 min. The wash was repeated with 10 ml NTGED-buffer + 0.01% (v/v) TritonX-100 and the suspension of inclusion bodies was aliquoted into 5 equal portions (typical yields are between 30 and 40 mg per aliquot) in 2-ml vials prior to centrifugation in a Beckman Microfuge®18 centrifuge at maximum speed. Supernatants were removed by pipetting and inclusion bodies were frozen at -20 °C until use.

### *2.3.5 Ni-NTA purification and IC5-derivatization of the β'-fragment*

One aliquot (typically 35 mg) of β' inclusion bodies were solubilized in 3 ml NTTwGu-buffer + 5 mM imidazole and incubated at room temperature for 15 minutes. The precipitate was spun down in a microfuge at 18,000 g (14,000 rpm) for 5 min and the supernatant was loaded on a BioRad column (10 ml PolyPrep, 0.8x4 cm) with 0.5 ml Ni-NTA matrix (Qiagen) previously equilibrated with 5 ml NTTwGu-buffer + 5 mM imidazole. To remove unbound protein, the column was washed with 5 ml NTTwGu-buffer + 5 mM imidazole. To reduce any disulfide bonds the column was washed with 5 ml of freshly prepared NTTwGu-buffer + 20 mM imidazole and 2 mM TCEP. TCEP is an odorless white powder that can be added as a solid just before use. It dissolves readily in aqueous solutions and is very stable unless chelators like EDTA are present (21). Excess TCEP and non-specifically bound protein were removed by washing with 3 ml NTTwGu-buffer + 20 mM imidazole which was saturated with $N_2$ to minimize exposure to $O_2$. The bound protein was

derivatized with IC5-maleimide by loading 2 ml freshly prepared NTTwGu-buffer + 20 mM imidazole and 0.1 mM IC5-PE-maleimide. IC5-PE-maleimide was dissolved in chloroform and divided into aliquots of 0.1 μmol which were then dried down in a Speed-Vac (SpeedVac SVC100H, Savant Instruments, Farmingdale, NY). The speedvac was equipped with a glass cover unaffected by organic solvents for removal of solvents from samples held in the microcentrifuge tubes. A refrigerated condensation trap is placed between the Speed-Vac and the vacuum pump, Edwards E2M-12 high vacuum pump, to trap solvents coming off the samples. The use of water is not recommended as it causes hydrolysis of the maleimide moiety. The flow-through was reloaded onto the column twice before excess dye was removed by washing with 3 ml NTTwGu-buffer + 20 mM imidazole. Derivatized protein was eluted with NTTwGu-buffer + 200 mM imidazole and stored denatured at -20°C at concentrations of around 2 mg/ml. The SDS-PAGE gel of the samples taken at different stages during the purification procedure can be seen in Figure 2.3 (Coomassie-stain and IC5-sensitive fluorescence scan).

The procedure was also done under non-denaturing conditions after refolding the β'-fragment into a 65-fold excess of TGE-buffer and binding to the Ni-NTA-resin. In another variation of the procedure, we refolded the the column-bound β'-fragment by washing with NTTw-buffer and continuing the procedure under non-denaturing conditions by omitting GuHCl in the buffers. This was done before the labeling reaction and in another experiment after labeling.

### *2.3.6 Purification and derivatization of σ70*

One aliquot of σ70 inclusion bodies (35 mg) was solubilized by resuspending in 5 ml TGE-buffer + 6 M GuHCl. To refold proteins the denaturant was diluted 100-fold by

dripping into 500 ml of chilled TGE-buffer + 0.01% TritonX-100 slowly stirring on ice. If precipitation occurred, the precipitate was removed by centrifugation at 25,000 g (15,000 rpm, SS-34 rotor) for 15 min at 4°C. The soluble refolded protein was then bound to an anion exchange resin by adding 1 g DE52 (Whatman) dry resin as a suspension in 5 ml TGE-buffer + 0.01% TritonX-100 directly into the mixture. After slow stirring for 30 min, the suspension was poured into an empty 25 ml Econo-Pack column (BioRad) and washed with 5 ml NTG-buffer + 0.01% TritonX-100. To reduce any disulfides formed by dimerization of sigma, 5 ml NTG-buffer + 2 mM Tris(2-carboxyethyl)phosphine (TCEP) + 0.01% TritonX-100 was loaded onto the column, followed by a wash with 5 ml NTG-buffer + 0.01% TritonX-100 to remove TCEP, which would otherwise interfere with maleimide labeling. To label σ70, 1 ml NTG-buffer + 0.01% TritonX-100 + 1 mM DTPA-AMCA is loaded onto the resin. The flow-through was reloaded onto the column 2 times to ensure maximum labeling. The Europium-complex was formed by loading 5 ml NTG-buffer + 0.01% TritonX-100 + 1 mM $EuCl_3$ onto the resin containing the derivatized protein. After a wash with 5 ml NTG-buffer + 0.01% TritonX-100, the labeled protein was eluted with TGE-buffer + 500 mM NaCl. The eluted fractions were analyzed by SDS-PAGE and pooled according to their purity and protein content. The pooled fractions of labeled σ70 were brought up to 50% glycerol or dialyzed against storage buffer (final protein concentration of about 2 mg/ml) and were stored at -20°C until use.

Labeling can also be done after purification and elution from the ion-exchange column (18) by adding the protein to an aliquot of the dye (same buffers and conditions). The procedure can be scaled down 10-fold. Excess label and Eu-ions are then removed by using a Pharmacia G50 spin-column.

### *2.3.7 Electrophoretic Mobility Shift (EMS) assay to test complex formation of labeled σ70 with β'-fragment*

The EMS assays were performed in 20 μl buffer containing 5% glycerol, 50 mM Tris-HCl, pH 8.8, 50 mM NaCl, bromphenol blue 0.005% (w/v). The standard protein concentrations were 0.25 μM σ70 (labeled protein) and 0.1 to 2 μM β'-fragment (labeled protein) but can be lowered to 20 nM and 10 nM, respectively at the detection limit of the Coomassie blue stain. Labeled σ70 was added to NTG-buffer first. In case of competition experiments unlabeled denatured β'-fragment was added subsequently. Denatured labeled β'-fragment was added last. The solutions were mixed well after addition of each component. The mixture was incubated for 5 min at room temperature, and then 15 μl were loaded on a pre-cast native PAGE-gel (12-well, 12%, Tris/glycine, NOVEX). The electrophoresis was run with pre-chilled buffers, gels and apparatus in the cold room (4°C) at constant voltage of 120 V (5-20 mA, variable) for 2.5 hours. The IC5-emission was scanned on a Storm system (Molecular Dynamics) in the red fluorescence mode. The Europium emission was measured using an Fotodyne UV light box ($\lambda_{excit.}$= 312 nm) with 6 seconds acquisition time. Total protein was stained with Coomassie blue stain using the Gel Code staining solution (Pierce) according to the procedure of the manufacturer and scanned with a Hewlett Packard flat bed scanner (ScanJet 6200C).

## 2.4 Results

### *2.4.1 Specificity of the labeling reaction*

The specificity of maleimide activated dyes towards reaction with cysteine residues at the applied conditions is commonly used in biochemistry. In order to ensure that labeling

under the chosen conditions occurs only at the desired position we tested mutant proteins that contain no cysteine residue under the same conditions and were not able to observe any labeling with maleimide containing fluorescent probes. Thus incorporation of such a probe into the single cysteine containing proteins was taken as evidence for the specificity of incorporation of the label into this position.

### *2.4.2 Labeling efficiency of the β'-fragment*

We tested the labeled sample for labeling efficiency by measuring the absorbance of the labeled sample (according to manufacturer's information, the molar absorptivity at 650 nm is approximately 90,000 $mol^{-1}cm^{-1}$). The derived concentration (1.8 mg/ml) compared with the protein concentration (2 mg/ml) measured by the Bradford method (Pierce). The SDS-gel in Fig. 2.3 (4-12% NuPAGE, MES buffer; NOVEX) of a purification under denaturing conditions shows no interference of the TCEP reduction with the binding of the histidine-tagged protein as well as a good purification of the labeled protein from the free dye. Labeling efficiencies were greater than 90% in all cases. Judging from the emission of the labeled protein and the free dye in the SDS gel (Fig. 2.3), the remaining content of free dye was less than 5%.

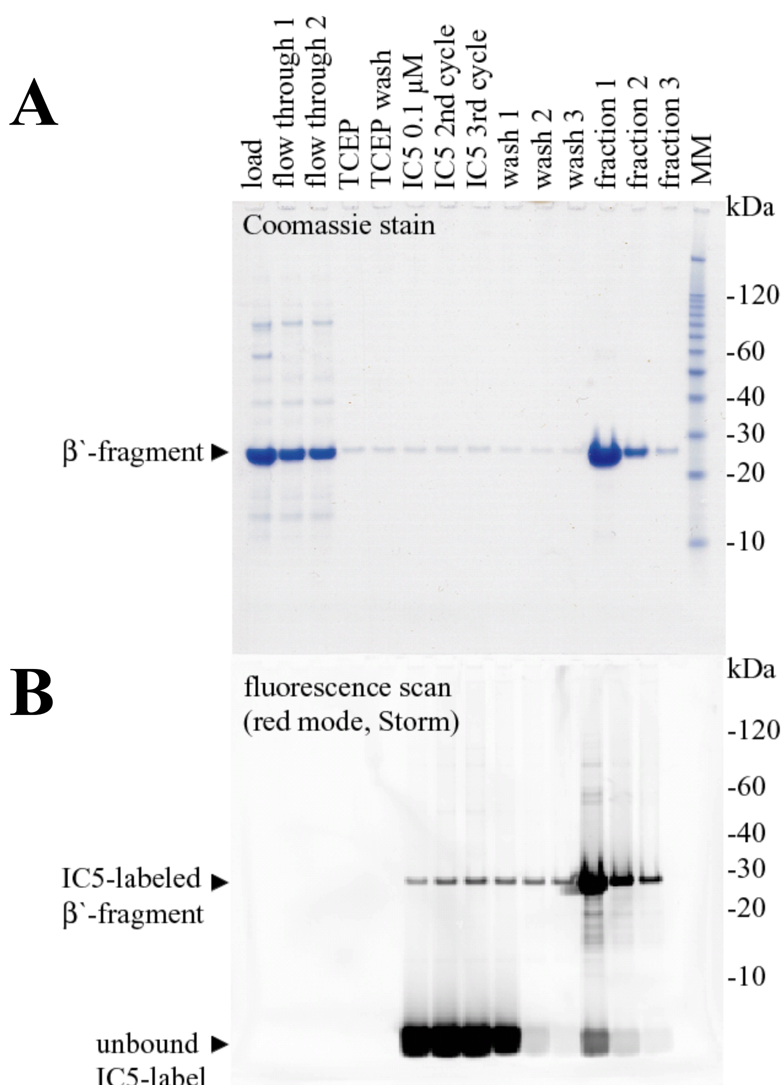


**Figure 2.3: SDS-PAGE gel of the β'-purification and derivatization steps. The Coomassie stain of gel (A) illustrates the purification of the β'-fragment throughout the procedure (at 6 M GuHCl). Purification from minor impurities can be observed as well as very low elution of the β'-fragment at any time except for the fractions 1 to 3 eluted with 200 mM imidazole. The IC5-scan (gel B) was performed with a Molecular Dynamics Storm system in the red fluorescence mode and thus shows the IC5-labeled proteins. It resembles the identical gel in (A) and was scanned before the staining procedure. Note the significant decrease of free label in the eluted fractions.**

### *2.4.3 Refolding prior to or after labeling of the β'-fragment*

Using a 0.8 ml Ni-NTA column volume, we were able to get a total of 9 mg of labeled protein in concentrations of typically 2 mg/ml using denaturing conditions throughout. When refolding the protein before or after the labeling step, yields go down dramatically (2.0 mg

protein, 22% yield compared to denaturing conditions). Also the labeled protein tended to precipitate further, when concentrations exceeded 0.2 mg/ml (concentrations of initially 0.5 mg/ml dropped more than 40% over 1 month at -20°C, or less than 1 week at 4°C). We found it to be best to keep the protein under denaturing conditions (6M GuHCl) and to refold the labeled protein directly into the assay when it was carried out (26). Still it was possible to refold the protein by 65-fold dilution into TGE-buffer followed by binding in batch mode. Yields and labeling efficiencies were similar to those under denaturing conditions, if the refolding efficiency was not considered since we saturated the Ni-NTA columns in all cases (see lane marked "flow through" on the SDS-gel in Figure 2.3). Furthermore we observed a slightly better overall solubility of the β'-fragment residues 100-309 compared to the β'-fragment residues 240-309 (data not shown).

### *2.4.4 Electrophoretic Mobility Shift (EMS) assay to test for the complex formation of labeled σ70 and β′*

The EMS assays clearly showed that the labeled as well as the unlabeled proteins could form a complex that migrates slower in the native gel than σ70 alone (Figure 2.4). The different scanning techniques also confirmed the identity of the bands in the EMS assay. Furthermore the EMS assay showed that unlabeled β'-fragment can compete for the labeled σ70. Thus unlabeled β'-fragment itself represents a positive control for an agent able to interfere with the binding of labeled β'-fragment and σ70 in the assay. We could further show that it was possible to shift 100% of the labeled σ70 with the complex. This suggests that all the labeled σ70 was active for binding under these conditions. The electrophoresis could be run up to 12 hours under described conditions (data not shown). Although the bands got more

diffuse with increasing electrophoresis time, the band of the complex never disappears indicating a low level of dissociation under these conditions.

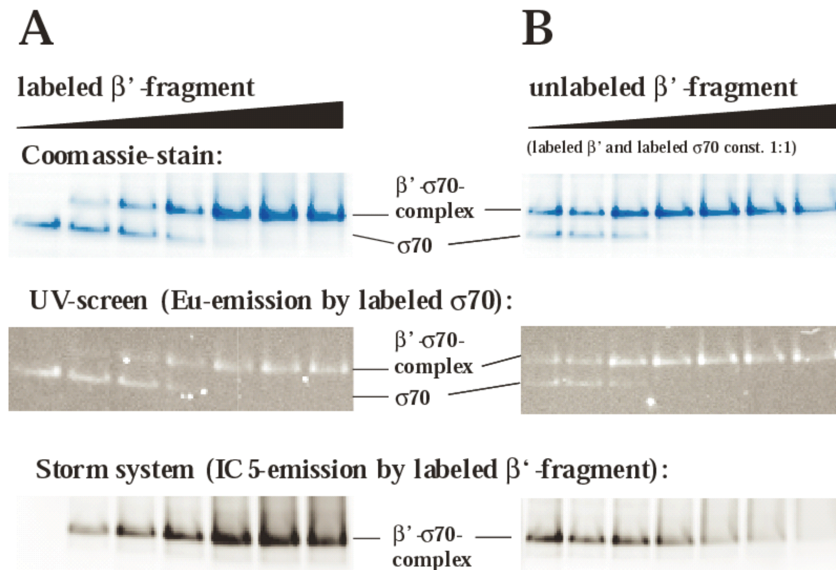


**Figure 2.4: The result of two EMS assays. (A) shows increasing amounts of labeled β'-fragment (0, 0.1, 0.25, 0.5, 1, 1.5, 2 µM) that can shift all the labeled σ70 (250 nM) into the upper band representing the complex in the Coomassie-stained gel at the top. The middle frame represents the same gel, but was acquired with a UV-box and an orange filter on the camera that can only visualize the Eu-emission due to the excitation wavelength (312 nm). It confirms that both bands contain Eu-labeled sigma. The bottom frame of the same gel, was taken with a Storm imager (Molecular Dynamics) that can only visualize the IC5-label. It confirms that only the upper band contains labeled β'-fragment. The free β'-fragment runs as a diffuse band barely migrating into the gel (not shown). In (B) a sample of labeled β'-fragment and σ70 in a ratio of 1:1 (250 nM) was taken but prior to mixing, an increasing amount of unlabeled β'-fragment (0, 0.1, 0.25, 0.5, 1, 1.5, 2 µM) was added to the labeled σ70. The unlabeled β'-fragment can clearly compete for the labeled σ70 as can be seen by a loss of the band in the bottom gel of (B).**

## 2.5 Discussion

Using fluorescence to monitor sigma binding to DNA has been shown to be an effective and very sensitive method (18, 27). In order to use the same technique to monitor sigma binding to RNAP, we labeled a fragment (residues 100-309) of the β'-subunit of RNAP that contained a N-terminal HMK-site and a $His_6$-tag with IC5-maleimide. A well-characterized σ70(442C) mutant (18) with all natural cysteine residues mutated to serine residues and serine 442 mutated to cysteine was derivatized with a DTPA-AMCA-maleimide

Eu-complex. It was shown to be as active in *in vitro* transcription as the wildtype protein. We used the labeled protein to test binding of the labeled β'-fragment. We were able to show with EMS assays that the labeled proteins can bind to each other in all combinations with and without the label. As controls the unlabeled proteins were tested to determine if they could compete with their labeled counterpart. In the EMS assays unlabeled β'-fragment was able to compete with the labeled β'-fragment for binding to labeled σ70. From these data we conclude that the assay can be used to monitor σ70-to-β'-binding and that the presence of the fluorophore on each binding partner has no significant effect on its binding properties. In our studies we started with inclusion bodies. In the case of σ70, our results favor refolding prior to purification and derivatization to achieve maximum yields and labeling efficiencies. However the stability of some proteins like the β'-fragment made it more effective to label under denatured conditions and keep the labeled protein denatured until use. In general the procedure should be useful for most soluble proteins that are stable under these conditions.

The described procedures represent a rapid and efficient way to reduce and label proteins with a single cysteine on either Ni-NTA (for histidine-tagged proteins) or anion exchange columns (for proteins with acidic sites). We could show that TCEP can be used in combination with Ni-NTA resin for reduction of proteins prior to their derivatization with maleimide derivatives of fluorescent probes. The procedure presented offers a quick and efficient alternative to other currently used procedures that require dialysis or further chromatographic steps to eliminate free fluorescent dye that can otherwise interfere with sensitive fluorescent assays like FRET and LRET assays.

We are actively using the labeled proteins for in vitro assays and have developed a high-throughput screen based on LRET (to be published elsewhere). In these assays we could show that both proteins are active in binding by measuring the IC5-emission resulting from

LRET upon their binding to each other. Furthermore we could show that this signal could be eliminated by addition of unlabeled σ70 that can compete with labeled σ70 for labeled β'-fragment (data not shown). This could be shown accordingly for unlabeled β'-fragment and confirms results from the EMS assays described in this paper. The assay is now being used to screen libraries of natural and synthetic compounds as well as for studying sigma binding to core RNAP.

## 2.6 Acknowledgments

We would like to thank Kit Foley and Dr. Nancy Thompson for their support in the lab and Vladimir Svetlov for helpful advice throughout this work.

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# Chapter 3

## LRET-Based High-Throughput Screening Assay for Inhibitors of Essential Protein-Protein Interactions in Bacterial RNA Polymerase

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My contributions are that I performed all the experiments and wrote the manuscript. Tomasz Heyduk provided the Europium label used in this study.

R. R. Burgess is the principal investigator.

### 3.1 Summary

The binding of sigma factors to core RNA polymerase is essential for specific initiation of transcription in eubacteria and is thus critical for cell growth. Since the responsible protein-binding regions are highly conserved among all eubacteria but differ significantly from eukaryotic RNA polymerases, it is a promising target for drug discovery. A homogeneous assay for sigma-binding to RNA polymerase (*E. coli*) based on luminescence resonance energy transfer (LRET) was developed using an europium-labeled sigma70 and IC5-labeled fragment of the beta-prime subunit of RNA polymerase (amino acid residues 100-309). Inhibition of sigma-binding was measured by the loss of LRET through a decrease in IC5-emission. The technical advances offered by LRET resulted in a very robust assay suitable for a high-throughput screening and was successfully used to screen a crude natural product library. We illustrate this method as a powerful tool to investigate any essential protein-protein interaction for basic research and drug discovery.

**3.2 Introduction**

The need for new antimicrobial drugs has become an obvious scientific challenge since inappropriate use and clinical conditions have favored selection for strains resistant to an increasing number of antibiotics. In order to accelerate the pace in which we compete with nature, high hopes lie in the exploitation of recent advances in genomic and proteomic research. Due to increasingly detailed structural and mechanistic information about proteins involved in the central processes of the life cycle of a cell like replication, transcription and translation, rationally designed assays can be developed to find inhibitors of very specific and vulnerable targets within these biochemical machines. With this paper we illustrate the development of a LRET-based high-throughput screen for antimicrobial drugs based on the identification of a crucial protein-protein interaction in RNA polymerase (RNAP).

Like the ribosome and DNA polymerase, the bacterial transcription machinery appears to offer an attractive target for drug discovery and rational drug design. Any inhibitor of the core RNAP assembly with a major sigma factor to form the holoenzyme would inhibit the initiation of transcription and therefore would prevent growth and eventually survival of a cell. Our group and others have previously identified by biochemical methods the regions in *E. coli* RNA polymerase (a coiled-coil region between amino acid residues 260-309 in the β'-subunit of *E. coli*) (1, 2) and its major sigma factor σ70 (region 2.2) largely responsible for sigma-core interactions (3, 4). These results have now been confirmed by the structures of *T. aquaticus* holoenzyme (5, 6). Each protein has a remarkably conserved (>80%) amino acid sequence (7, 8) among all known eubacteria within these regions. These homologies suggest a highly conserved structure and function within the holo form of RNAP, which is crucial for a correct initiation of transcription. Any inhibitor of this interaction can thus be expected to

be a broad-spectrum antibiotic. This is supported by the fact that induction of expression of the β'-fragment (residues 260-309) stops cell growth in *E. coli*. (9). No σ70 homologue has been found in archaea and mammalian cells except for sigma factors in mitochondria (10) and chloroplasts (11). However, they do not show a significant homology to their bacterial counterparts. This fact implies that there is very little chance of a potential new antibiotic interfering with eukaryotic RNAP assembly, which could otherwise lead to serious side effects, if it were used as a drug.

In order to screen for inhibitors of RNAP assembly with sigma, a more simple, fast and reliable assay than the ones currently available (12) was developed for the formation of the σ70-β'-complex of *E. coli*. We decided to use LRET (Luminescence Resonance Energy Transfer), a recent modification of FRET, that can create the desired signal upon protein binding (13-15). The more general term luminescence instead of fluorescence (as in FRET) indicates that lanthanide emission is technically not considered as fluorescence (i.e., arising from a singlet to singlet transition). The details of LRET have been elegantly described in recent reviews by Selvin and Heyduk (16, 17) and will be covered only briefly here. A quantitative description of the effect is based on the Förster theory that describes the decrease of energy transfer as inversely proportional to sixth-power of the distance between the two dyes and is applicable for FRET and LRET. The key differences to FRET result from prolonged fluorescent lifetimes of the lanthanide-based donor fluorophores like Eu and Tb (> microseconds up to several milliseconds) compared to the short lifetime of most organic-based fluorophores used in FRET like Cy5 or IC5 (picoseconds up to a microsecond), so that LRET offers mainly technical advantages over FRET.

Heyduk and co-workers have used LRET to measure DNA binding to σ70 in holoenzyme using an Eu-chelate as donor and Cy5 as an acceptor (18). They have been able to show its applicability within this system. We adapted the principle by exchanging the Cy5-labeled polynucleotide for an IC5-labeled β'-fragment (residues 100-309 N-terminally fused to a heart-muscle kinase (HMK) recognition site and a $His_6$-Tag). For the resulting homogenous assay, we labeled σ70 with an Europium-DTPA-AMCA complex as a donor and the HMK-$His_6$-β'(100-309)-fragment with the Cy5-analogue IC5-maleimide (Dojindo) (purification and labeling procedures described in Bergendahl et al. 2002 (19)). It is possible to monitor complex formation between σ70 and β' simply by looking at the long-lived emission of the acceptor (IC5) due to LRET as an optically measurable signal of complex formation. Measuring time-resolved fluorescence allows one to start signal acquisition after the background fluorescence (potentially from a natural product) and the intrinsic acceptor fluorescence have decayed so all short-lived background fluorescence can be excluded from the measurement. This leads to a highly favorable signal-to-noise ratio and a higher sensitivity and confidence. This is especially important when working with natural product samples which often produce a high background fluorescence. The principle of the assay and the structure of the dyes are described in Figure 3.1.

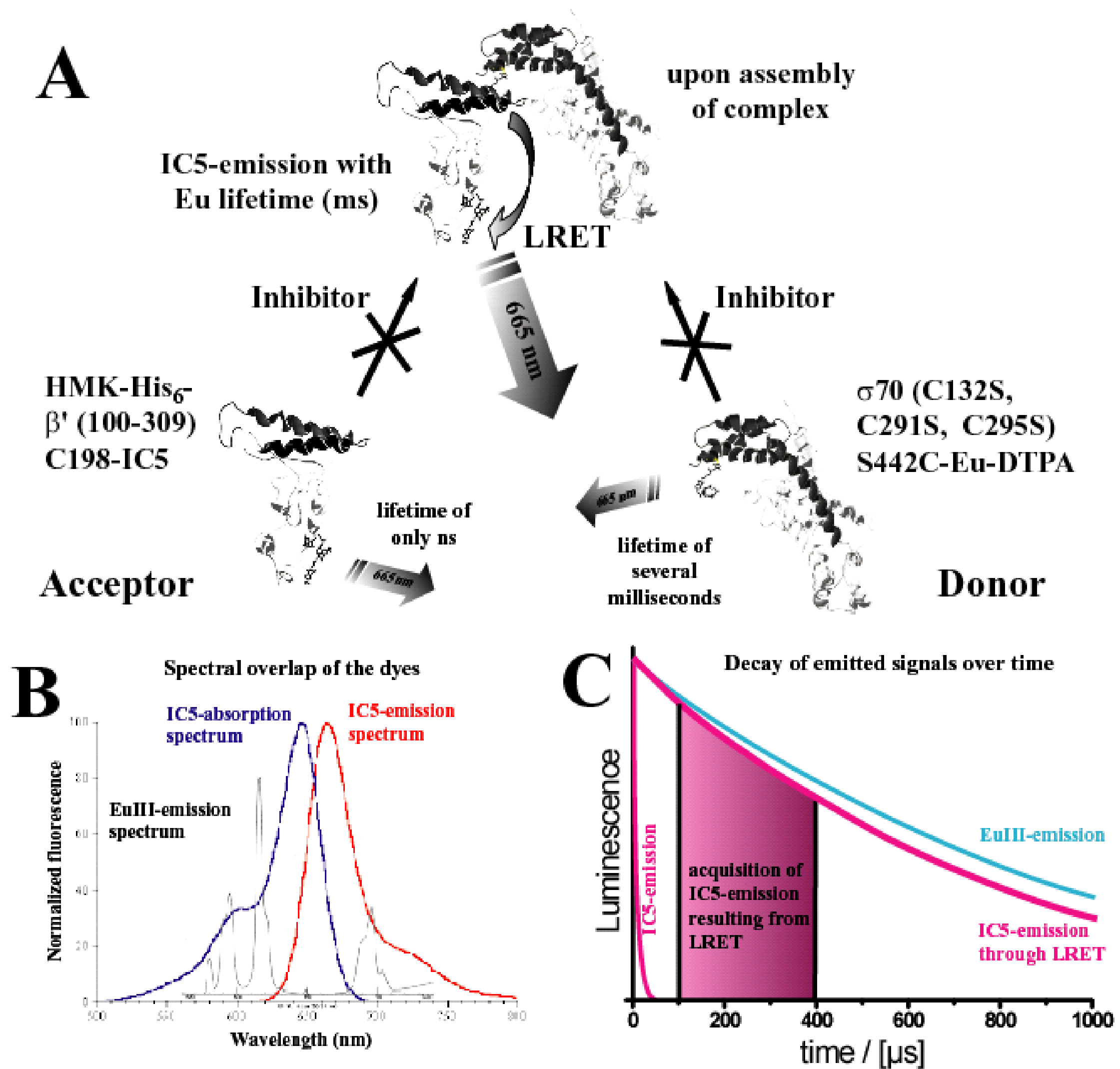



**Figure 3.1: Principle of the LRET assay A) The scheme above shows the principle of how the LRET signal in the assay is created upon binding of the labeled proteins β'-100-309 and σ70. The fluorescence of the IC5-labeled β'-fragment has decayed during the delay of the data acquisition 50 microseconds after excitation at 320 nm. Only the Eu-emission of labeled σ70 and the sensitized IC5 emission in the complex can be observed after the delay due to the characteristic long Eu-luminescence lifetime of over 1 millisecond. B) The spectra of the fluorophores that were used to derivatize the proteins are shown: The emission spectrum of the Eu-chelate (Eu-DTPA-AMCA-maleimide) is shown in black. The the absorption spectrum (blue) and the emission spectrum (red) of IC5-PE-maleimide are superimposed to show the spectral overlap between these dyes that facilitates LRET. C) In the diagram the luminescence is plotted versus the time. The graphs show the intensity of the luminescence emitted at 615 nm and 665 nm over time. Where as the intrinsic IC5-emission has decayed long before the data acquisitions starts, the emission from the IC5 sensitized by LRET goes on for over 1 ms. That way only the IC5-emission due to LRET is acquired in the data acquisition window, where as the background emission and the intrinsic IC5-fluorescence is excluded.**

The assay can be performed in a multi-well plate and measured by a multi-plate reader to accomplish a high-throughput of a large number of samples from any chemical library in an automated way. Typical reaction volumes are 10-200 µL where the components including the test substances are mixed directly in the multi-well plate before the plate is measured in the reading device. The very sensitive nature of such a fluorescence-based assay (typically in the low nanomolar range) provides good accuracy and signal-to-noise ratio, avoiding false positive hits in the measurement. We have chosen to screen a marine sponge library (20) since it has been successfully screened for cytotoxic compounds. Due to its complexity it demanded a high performance of the assay and was therefore well suited to evaluate its applicability. We show the robust nature of the assay and its suitability for use as a high-throughput screen.

## 3.3 Experimental procedures

### *3.3.1 Materials and chemicals*

Overproduction, labeling and purification of HMK-$His_6$-β'(100-309) and sigma70(442C) were described in (19). The origin of the natural product library comprises privately collected samples of marine sponges (20) extracted with acetone and ethylacetate. The samples (2 to 5 mg dry weight) were dissolved in 1 ml methanol and diluted 1:50 in DMSO for the assay. All chemicals used were purchased from Sigma unless otherwise indicated in the text. A multi-plate reader (Wallac, $VictorV^2$ 1420) was used to perform the LRET assay. Buffers: NTG-buffer: 50 mM NaCl, 50 mM Tris, 5% glycerol; pH 7.9; Storage buffer: 50 mM Tris-HCl, 10 µM EDTA, 0.5 M NaCl, 10% glycerol, pH 7.5; TNTw-buffer: 6 M GuHCl, 50 mM Tris-HCl pH 7.9, 500 mM NaCl, 0.1 % (v/v) Tween20, 400 mM imidazole. Core RNA polymerase was prepared according to Thompson et al. (21).

### *3.3.2 LRET assay to test for the inhibition of protein-protein interaction between labeled σ70 and the β′-subunit*

The assay was performed in NTG-buffer (200 µL total volume) plus 2.5% DMSO (when library samples are used) with 40 nM σ70* (labeled protein) and 30 nM β'*-fragment (labeled protein). All assays were performed with refolded σ70*. In the standard assay the β'*-fragment was added in denatured form directly into the reaction mixture, which by 10-fold dilution of GuHCl to 0.15 M in the final assay allowed instant refolding. This was done to prevent precipitation of the refolded labeled protein upon storage before the assay (19). In order to assure that refolding occurs and to confirm the results from the experiments with denatured protein being added, some assays were carried out by adding β'*-fragment in its refolded state.

A stock solution (200 nM) of σ70* was prepared prior to the assay by 1:200 dilution with NTG of labeled protein (40 µM) stored at -20°C in Storage buffer. A stock solution (1.25 µM) of the β'*-fragment was prepared by dilution of labeled denatured protein (75 µM in TNTw-buffer containing 6 M GuHCl) to 1 M GuHCl with NTG and NTG + 6 M GuHCl. First, 10 µl of that σ70*-stock solution was mixed with NTG buffer (amount adjusted to give a final volume of 200 µl), then the potential inhibitor (5 µL in DMSO), salt or solvent was added and finally the 5 µl of denatured labeled β'*-stock solution was added. Salts or denaturants were dissolved in NTG buffer according to the desired final concentration to maintain standard buffer conditions. Mixing (pipetting up and down three times) after addition of each component was very important for reproducible results. The mixture was incubated for 30 min at RT and measured in a 96-well plate (Costar 3650) with a multi-plate reader (Wallac, Victor$V^2$ 1420). For this time-resolved fluorescence measurement, the

manufacturer's protocol (LANCE high count 615/665) was used (excitation was with 1000 flashes at 325 nm; measurement was delayed by 100 µs and data acquired for 50 µs at 615 and 665 nm).

In fluorimetric measurements it is common to use a second emission wavelength as an internal standard. This allows correction for instrument noise, but also to normalize the signal for the actual amount of donor in this particular case. This is possible, since donor and acceptor emission wavelengths are well separated and can be acquired separately with the multi-plate reader. The IC5-emission is corrected for the very small amount of signal from the Eu-emission band (by cross talk measurement of a standard) and then divided by the intensity of the Eu signal. The normalization can be included into the overall measurement protocol and is described by the manufacturer of the multi-plate reader (Wallac). Fortunately the nature of this method allows us to differentiate the loss of signal due to inhibition of protein-protein binding from simple absorption caused by the inner filter effect of the substance. This will help us to identify false positives in the actual high throughput screen.

### *3.3.3 In vitro transcription*

The conditions and procedure in the *in vitro* transcription assay were according to those published by Landick et al. (22) except for omitting BSA since it could bind potential hydrophobic inhibitors (Assay conditions: 100 mM NaCl, 25 mM Tris HCl pH 7.9, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.15 mM ApU, 20 nM template DNA, 20 nM core RNAP, inhibitor in final concentration 2% DMSO, 10 nM σ70, 25 µM ATP and CTP, 10 µM GTP, [$\alpha$-$^{32}$P]GTP 20 µCi/100 µl, added in the order listed). As template we amplified a 694-bp fragment from the plasmid pCL185 by PCR using the primers 5'-GTT TTC CCA GTC ACG AC-3' and 5'-CAG TTC CCT ACT CTC TCG CAT G-3'. Omitting

UTP in the reaction mixture and initiating with ApU dinucleotide results in a halted complex at position +16 and the transcribed RNA oligonucleotide aug gag agg gac acg g. The assay tests for the ability of RNA polymerase holoenzyme (core plus sigma factor) to recognize a sigma70-dependent promoter (T7A1; located after base pair 280 of the 694 bp PCR fragment) and to initiate as well as elongate to yield the 16-mer. Failure of sigma binding to core would result in a lack of product.

### 3.4 Results

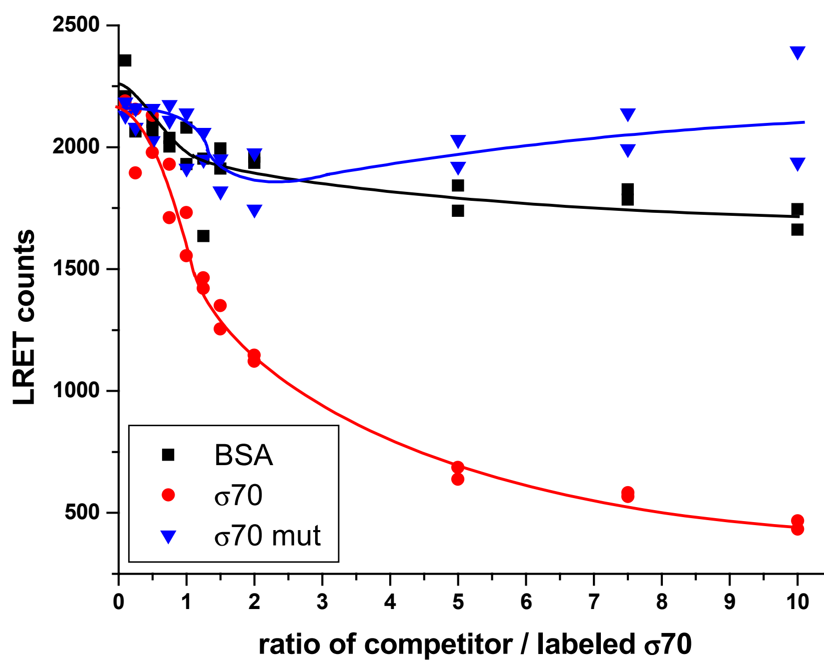
Labeled as well as the unlabeled proteins were shown to be able to form a complex in EMS assays using native PAGE gels (19). The different scanning techniques also confirmed the identity of the bands in the EMS-assay. Furthermore the EMS assay showed that unlabeled β'-fragment can compete for binding to the labeled σ70. Thus unlabeled β'-fragment itself represents a positive control for an agent able to interfere with the binding of labeled β'-fragment and σ70 in the assay.

#### *3.4.1 Validation of LRET assay to test for the inhibition of protein-protein interaction of labeled σ70 and β' with unlabeled proteins*

The LRET assay provides a fast and reproducible alternative to the EMS assay (19) to monitor the formation of a protein-protein interaction between labeled σ70 and β'-fragment as well as its inhibition. All results of the EMS assay were reproducible by the LRET assay and as an example we were able to show competition of labeled σ70 binding to the β'-fragment by increasing amounts of unlabeled σ70 (Figure 3.2). As a very important feature of the assay, the signal-to-noise ratio was between 10 and 11. The limit of detection within the

assay using the described instrument was 1 nM of labeled σ70. A maximum of 250 nM labeled σ70 could be applied to the assay before diffusion controlled LRET occurred.

**Figure 3.2: Competition of unlabeled σ70, a non-binding σ70 mutant (D403R) (2) and BSA with labeled σ70 for binding to the β'-fragment. Only the σ70 (wt) competes for binding to the β'-fragment while the other proteins show only non-specific binding resulting in a slightly lower LRET counts value.**




### *3.4.2 Influence of salt and organic solvents on the LRET assay*

In further experiments the dependence of salt (NaCl and GuHCl) and solvents (methanol, ethanol and DMSO) were characterized (Figure 3.3). As can be seen, the salt concentration has a

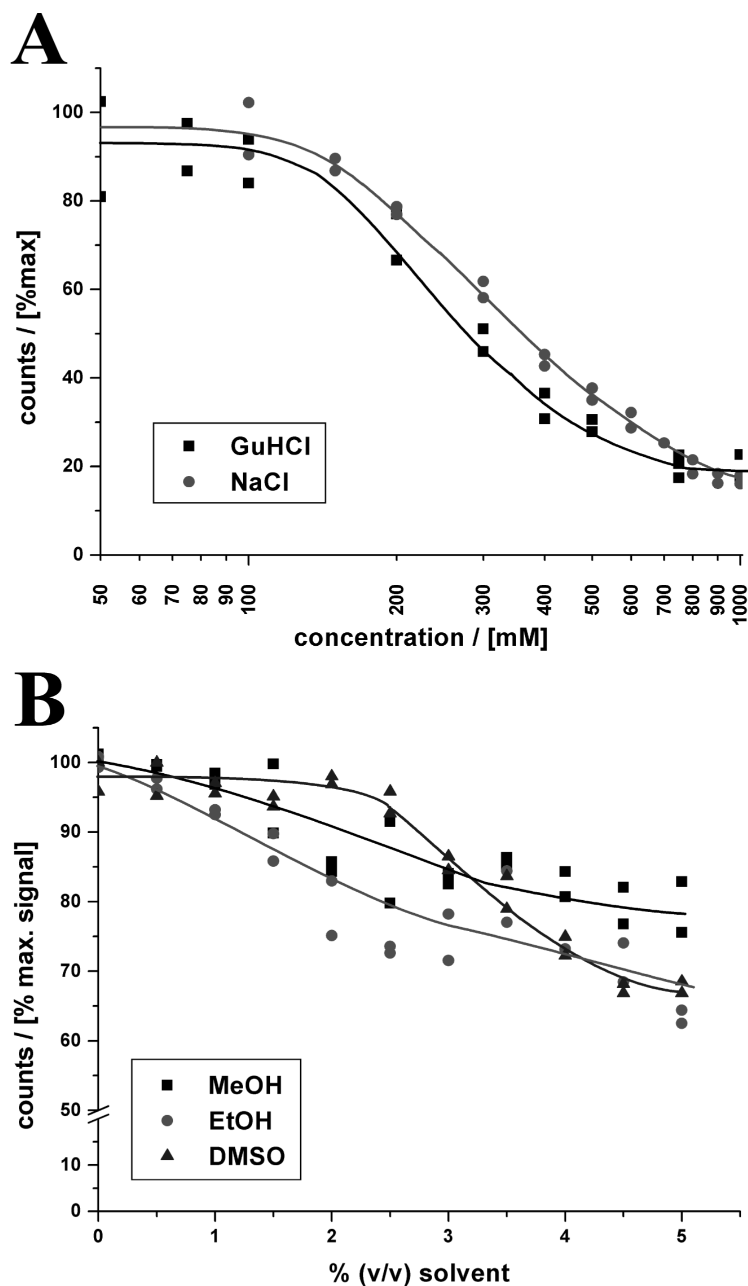



**Figure 3.3: Dependence of salt concentration and effect of solvent concentration on the LRET-assay. A: With increasing amounts of salt (NaCl, GuHCl), the LRET-signal significantly decreases which should be due to the decreased amount of σ70/β'-complex formed. B: Increasing amounts of DMSO, methanol and ethanol (0-5%) mixed with the assay buffer prior to the addition of proteins.**

major effect on the assay, since the signal drops to 50% when the NaCl concentration is increased from 100 to 400 mM. It is known that this β'-fragment interaction with σ70 is weakened by increased NaCl concentration. On the other hand DMSO as a common solvent for natural products to be tested has no significant effect on the assay. The signal in the

LRET-assay was not critically affected by the amount of DMSO present up to 2.5%. In the same experiment ethanol and methanol showed a more significant effect over the range of 1 to 5%.

### *3.4.3 Performance of the LRET assay in a preliminary screen of 100 sponge extracts*

The concentration of inhibitor in the extracts of marine sponges was estimated to be less than 1 µM (assuming a 2 mg sample was 100% a single component of an approximate molecular weight of 1000 dissolved in 1 ml methanol, diluted 1:50 in DMSO and 5 µl applied to the reaction mixture (200 µl) in the screening assay). Out of 100 samples tested, the sample D7 turned out to be the only extract to reduce the signal by roughly 90% in the assay. Samples E1, E12, G1, H4 and H6 showed a loss of signal of more than 50% which served as an arbitrary threshold (between 55 and 75%). Most samples (like A7 and B12) were significantly less active in inhibition of binding judged by the loss of the LRET signal (Figure 3.4). The variance of the obtained values for the majority of samples spread over a wide range of up to 40% loss of signal.

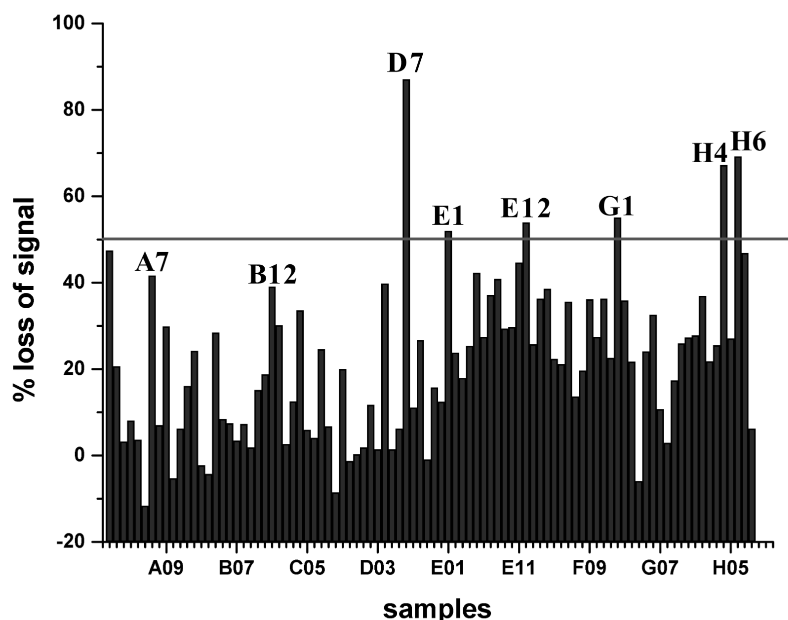



**Figure 3.4: Screen of 100 extracts from marine sponges with the LRET assay. Inhibition of σ70-binding to the β'-fragment was tested with 100 extracts from marine sponges (G. Marriott and J. Tanaka). Binding of σ70 to the β'-fragment could be significantly inhibited by fraction D7, while other fractions show lower inhibition under these conditions. Assuming a molecular weight of 1000 Da and 100% active content in a 2 mg sample, concentrations used are in the low µM range.**

### *3.4.4 Confirmation of hits by the LRET assay and by in vitro transcription*

The concentration of inhibitor in the extracts of marine sponges was examined over the range of 30 nM to 2 µM in the LRET assay. Samples A7, B12, E1, E12, G1, H4 and H6 showed no or very minor inhibition. The sample D7 turned out to be the only extract to give a clear inhibition curve over the examined range (Figure 3.5A). That was confirmed by *in vitro* transcription assays. As judged by densitometry of the electrophorograms of the gels, D7 has an $IC_{50}$ value of around 1 µM (Figure 3.5B) assuming the mass and content of the sample mentioned above. There was no significant difference between the $IC_{50}$ values determined by LRET and those derived from the *in vitro* transcription assays.

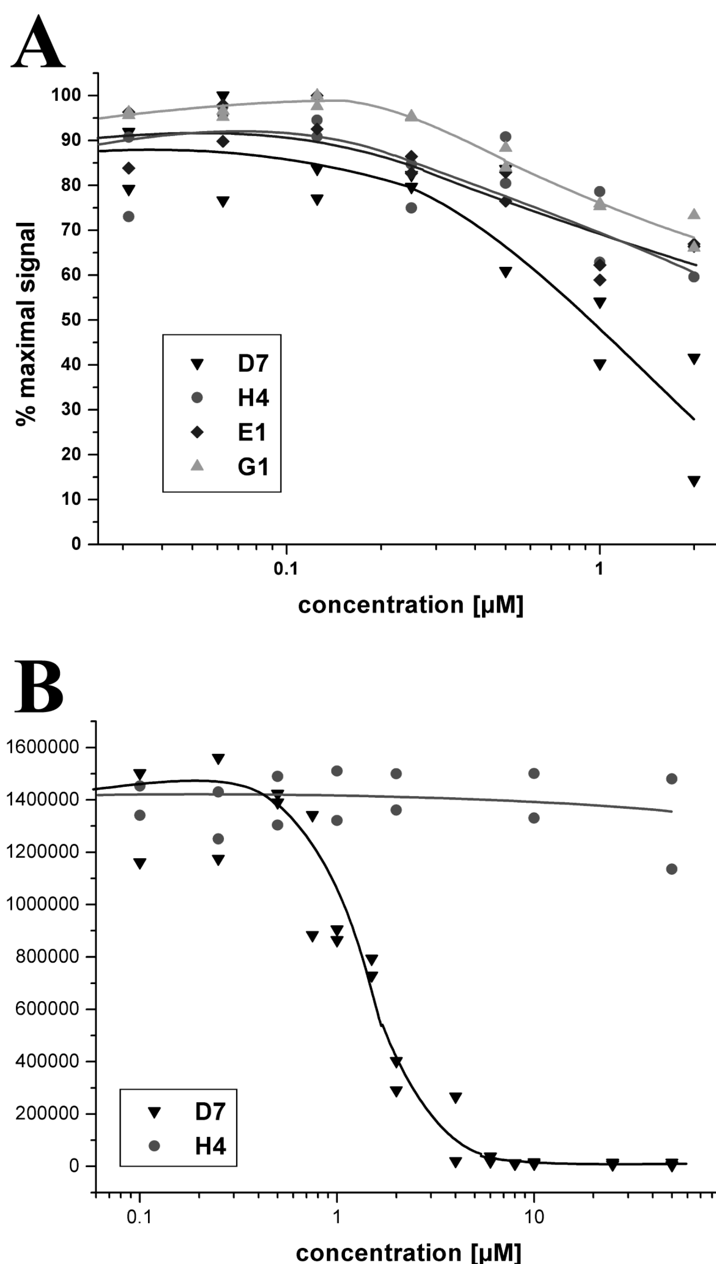



**Figure 3.5: Validation of the hits with LRET and *in vitro* transcription assays. A: Some candidates (D7, H4, E1, and G1) resulting from the screen were tested in the LRET assay over a range of 33 nM to 2 µM. B: To determine functional effect of an inhibitor of the σ70-β' binding, we tested the candidates in an *in vitro* transcription assay. The estimated concentrations in the assay are between 0.1 µM and 50 µM (E1 and G1 were similar to H4 and are not shown). The autoradiogram of the sequencing gel was used to quantify the transcription product from a halted complex using a "T-less cassette" as a template and omitting UTP in the reaction mixture (see methods). The resulting 16-mer can be separated on the gel and quantified using Molecular Dynamics software.**

**3.5 Discussion**

The use of luminescence resonance energy transfer to investigate protein-protein interactions in a homogenous assay has been described before along with its advantages and challenges. In spite of that there are not many publications that describe the development of an assay based on this technique and actually documenting its final application. In this work we describe a straight-forward approach to design a robust high-throughput assay which can be exploited for any other pair of proteins that appears to be a valid target for drug discovery.

There are several reasons to believe that the primary protein-protein interaction between bacterial core RNAP and sigma factors represents a prime target for drug discovery. The key to the potential of this target is the absolute requirement of sigma binding to core RNAP for the initiation of transcription; no bacterial cell can grow upon uptake of an inhibitor that effectively blocks this interaction. In addition to a very high bioactivity, a good specificity can also be expected since the binding region of both proteins is highly conserved among bacteria and is significantly different from any known eukaryotic analogue. This implies a very low probability for side effects to occur due to interference with human RNAP. The site itself offers another advantage over many potential and specific targets. Since the binding site on the β'-subunit of RNAP is suspected to interact with many if not all sigma factors of a bacterium, the development of resistance via point mutations against an inhibitor which binds to the β'-subunit of RNAP in the binding site is unlikely, since it may impair binding to any of the essential sigma factors. Due to the increasing incidence of antibiotic resistance and the growing need for new antibiotics, this has recently become a major issue in drug discovery.

Using luminescence resonance energy transfer (LRET) to measure sigma binding to core RNAP has been shown by Heyduk and coworkers to be an effective and very sensitive method. We used a well-characterized σ70(442C) mutant (18) with all natural cysteine

residues mutated to serine residues. Previous work also showed its preserved activity in *in vitro* transcription assays (18). Is derivatized with a DTPA-AMCA-maleimide Eu-complex that serves as a donor in the LRET assay. A fragment (residues 100-309) of the β'-subunit of RNAP with a N-terminal HMK-recognition site and $His_6$-tag fusion was derivatized with IC5-maleimide as LRET acceptor (19). We were able to show with electrophoretic mobility shift (EMS) assays (19) and with spectrometric measurements using time-resolved fluorescence that the labeled proteins can bind to each other in all combinations with and without the label. As controls, the unlabeled proteins were tested to determine if they could compete with their labeled counterpart. In both assays, EMS and LRET, unlabeled β'-fragment was able to compete with labeled β'-fragment for binding to labeled σ70. With this data we conclude that the assay can be used to monitor σ70 to β'-binding, and can be used to screen for inhibitors of this protein-protein interaction. The assay represents a fast and sensitive probe for this particular complex-formation. Substrates and material are either readily available or can be prepared in a simple and efficient procedures. All the labeled protein components show excellent stability during storage, a great advantage when screening large libraries with 10,000 to 100,000 or more substances. Furthermore the LRET assay has a very high sensitivity so that measurements can be performed at very low protein concentrations of 1 to 100 nM resulting also in a very low cost per screen.

The suitability of the assay for high-throughput screening of natural and combinatorial libraries was demonstrated by a screen of 100 extracts of marine sponges. The high demand on the performance of an assay when screening natural product libraries such as this, containing of very complex mixtures possibly more than 30 compounds becomes obvious by looking at the variance of the obtained values in the LRET assay. This variance occurs mainly due to flourophores in the mixtures that can absorb at any of the crucial wavelengths of the assay and is generally referred to as "inner-filter effect". The LRET-assay

turned out to be very sensitive and reliable. A very good signal-to-noise ratio of above 10 and the internal standard method help to distinguish between inhibition and fluorescence quenching by an inner filter effect of the test substance. This contributed enormously to avoiding false positive readings. Also the very good compatibility of the assay with the use of DMSO as a solvent to add test substances adds to its applicability as a high throughput screen. Since many natural products and most peptides or small molecules from combinatorial libraries have low solubility in water, it is necessary to use organic solvents. In this respect DMSO represents the most versatile and potent solvent and since it is often used in libraries, its compatibility is crucial for an assay. One sample could be identified by the LRET assay as an inhibitor containing extract and was confirmed by an *in vitro* functional assay. The assumptions made to estimate the concentration of the inhibitors should clearly overestimate the amount of active compound present so that it is fair to expect an inhibitory concentration of 10-100 nM or less. Initial studies on the ability of the D7 extract to inhibit cell growth of *E. coli* cells on culture plates were inconclusive due to limited amounts of available material (data not shown). Considering the complexity of the samples, we plan to carry out an automated screen of over 1500 more sponge extracts before deciding on which samples to characterize in detail.

In the case of natural product libraries, a positive hit could represent a new class of antibiotic, since no substance is known with such a mode of action. On the other hand the screen will help to identify known antibiotics, for which the mode of activity has not yet been identified or that reveal more than one activity. Together with positive hits from combinatorial libraries, these substances can then serve as lead structures to design and tailor a new compound with desirable characteristics such as: high activity, specificity, stability, ability to enter the cell and low side effects, costs, chance and likelihood of resistance

development on the other hand. In addition this assay can serve as a powerful tool to investigate the relative binding of different sigma factors and sigma factor mutants to core.

### 3.6 Acknowledgments

We would like to thank Katherine Foley, Katherine Coleman and Dr. Nancy Thompson for their support in the lab. We also thank Junichi Tanaka and Gerard Marriott for providing the natural product library. This work was supported by NIH Grant GM28575 to RRB.

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# Chapter 4

# Determination of Equilibrium Binding Constants for Sigma-Binding to Core RNA Polymerase by LRET

My contributions are that I developed labeling procedures, assay and data evaluation. The experiments were performed in collaboration with Larry C. Anthony from this lab who also provided purified $\sigma^{32}$.

## 4.1 Summary

The work in this  summarizes the evaluation of an LRET-assay for its capacity to measure equilibrium binding constants. The $K_{Diss}$ for $\sigma^{70}$ and $\sigma^{32}$ binding to core RNA polymerase from *E. coli* were determined using the proteins randomly labeled with the amine-reactive dyes IC3-OSu as LRET acceptor or the terbium chelate Tb-cs124-DTPA-NCS as LRET-donor. For $\sigma^{70}$ binding to core RNA polymerase a $K_{Diss}$ of 0.5 +/- 0.2 nM was obtained  and a $K_{Diss}$ of 28 +/- 10 nM for $\sigma^{32}$ by the use of this assay. These values are in good correlation with previously described results and therefore confirm the value of this LRET-based assay to determine binding constants.

**4.2 Introduction**

The determination of equilibrium binding constants for the interaction of sigma factors with core RNA polymerase has been a challenge since the discovery of the first sigma factor from *E. coli* in 1969 by Burgess et al. (1). Detailed information about the strength of the interaction under physiological relevant conditions would be needed to elucidate possible competition between the seven different sigma factors for core RNA polymerase in *E. coli*. This knowledge combined with reliable measurements of protein levels and would be an important step towards understanding the global transcriptional regulation of gene expression controlled by sigma factors.

Several attempts have been made in the past and a few binding constant determinations are published. The results of these publications are summarized for $\sigma^{70}$ and $\sigma^{32}$ in Table 1, which we decided to focus on in this work. Some of the results shown in Table 1 were obtained through non-homogeneous assays so that they suffer from a potential error since they do not represent equilibrium conditions as described in the introductions. This applies for studies using surface plasmon resonance (SPR) (2), size exclusion chromatography (SEC) (3), high performance liquid chromatography (HPLC) (4), ultracentrifugation (UC) (5) and glycerol gradient ultracentrifugation (GGUC) (6). Values for $K_{Diss}$ obtained from *in vitro* transcription (*iv*Tr) (6) and fluorescence quenching experiments (FQ) (4, 7) should be considered homogeneous and could enable measurements at equilibrium conditions.

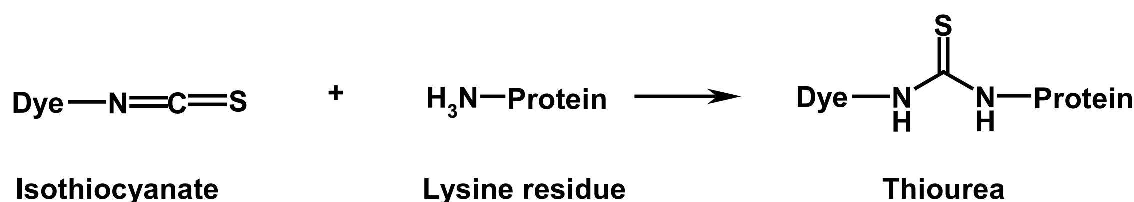
**Table 1: Previously published binding constants for binding of $\sigma^{70}$ and $\sigma^{32}$ to core RNA polymerase from *E. coli*. (ND: not determined)**

| Method / Factor | SPR (2) $K_{Diss}$ in nM | UC (5) $K_{Diss}$ in nM | HPLC (4) $K_{Diss}$ in nM | FQ (7) $K_{Diss}$ in nM | GGUZ / *iv*Tr (6) $K_{Diss}$ in nM | SEC (3) $K_{Diss}$ in nM |
|---|---|---|---|---|---|---|
| $\sigma^{70}$ ($\sigma^D$) | 190 | ND | ≤ 10 | 1 to 0.3 | ND | 0.26 |
| $\sigma^{32}$ ($\sigma^H$) | ND | < 2000 | ND | ND | 33 (GGUZ)<br>1 (*iv*Tr) | 1.24 |
| **Assay-Conditions** | | | | | | |
| pH | 7.4 | 8.0 | 6.5 | 7.5 | 7.9 | 7.6 |
| Temp., °C | 20 | 20 | 20 | 22 | 4 | 20 |
| NaCl, mM | 150 | 200 | 250 (KCl) | 200 (KCl) | 100 | 200 |
| Glycerol, % (v/v) | 0 | 10 | 0 | 0 | 15-35 | 5 |
| max. conc. of holo | 490 nM | 2.5 µM | 1 µM | 40 nM | 100 nM | 400 nM |

The purpose of the investigations presented in this  is to show if the LRET-binding assay, presented in Chapter 3 of this thesis, is applicable to the determination of equilibrium binding constants ($K_{Diss}$) for the sigma-to-core interaction. The studies described in Chapter 3 showed that the sensitivity as well as the detection limits would be suitable for measuring binding near nM concentrations. This would be desirable since working at concentrations near the binding constant will allow most accurate evaluation of the data and the obtained values for $K_{Diss}$ of $\sigma^{70}$-binging to core were mostly in the nM range. Several changes to the LRET-assay described in Chapter 3 have been made. Instead of the β '-fragment (100-309) we now used core RNA polymerase. Controlled labeling of core with a single fluorophore was investigated using intein-mediated expressed protein ligation (8) with the use of an IC5-labeled cysteine (synthesized following the synthesis strategy similar to the one described in Mukhopadhyay et al. (9)). Since this approach resulted in very low yields of fluorescence

labeled protein unsuitable for this investigations, a different approach was chosen. We decided to randomly label sigma factors and core RNA polymerase from *E. coli* using amine-reactive fluorescent probes. The RNA polymerase subunits contain the following number of lysine residues: $\alpha$ 16, $\beta$ 80, $\beta'$ 87, $\sigma^{70}$ 34 and $\sigma^{32}$ 16. Not all of them will be accessible for derivatization, but a large number of sites are expected to be available for fluorescence labeling. Succimidyl ester (OSu) and isothiocyanates (NCS) were used for selective labeling of the primary amines in lysine residues (10). The reaction scheme for these derivatizations is shown in Figure 4.1.

A) Reaction of a primary amine with an isothiocyanate:




B) Reaction of a primary amine with a succinimidylester:

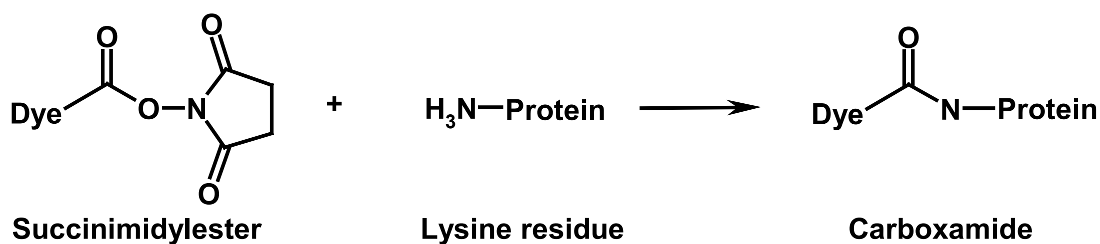



**Figure 4.1: Labeling reactions used for fluorescence labeling of $\sigma^{70}$ and core RNA polymerase**

For availability reasons we switched from the europium chelate to the terbium chelate carbocystryl24-diethyletriamine-pentaacetate-phenylalanine isothiocyanate terbium(III)

(cs124-DTPA-Phe-NCS•$Tb^{3+}$) and its compatible acceptor IC3-OSu. The structures of these dyes and a diagram explaining the principle of LRET with these dyes is shown in Figure 4.2.

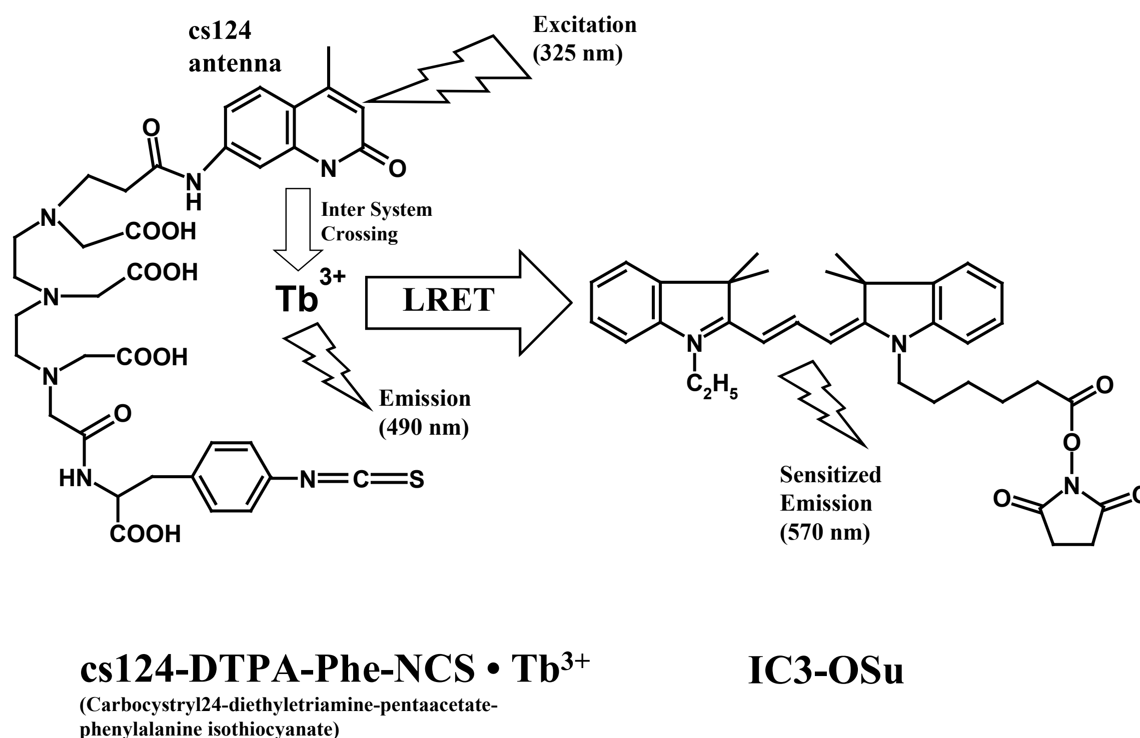



**Figure 4.2: Principle of LRET with Tb-donor and IC3-acceptor. The $Tb^{3+}$-ion is excited by an inter system crossing from the antenna molecule (carbostyril cs124) that can be excited by UV light at 325 nm. The terbium can now fluoresce with its particular emission spectrum, which contains a maximum at 490 nm applicable for observation in this setup. If the acceptor fluorophore IC3 is in close proximity (< 10 nm) it can act as a donor transferring its energy by LRET. That sensitizes IC3 which now fluoresces with a lifetime enormously increased from 1 ns to about 2 ms corresponding to the Tb-ion.**

Emission and absorption spectra of IC3 and the Tb-chelate are shown in Figure 4.3. The maximum emission of the Tb-ion overlaps perfectly with the absorption spectrum of the IC3. That makes it a very good LRET-pair, enabling maximum energy transfer, which at the same time leads to an optimal signal.

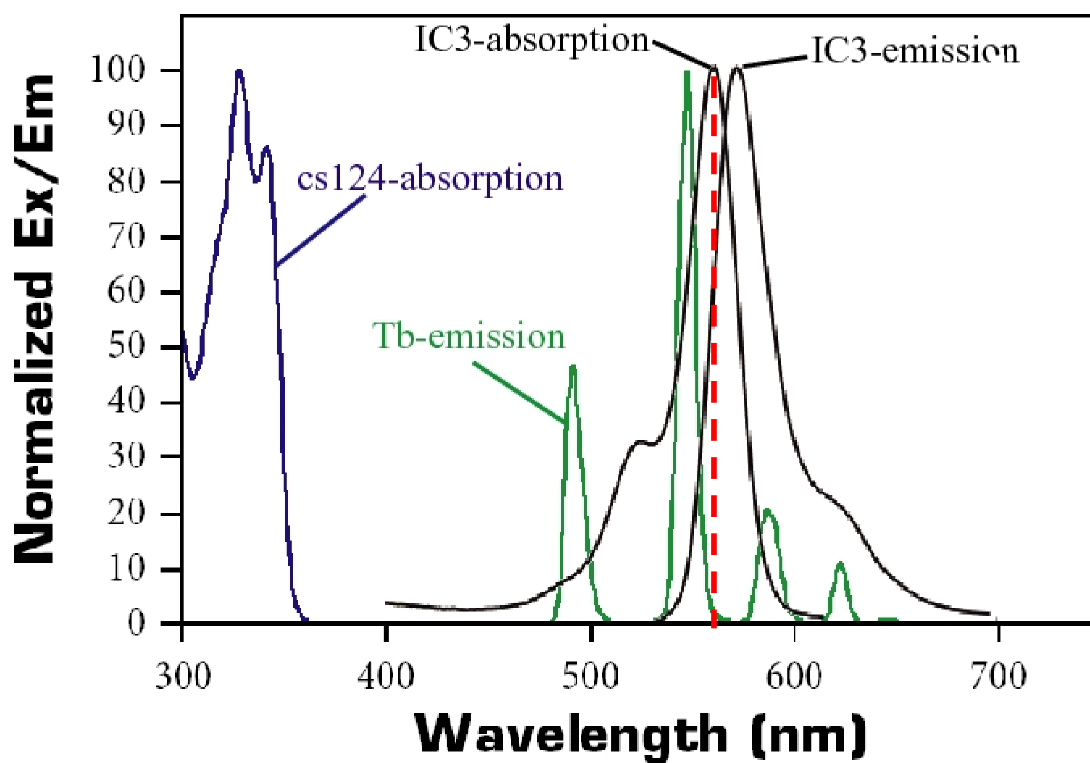
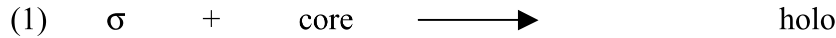



**Figure 4.3: Emission and absorption spectra of Tb and IC3 from the used dyes. The good overlap of the Tb-emission spectrum (green) and the IC3-absorption spectrum (left, black) leads to very good LRET. Also the maximum of the IC3-emission (indicated by the dotted red line) allows for good detection, since it contains minimal contribution by the Tb-emission (crosstalk).**

In order to fit the data from the LRET-assay for determination of $K_{Diss}$ we performed the following calculations based on the definition of the binding constant. Assuming a bimolecular binding reaction (all considered as correspondingly labeled):

(1) σ + core  holo

therefore $K_{Diss}$ is defined by:

$$(2) \qquad K_{Diss} = \frac{[\sigma] \quad [core]}{[holo]}$$

with

(3) $[\sigma]$ = concentration of free σ (at equilibrium)

(4) [core] = concentration of free core (at equilibrium)

(5) [holo] = concentration of free holo (at equilibrium)

(6) $[\sigma]_0$ = starting concentration of σ

(7) $[core]_0$= starting concentration of core

follows:

$$(8) \qquad K_{Diss} \cdot [holo] = ([\sigma]_0 - [holo]) \cdot ([core]_0 - [holo])$$

Since the LRET assay is an indirect measure of [holo] and $[\sigma]_0$ is kept constant while varying $[core]_0$ in every separate experiment, equation (8) has to be rearranged to get [holo] in dependence of $[core]_0$.

$$(9) \qquad [holo]^2 + (-[\sigma]_0 - K_{Diss} - [core]_0) \cdot [holo] + [\sigma]_0 \cdot [core]_0 = 0$$

Solving the quadratic equation (9) results in:

(10)

$$y = \frac{(-[\sigma]_0 \quad - K_{Diss} \quad - [core]_0)^2}{2} \pm \sqrt{\frac{(-[\sigma]_0 \quad - K_{Diss} \quad - [core]_0)^2}{4} \quad - [\sigma]_0 \quad [core]_0}$$

Normalization was done with equation (11).

(11) y = (LRET signal) / (maximum LRET signal) · 100

Equation (10) was used to fit the data from the LRET assay using Origin 7.0 (Microcal Software Inc., Northampton, MA).

## 4.3 Experimental procedures

### *4.3.1 Materials and chemicals*

A multi-plate reader (Wallac, VictorV$^2$ 1420) was used to perform the LRET assays. The assays were done in Costar 3915 8x12 multiwell plates. IC3-OSu was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, USA). cs124-DTPA-Phe-NCS•$Tb^{3+}$ was a generous gift from PanVera (Madison, WI). All other chemicals were purchased from Sigma unless otherwise indicated in the text. Purified RNA polymerase was made as described in Thompson et al. (11). Purified sigma factors were made as described in Anthony et al. (12).

### *4.3.2 Buffers*

10x PBS buffer: 1.55 M NaCl, 10.6 mM $KH_2PO_4$, 29.6 mM $Na_2HPO_4$•7 $H_2O$, pH 7.4; carbonate buffer: 1.7 g $Na_2CO_3$, 2.8 g $NaHCO_3$ in 100 ml $H_2O$ at pH 8.5; Blotto: 1% (v/v) non-fat dry milk powder in PBS-buffer. loading buffer: 50 mM NaCl, 50 mM Tris HCl pH 7.9, 5% glycerol. NTG buffer: 300 mM NaCl, 50 mM Tris HCl pH 7.9, 5% glycerol.

### *4.3.3 Fluorescence labeling of $\sigma^{70}$, $\sigma^{32}$ and core RNA polymerase*

The proteins were dialyzed into carbonate buffer. The protein concentration was adjusted by dilution with carbonate buffer to 0.5 mg/ml. In the case of core RNA polymerase, 1 mg of IC3-OSu was dissolved in 1 ml of chloroform and divided into 10 aliquots of 100 µl each in 1.5 ml Eppendorf vials. The chloroform is evaporated in a Speed Vac (5-10 minutes) and the vials are stored in sealed, light-excluding bag at -20°C. These aliquots were further divided to give 10 µg per aliquot. One aliquot of 10 µg IC3-OSu were dissolved in 10 µl DMSO (1.3 nmol/µl) and 2 µl were added to 1 ml of core RNA polymerase (0.5 mg/ml; 1.3 nmol) to give a 2-fold ratio of IC3-OSu to protein. While the reaction was incubated for 30 minutes at room temperature (24°C), a gravity flow column was prepared with 1 ml DEAE cellulose (DE-52, Whatman) and equilibrated with the loading buffer. The labeling reaction was diluted 10-fold with loading buffer and then loaded onto the resin. After washing with 10 ml loading buffer, the labeled protein was eluted with NTG-buffer. This was done to separate the labeled proteins from the unbound label which could interfere with the LRET assay. The same procedure was performed with $\sigma^{70}$ and $\sigma^{32}$ using cs124-DTPA-Phe-NCS•$Tb^{3+}$ (MW: 909 g/mol) with the same concentrations for labeling. Ion exchange chromatography was used in place of dialysis, since we observed very poor dialysis efficiency for the cyanine dyes and the Tb-chelate.

### *4.3.4 LRET assay*

The LRET-binding assays were performed in NTG buffer at (20°C). The concentration of sigma factor was kept constant at 10 nM. Core concentrations were varied to 0, 0.5, 1, 5, 10, 20, 25, 40, 50, 60, 75, and 100 nM. After 5 minutes incubation at 20°C the samples were measured. As protocol for the plate reader we used a LANCE template

provided by the manufacturer. The excitation was done with 1000 flashes at 325 nm and the emissions at 490 nm (Tb-specific) and 570 nm (IC5-specific) with a 50 µs time delay after excitation and a counting period of 50 µs. The LANCE correction offered by the manufacturer's software was used to correct for background and crosstalk.

### 4.4 Results

The results of the assay are presented graphically in Figure 4 and 5.

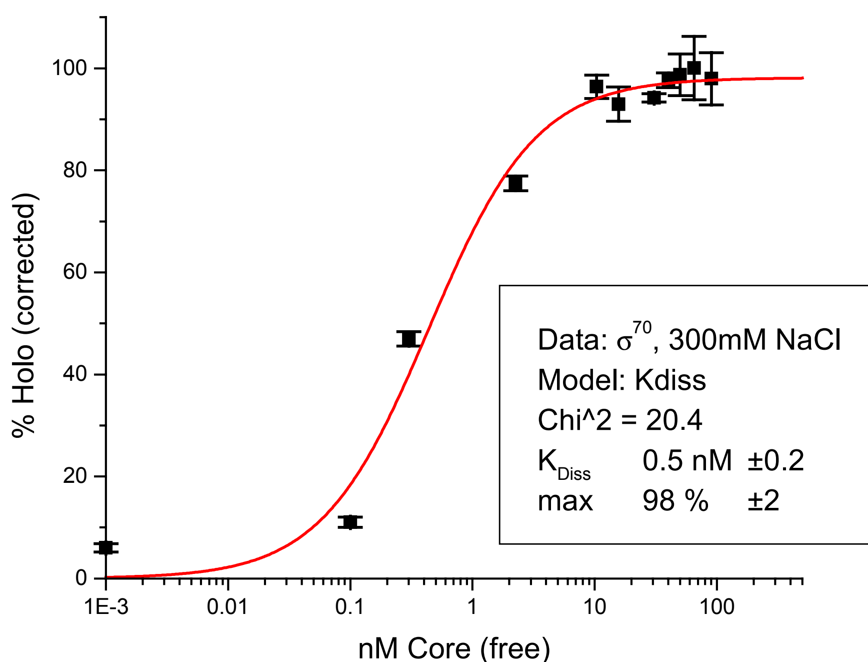



**Figure 4.4: Fit of the binding assay data with $\sigma^{70}$ at 300 mM NaCl. The box in the corner to the right summarizes the fitting data including chi-values, the binding constant $K_{Diss}$ and the fitted maximal value at saturated holo.**

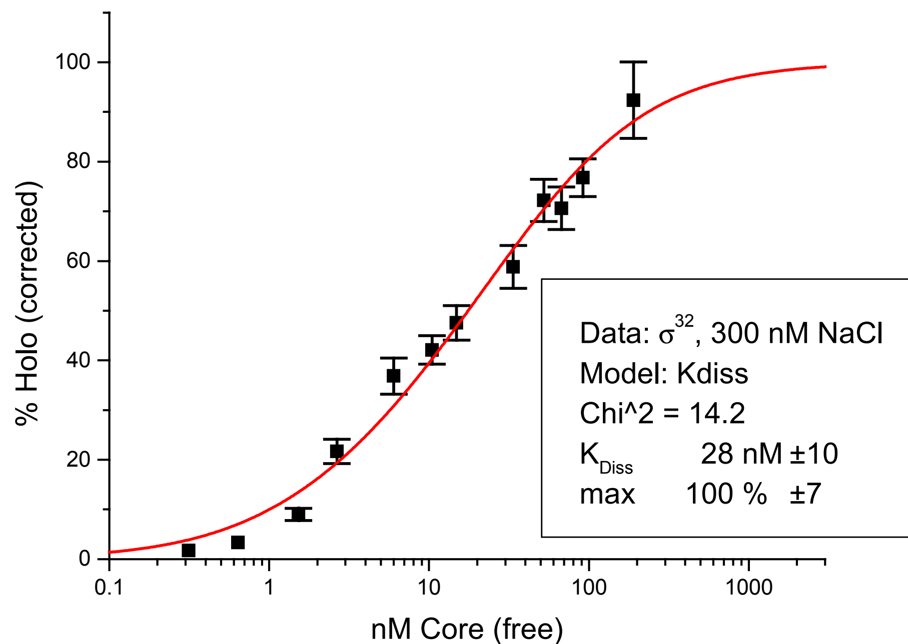



**Figure 4.5: Fit of the binding assay data with $\sigma^{32}$ at 300 mM NaCl. The box in the upper right corner summarizes the fitting data including chi$^2$-values, the binding constant $K_{Diss}$ and the fitted maximal value at saturated holo.**

According to the data, $\sigma^{70}$ binds core with a binding constant $K_{Diss}$ of 0.5 nM (+/- 0.2 nM) and $\sigma^{32}$ binds core with a binding constant $K_{Diss}$ of 28 nM (+/- 10 nM) at 20°C, 50 mM Tris HCl pH 7.9, 300 mM NaCl and 5% glycerol.

## 4.5 Discussion

Many different techniques have been used to determine the equilibrium binding constants for sigma-binding to core RNA polymerase. The evaluation and especially the comparison of the results has to be treated with great caution, since many parameters like salt concentration, temperature and pH influence the binding constant. Additionally some non-homogeneous techniques are limited to the fact that they disturb the binding equilibrium by separating the free components from their complex or through adsorption phenomena. The

conditions in this investigations were based on the experience gained by the previously published work. We have chosen to work at 300 nM salt (NaCl) to avoid aggregation of core and holo forms of RNA polymerase that was described by Gill et al. (4). Other parameters like pH (7.9) and temperature (20°C) were commonly used in the described studies.

The values for $K_{Diss}$ obtained in this study was 0.5 +/- 0.2 nM for $\sigma^{70}$ and 28 +/- 10 nM for $\sigma^{32}$. Although the confidence levels of the fitting procedure indicated by the chi-square values were not satisfactory, these numbers are well within the range of previously reported results. Especially those values that were obtained with homogenous techniques for $\sigma^{70}$ by Gill et al.(4) and Wu et al. (7) and for $\sigma^{32}$ by Joo et al. (6), should be taken to compare these new results (see Table 1). Judged by the results from the LRET assay and their similarity to previous published numbers this technique is well suited to study binding constants of nanomolar magnitude. It will be difficult to use the assay for weaker interactions, since limitations in the workable range does currently not allow to use higher than 200-300 nM concentrations of dye due to interference by diffusion-controlled LRET. Hence it will be impossible with the current method to reach saturation if proteins with binding constants of larger than 20 nM are investigated. Unfortunately just that region is very important for a good fitting evaluation and hence important for the reliability of the data obtained.

In order to use this LRET assay for the determination of new values for sigma-to-core binding constants with enhanced reliability, a few but very important improvements of the assay have to be pursued. It will be necessary to achieve better chi-square values, possibly through a greater number of measurements. If that is impossible, the fitting procedure has to be evaluated and possibly a different equation has to be used that describes the binding process more accurately. Gill et al. (4) and others agree that the assumption of a perfect bimolecular binding process is already a compromise, since it is known that there are multiple allosteric rearrangements upon binding of sigma to core that will certainly have an

effect on the way in which the process has to be described quantitatively. A few technical improvements could be considered. To avoid adsorption to the plate walls low affinity "Non-binding Surface" black opaque microplates should be chosen, that are especially treated with a proprietary polyethylene oxide-like material especially recommended for such fluorescence assays. Gill et al. (4) have also stated that heterogeneously fluorescence labeled proteins increase the uncertainty of such assays, so that it might be necessary to improve single labeling techniques and investigate the use of single labeled proteins. An extensive study of all seven sigma factors from *E. coli* under several conditions will be necessary to extend this study in order to obtain values with sufficient confidence levels and to gain new knowledge about sigma binding to core RNA polymerase. The ease of the procedure makes it a highly desirable tool for these measurements.

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# Chapter 5

## A Cross-Reactive Polyol-Responsive Monoclonal Antibody Useful for Isolation of Core RNA Polymerase from Many Bacterial Species

This Chapter has been submitted for publication as:

Bergendahl, V., Thompson, N. E., Foley, K. M., Olson, B. M., and Burgess, R. R. A Cross-Reactive Polyol-Responsive Monoclonal Antibody Useful for Isolation of Core RNA Polymerase from Many Bacterial Species. *Appl Environ Microbiol*.

My contributions are that I performed all the experiments and wrote the manuscript. Nancy E. Thompson and Katherine M. Foley introduced me into the methods for making of PR-MAbs. Brian M. Olson helped with the purification of RNA polymerase using PR-MAb 8RB13 in immunoaffinity chromatography during his work as an undergraduate student in the lab.

R. R. Burgess is the principal investigator.

## 5.1 Summary

The use of antibodies for protein purification is a powerful technique but the release of the target protein in its active form is often difficult. So called “polyol-responsive” monoclonal antibodies (PR-MAbs) have a feature that allows elution of the antigen under very gentle conditions, so that even multi-subunit proteins can be released in their active form. In this work a PR-MAb, 8RB13, was isolated that can purify RNA polymerase (RNAP) from many different bacterial species. High specificity towards RNAP with a broad species cross-reactivity was achieved by immunization with RNAP from *Escherichia coli* and screening with *Bacillus subtilis* RNA polymerase. The isolated MAb could detect the beta subunit of RNA polymerase from 11 out of 13 species tested on a Western blot. Four of these species *E. coli*, *B. subtilis*, *Pseudomonas aeruginosa* and *Streptomyces coelicolor* were subjected to immunoaffinity purification yielding RNA polymerases that were active in *in vitro* transcription and seemed to be primarily core polymerase, lacking sigma subunits.

## 5.2 Introduction

The purification of proteins using antibodies has been an important tool in protein purification. Monoclonal antibodies (MAbs) can be selected for high specificity and very strong binding to their antigen. This allows immunoaffinity binding and purification of a target protein from a very complex mixture like whole cell extracts even if it is present only in very low concentrations. A major disadvantage can be the fact that very harsh conditions like denaturants, detergents or extreme pH are usually required to release the desired protein from the antibody. Our lab has developed a method for protein purification using a remarkable property of certain MAbs that are called "polyol-responsive" (1-5). In the case of polyol-responsive MAbs (PR-MAbs), the antigen can be eluted by applying polyol-containing buffers (ethylene glycol, 1,2-propanediol or 2,4-butanediol) and salt, so gentle that even large protein complexes like holo RNA polymerase from *E. coli* (6 subunits, about 450 kDa) or RNA polymerase II from *Saccharomyces cerevisiae* (12 subunits, about 515 kDa) are preserved (6).

PR-MAbs were made by standard hybridoma methods. MAbs can be easily screened for polyol-responsiveness and cross-reactivity by a modified enzyme-linked immunoabsorbent assay (ELISA) that has been described as an ELISA-elution assay (2). We find that 5-10% of the MAbs produced in a fusion of mouse spleen and plasmacytoma cells turn out to be polyol-responsive, depending on the fusion and antigen. An immunoaffinity column to purify RNA polymerase from cell extracts can be made by covalently attaching purified MAbs from ascites fluid or hybridoma culture to a cyanogen bromide (CNBr)-activated resin. The cell extracts are pretreated with polyethyleneimine (PEI) and ammonium sulfate precipitation. Resolubilized ammonium sulfate precipitates are loaded onto the

immunoaffinity column and can be eluted under very gentle conditions using high salt and a polyol like 1,2-propanediol.

Here we describe the isolation and use of a polyol-responsive antibody that reacts with RNA polymerase from different bacteria. MAb-producing hybridoma cells were made from an *E. coli* RNA polymerase-immunized mouse. The MAb-containing culture supernatants from hybridoma cells were screened for recognition of *B. subtilis* RNA polymerase by ELISA and thus for their cross-reactivity between RNA polymerases from *E. coli* and *B. subtilis*. Two MAbs were tested for cross reactivity with RNA polymerase from 12 bacterial species in Western blots. The PR-MAb 8RB13 was subjected to immunoaffinity purification of RNA polymerases from *E. coli*, *B. subtilis*, *Pseudomonas aeruginosa* and *Streptomyces coelicolor*.

## 5.3 Experimental procedures

### *5.3.1 Materials and chemicals*

Media ingredients were bought from DIFCO. CNBr-activated Sepharose was purchased from Sigma. Disposable columns were purchased from Bio-Rad. DEAE-cellulose discs were purchased from McLeester Research Equipment, Inc. (Madison, WI). [$\alpha$-$^{32}$P]UTP was purchased from NEN. Calf thymus DNA was purchased from Roche. All chemicals used were purchased from Sigma unless otherwise indicated in the text. Core RNA polymerase for immunization of mice was prepared from *E. coli* according to Thompson et al. (3).

***5.3.2 Strains***

*Escherichia coli* K12 (MG1655, gift from Frederick R. Blattner, UW Madison), *Bacillus subtilis* ATCC 6051, *Streptomyces coelicolor* A3(2) (gift from Prof. Sir David Hopwood, John Innes Centre, Norwich, UK), *Shigella boydii* ATCC 9207, *Pseudomonas aeruginosa* ATCC 10145, *Pseudomonas fluorescens* ATCC 13525, *Pseudomonas putida* ATCC 12633, *Agrobacterium tumefaeciens* ATCC 15955, *Klebsiella pneumoniae* ATCC 13883.

***5.3.3 SDS PAGE samples***

*Rhodobacter sphaeroides* (whole cell extract, gift from Tim Donohue, UW Madison), *Anabena sp.* 7120 (purified RNA polymerase, gift from Robert Hazelkorn, University of Chicago), *Streptococcus pneumoniae* (whole cell extract, gift from Elizabeth Campbell, Rockefeller University, New York), *Bacillus subtilis* (purified RNA polymerase, gift from Glenn Chambliss, UW Madison).

***5.3.4 Media***

LB medium (Lennox): 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, 5 g NaCl in 1 l $H_20$; Brain Heart Infusion (BHI) medium: 37 g BHI in 1 l $H_20$; Yeast Extract-Malt Extract (YEME) Medium: 5 g Bacto Peptone, 3 g Bacto Yeast Extract, 3 g Oxoid Malt Extract, 10 g glucose, 340 g sucrose in 1 l $H_20$, 2 ml of 2.5 M $MgCl_2 \cdot 6H_2O$ added after autoclaving.

### *5.3.5 Buffers*

Lysis buffer: 200 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA; 0.1 mM DTT, 5% glycerol, TE buffer: 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA; PR-equilibration buffer: 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 150 mM NaCl; PR-wash buffer: 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 500 mM NaCl; PR-elution buffer: 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA, 700 mM ammonium sulfate, 40% 1,2-propanediol; storage buffer: 200 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1 mM EDTA; 0.1 mM DTT, 50% glycerol.

### *5.3.6 Cell growth and cell extract preparation*

All cells subjected to Western blot assays or purification were harvested during late logarithmic growth phase. *B. subtilis* ATCC 6051 was grown in BHI medium at 37°C, *S. coelicolor* A3(2) was grown in YEME medium at 30°C for 2 days. All other cells were grown in LB medium at 37°C. Cells were isolated by centrifugation, resuspended in 10 ml lysis buffer, lysed by incubation with 100 µg hen lysozyme per ml on ice for 30 min and sonication. SDS PAGE gels of cell extracts were loaded with approximately 1 µg of total protein.

### *5.3.7 Isolation of MAb 8RB13*

The procedure used to make monoclonal antibodies 8RB13 and 8RC8 followed the one described in Thompson et al. (3). Briefly a mouse was immunized with 10, 15 and 20 µg of purified core from *E. coli* in 2-week intervals. The spleen was harvested 4 weeks after the last immunization and 3 days after boosting the mouse with an injection of 100 µg of core. Isolated spleen cells were fused with plasmacytoma cells (cell line NS1). After the hybridoma cells were plated in selected medium, they were selected for antibody production by ELISA

and Western blots for reactivity towards core RNAP from *B. subtilis* and *E. coli*. With the ELISA-elution assays we determined the polyol responsiveness. In this ELISA-derived assay, we washed the multi-well plates with PR-elution buffer. Under these conditions most PR-responsive MAbs are washed away from their antigen. The subsequent detection with secondary antibody conjugated to peroxidase shows a decreased signal of 50% or less for PR-responsive MAbs. Selected cells were cloned by dilution and expanded for MAb production in culture or ascites production.

***5.3.8 Preparation of an 8RB13 immunoaffinity column***

The procedure used to make an immunoaffinity column with 8RB13 followed the one described in Thompson et al. (3). Briefly the antibody was purified by 45% saturated ammonium sulfate precipitation and passage through a DE52 column at pH 6.9. The minor impurities that are still present at this point do not interfere with the final immunoaffinity chromatography step. To make an 8RB13 immunoaffinity column, 9 mg of purified antibody (judged by absorption using a molar absorptivity of 200,000 $M^{-1}$ $cm^{-1}$) was conjugated per 1 g of dry CNBr-activated Sepharose to result in a final level of 2.5 mg MAb per ml packed wet resin.

***5.3.9 Immunoaffinity chromatography with 8RB13***

The procedure used for immunoaffinity chromatography with 8RB13 followed the one described in Thompson et al. (3). Briefly nucleic acids and proteins are precipitated by adding 10% (v/v) PEI to a final concentration of 0.2% (v/v) PEI at low salt (0.1 M NaCl) to a 10 ml cell extract of 5 g cells (wet weight). RNA polymerase was eluted from the PEI-pellet with 1 M NaCl and precipitated with 35 g ammonium sulfate per 100 ml. The ammonium

sulfate pellet was dissolved in TE buffer to give 150 mM salt (judged by conductivity) and loaded on a 2 ml 8RB13-immunoaffinity column equilibrated with PR-equlibration buffer. After washing with PR-wash buffer, RNA polymerase is eluted with PR-elution buffer that contains 40% 1,2-propanediol and 0.7 M ammonium sulfate that releases the antigen from the PR-MAb.

### *5.3.10 Nonspecific in vitro transcription*

The conditions and procedure in the *in vitro* transcription assay were: 100 µl total volume, 100 mM NaCl, 25 mM Tris HCl pH7.9, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, 2 µg calf thymus DNA, 50 nM core RNA polymerase, 200 µM ATP, GTP and CTP, 50 µM UTP, and 0.1 µCi/µl [α-$^{32}$P]UTP (12 µM). Reactions were incubated at 37°C for 10 min, before they were quenched with 25 µl of stop mix (0.1 M EDTA, 5% SDS). 75 µl of the quenched reaction was applied to DEAE-cellulose discs and dried under a heat lamp. The dried discs were washed three times with 350 mM $Na_2HPO_4$ and once each with $H_2O$ and with 95% ethanol. The dried discs were subjected to scintillation counting using 5 ml of BioSafe II mix. The assay tests for the ability of core RNA polymerase to transcribe from denatured DNA showed its nonspecific transcriptional activity.

## 5.4 Results

The fusion of spleen cells taken from a mouse immunized with RNA polymerase from *E. coli* with plasmacytoma cells yielded approximately 300 isolated hybridoma cell lines. Two cell lines, secreting 8RC8 and 8RB13 (8th fusion to core in the lab, MAb for RpoC and RpoB from clone 8 and 13, respectively), were isolated after screening for cross-reactivity between *E. coli* and *B. subtilis* RNA polymerase. The effective ammonium sulfate and 1,2-propanediol concentrations were determined by the ELISA-elution assay (Figure

5.1). The results from the ELISA-elution assay can differ from the behavior of the immobilized antibody in immunoaffinity chromatography. Thus we have chosen to use slightly higher salt and polyol concentrations, than suggested by the assay, in order to ensure efficient elution of RNA polymerase from an 8RB13 column.

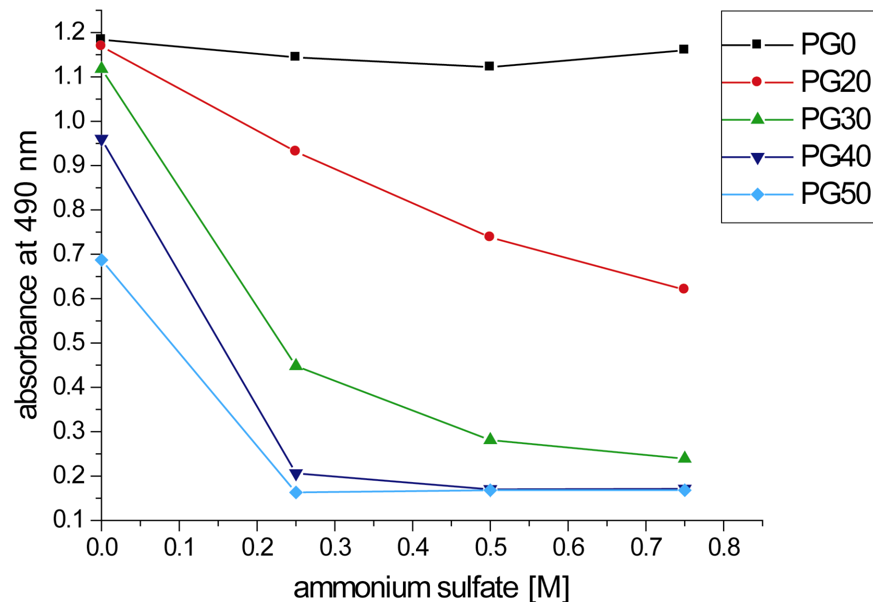


**Figure 5.1: ELISA-elution assay for 8RB13. In the ELISA-elution assay we tested 8RB13 with 1,2-propanediol concentrations at 0, 10, 20, 30, 40 and 50% (v/v) at ammonium sulfate concentrations of 0, 0.25, 0.5 and 0.75 M. Note that the release is a combined effect of salt and 1,2-propanediol.**

The isotypes of both MAbs were identified by an ELISA-based test as being IgG1 (data not shown). 8RC8 recognizes the beta prime subunit (RpoC) and 8RB13 recognizes the beta subunit (RpoB) of RNA polymerase as determined by western blots (data not shown). They showed broad cross-reactivity to different bacteria as follows: *E. coli* (8RC8/8RB13), *B. subtilis* (8RC8/8RB13), *Shigella boydii* (8RC8/8RB13), *Rhodobacter sphaeroides* (8RC8), *Pseudomonas aeruginosa* (8RC8/8RB13), *Pseudomonas fluorescens* (8RC8/8RB13), *Pseudomonas putida* (8RC8/8RB13), *Agrobacterium tumefaeciens* (8RC8/8RB13), *Anabena*

*sp.* (8RB13), *Klebsiella pneumoniae* (8RC8/8RB13), *Streptococcus pneumoniae* (8RC8) (Figure 5.2) and *S. coelicolor* (8RB13, data not shown).

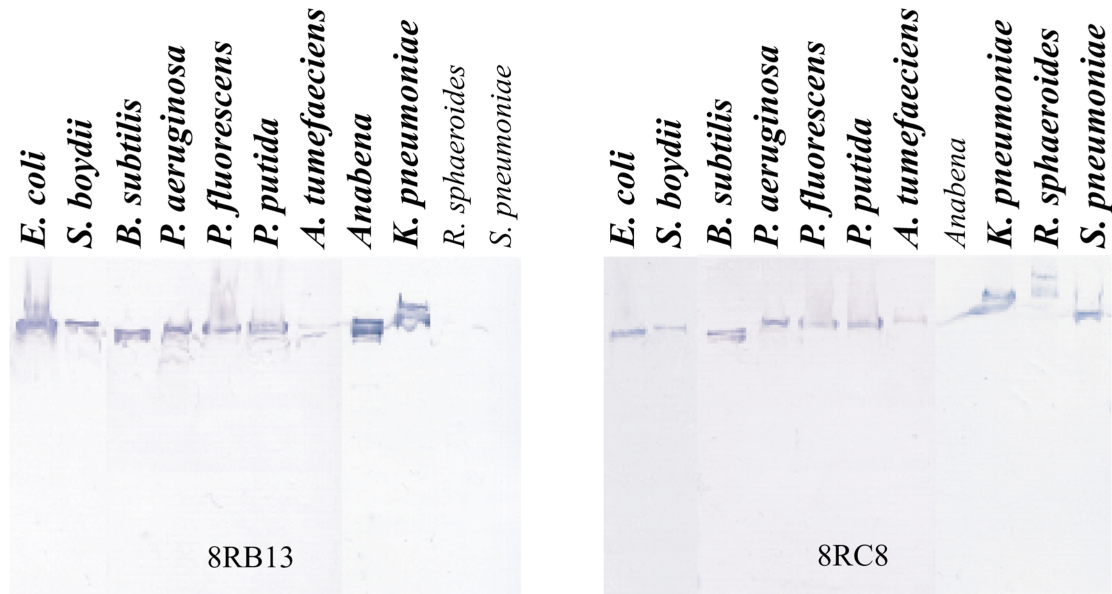


**Figure 5.2: Western Blots with 8RC8 and 8RB13 reacting with RNA polymerases from different species. Blotted RNA polymerases were incubated in the supernatants of the hybridoma clones for 1 hour and then subjected to the regular ECL kit procedure (Amersham). The names of species with RNA polymerase cross-reactive to the MAbs are indicated by bold letters.**

The hybridoma cell lines producing 8RC8 and 8RB13 were injected into and grown in the peritoneum of mice in order to obtain ascites fluid that contains a high concentration of MAb. These were then purified by ammonium sulfate precipitation followed by anion exchange chromatography with a DE-52 resin. The minor impurities that are still present at this point do not interfere with the final immunoaffinity chromatography step. The MAbs were then immobilized on a cyanogen bromide-activated Sepharose column. This immunoaffinity column was used to purify the antigen from cell extracts after removal of DNA by PEI precipitation and a 0-45% saturated ammonium sulfate cut. A typical elution

from a NT73 immunoaffinity column (NT73 is a polyol-responsive MAb, used for purification of *E. coli* RNA polymerase) is shown in Figure 5.3 last lane.

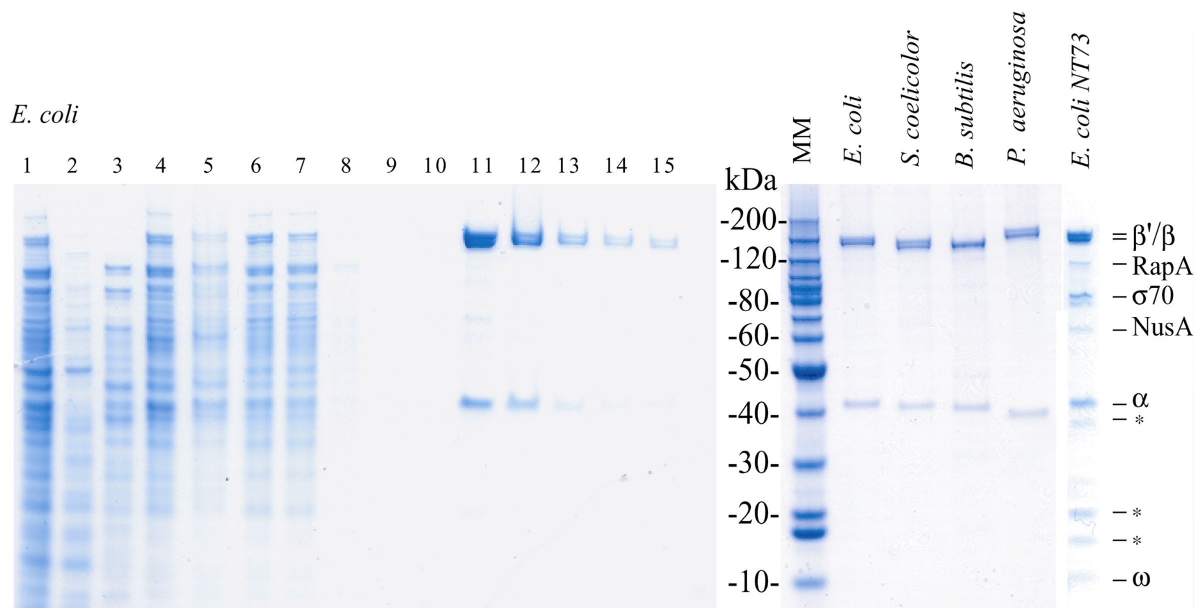


**Figure 5.3: Comparison of an immunoaffinity purification of RNA polymerase from *E. coli*, *S. coelicolor*, *B. subtilis* and *P. aeruginosa*. The left gel shows the different samples taken during the purification of RNA polymerase from *E. coli* with 8RB13 (left). Lanes: 1-whole cells, 2-insoluble pellet, 3-cell extract, 4-ammonium sulfate pellet, 5-ammonium sulfate supernatant, 6-PEI pellet, 7-load, 8-wash, 9-15-elution fractions. To the right, the RNA polymerase from *E. coli*, *S. coelicolor*, *B. subtilis* and *P. aeruginosa* purified with 8RB13 are shown. The rightmost lane shows the RNA polymerase from *E. coli* purified with PR-MAb NT73. The transcription factors bound to the RNA polymerase complexes are purified as a complex by NT73. The 8RB13 column binds only the core enzyme of *E. coli*. Positions of polymerase subunits are indicated. Asterisks indicate unknown proteins.**

Only 8RB13 of the two cross-reactive MAbs turned out to be capable of binding RNA polymerase in solution, so that it could be used in immunoaffinity chromatography. We tested an 8RB13 column with extracts of *E. coli*, *S. coelicolor*, *B. subtilis* and *P. aeruginosa* for RNA polymerase purification. The SDS PAGE gel shown in Figure 5.3 was loaded with samples taken during the purification and elution fractions from the *E. coli* preparation. Samples of the purified RNA polymerases from the other organisms are shown to the right. We got yields of 0.1 to 0.75 mg of almost pure core RNA polymerase per 1 g of wet cells that was surprisingly free of sigma70 as tested by Western blot using 2G10, a MAb cross-reactive

for major sigma factors from different species (7) as primary antibody (data not shown). The activity of the purified RNA polymerases was tested in an *in vitro* transcription assay. All samples were active for transcription (Table 1).

**Table 1: Summary of core RNA polymerase-purifications from 4 different bacteria using immunoaffinity chromatography with the PR-MAb 8RB13, illustrating activity and yield of each preparation.**

| Organism | Relative Non-specific Transcription (% activity of *E. coli*) | Yield (mg/g wet cells) |
|---|---|---|
| *E. coli* | 100 | 0.5 |
| *B. subtilis* | 13 | 0.4 |
| *P. aeruginosa* | 43 | 0.75 |
| *S. coelicolor* | 8 | 0.1 |

Note that the conditions used were optimized for *E. coli* RNA polymerase transcription and are probably not optimal for transcription activity of the other RNA polymerases.

## 5.5 Discussion

Polyol-responsive MAbs have been shown to be a valuable tool to purify RNA polymerase from *E. coli* and eukaryotic RNA polymerase II (4, 8). In this work we isolated two new MAbs, 8RB13 and 8RC8, with cross-reactivity to a number of prokaryotic RNA polymerases. We could show cross-reactivity of 8RC8 and 8RB13 on a Western-blot for the beta prime-subunit and to the beta-subunit of RNA polymerase, respectively, for *E. coli* (8RC8/8RB13), *B. subtilis* (8RC8/8RB13), *S. boydii* (8RC8/8RB13), *R. sphaeroides* (8RC8), *P. aeruginosa* (8RC8/8RB13), *P. fluorescens* (8RC8/8RB13), *P. putida* (8RC8/8RB13), *A. tumefaeciens* (8RC8/8RB13), *Anabena sp.* (8RB13), *K. pneumoniae* (8RC8/8RB13) and *S. pneumoniae* (8RC8). The cross-reactivity of the antibodies can be explained by the assumption that the epitope of each is a highly conserved motif in the recognized subunit of

RNA polymerase. Although 8RB8 was polyol-responsive in the ELISA elution assay, it could not pull RNA polymerase out of cell extracts and thus could not be used for immunoaffinity chromatography. This is likely because the epitope of 8RC8 is not exposed in its native form but is exposed in its immobilized form, when it is bound to the polystyrene surface of the multi-well plate.

The MAb 8RB13 was able to purify RNA polymerase from all 4 tested bacteria *E. coli*, *B. subtilis*, *P. aeruginosa* and *S. coelicolor* in a short immunoaffinity chromatography procedure. In addition, 8RB13 also gives a very pure core RNA polymerase without the need of using a BioRex70 column to remove the bound transcription factors. We tested the RNAP preparations by Western blots using an antibody (2G10) (7) highly sensitive and cross-reactive to the major sigma factor from different bacterial species. We were able to detect only a negligible amount of sigma70 or its equivalent in all four preparations. The reason for discrimination for the core enzyme by 8RB13 could be either a steric or allosteric effect of the MAb on RNA polymerase that either prevents holo RNA polymerase from binding or that releases sigma factors from the holoenzyme, respectively. The cross-reactivity of 8RB13 to *Anabena* is remarkable, since it belongs to the family of cyanobacteria and is therefore phylogenetically very different. The cross-reactivity nevertheless points to a significant similarity that was conserved over a broad range of evolution. However we did not see cross-reactivity of 8RB13 with yeast, wheat germ and calf thymus RNA polymerase in a Western blot (data not shown).

In future experiments we will determine the epitope recognized by 8BR13 and its effect on RNA polymerase activity in *in vitro* transcription experiments. That way we can study the structural as well as mechanistic effects that 8RB13 may have on RNA polymerase. 8RB13 has been shown to be valuable for purification of mutants and chimeras of RNA polymerase that were not amenable to purification with the conventional purification

strategies (unpublished results from this lab). Additionally we will evaluate 8RB13 for its utility to purify RNA polymerase from other species like *Helicobacter pylori*, *Mycobacterium tuberculosis, Staphylococcus aureus* and *Yersinia pestis* that have pharmacological relevance. We have developed a screen for inhibitors of the sigma to core RNA polymerase interaction for the *E. coli* machinery (9, Chapter 3). With the purified enzymes we can test the effect of these inhibitors on other systems and thus evaluate their cross-reactivity.

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# Chapter 6

## A Fast Western Blot Procedure Improved for Quantitative Analysis by Direct Fluorescence Labeling of Primary Antibodies

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Bergendahl, V., Glaser, B. T., and Burgess, R. R., A Fast Western Blot Procedure Improved for Quantitative Analysis by Direct Fluorescence Labeling of Primary Antibodies. *J Immunol Methods*.

My contributions are that I performed all the experiments and wrote the manuscript. Bryan T. Glaser helped with the Western blots during his rotation period as a graduate student.

R. R. Burgess is the principal investigator.

## 6.1 Summary

The procedures for Western blots have been around for a long time and recent developments have increased the sensitivity for luminescent techniques so that the need of radioactive probes has been limited to only a few applications. Nevertheless most protocols require more than 6 hours and are often performed over more than a day. The majority of techniques require a secondary antibody conjugated to an enzyme that catalyzes a color reaction in order to amplify a detectable signal. However both processes, the binding of a secondary antibody and the catalyzed reaction with the dye, are sources for errors and the latter is disadvantageous for a signal that is linear over a larger range of detected antigen. In order to improve the procedure most commonly used for quantitative analysis and convenience, we investigated the use of fluorescence labeling of primary antibodies against *E. coli* RNA polymerase subunits (β', σE and σFecI) and their use in Western blots. We achieved a sensitivity (< 1 ng detectable protein) comparable to most luminescent techniques. Additionally we reduced the procedure time significantly to less than 1 hour after SDS PAGE and transfer to a membrane. Above all we obtained a linear signal over the range of 1 ng to 1µg of protein making quantitative analysis of Western blots easier and more reliable.

## 6.2 Introduction

Western blots are widely used to detect proteins in biological samples with very high sensitivity. The most common and established procedures for Western blots involve SDS-PAGE to separate the proteins according to their size, a transfer to a membrane for immobilization and the use of two antibodies for their detection. The primary antibody specifically recognizes the protein of interest on the membrane. It is applied to the blot after it was blocked with either BSA or non-fat dry milk to saturate the membrane with protein to prevent unspecific binding of the antibody to the membrane. The secondary antibody (for example, goat anti-mouse IgG) recognizes the primary antibody according to its origin (for example mouse), since it generally binds to all immunoglobulins from the particular organism that the primary antibody was isolated from. The second antibody is typically conjugated with an enzyme catalyzing a reaction that turns a colorless compound into a detectable dye and can be used universally with compatible primary antibodies. Older procedures used radioactively-labeled secondary antibodies for sensitivity purposes (1), but recent developments make use of luminescent systems that reach similar performance without the disadvantages of working with radioactivity (2, 3). Both non-radioactive systems make use of an enzymatic reaction that amplifies the signal through a turnover of detectable substrates at the site of recognition. However this strategy poses a significant caveat for quantitative purposes, since it involves formation of a ternary complex at the recognized site and an amplification step through an enzymatic reaction. Both processes contribute to a potential variance of the signal created by a specific amount of sample. Additionally the linearity of the signal over an applicable range of sample amount is compromised especially by the amplification reaction (4).

In this work we describe an improved procedure for Western blots that can be performed in 1 hour or less after SDS-PAGE and transfer to the membrane. That not only results in a significantly shortened procedure, but also in an improved quantitative analysis with a linear range of detectable signal obtained from 1 ng to 1 μg of protein loaded on the initial SDS-PAGE gel. In order to achieve better reproducibility, signal-to-noise ratios and larger workable linear ranges for the quantitative use of Western blots, we investigated direct fluorescence labeling of the primary antibody. Fluorescence labeling of antibodies is commonly used procedure in fluorescence microscopy but surprisingly has not been used much in regular Western blot procedures. As a label, we used the cyanine dye IC5 as a bright fluorophore to achieve good sensitivity. Labeling was facilitated through a succinimide moiety linked to the dye, that reacts rapidly and selectively with lysine residues of the antibody in aqueous solutions under the conditions used. The fact that a secondary antibody and the addition of a substrate to develop the signal is no longer needed, significantly cuts down the time needed for this procedure, yet achieves limits of detection similar to most common luminescent techniques.

## 6.3 Experimental procedures

### *6.3.1 Materials and chemicals*

IC5-OSu was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD, USA). Pre-casted 4-12% NuPAGE gels were purchased from Invitrogen. The MAbs NT73 (anti β'-subunit of core RNA polymerase), 1FE16 (anti σFecI) and 1RE53 (anti σE) used for labeling were produced in our lab as described elsewhere (6). Nitrocellulose 0.04 μm pore size was purchased from Schleicher&Schuell. All other chemicals were purchased from Sigma unless otherwise indicated in the text. Purified RNA polymerase was made as

described in Thompson et al. (7). Purified sigma factors were made as described in Anthony et al. (6).

### *6.3.2 Buffers*

10x PBS buffer: 1.55 M NaCl, 10.6 mM $KH_2PO_4$, 29.6 mM $Na_2HPO_4 \cdot 7\ H_2O$, pH 7.4; carbonate buffer: 1.7 g $Na_2CO_3$, 2.8 g $NaHCO_3$ in 100 ml $H_2O$ at pH 9.5; Blotto: 1% (v/v) non-fat dry milk powder in PBS-buffer.

### *6.3.3 Fluorescence labeling of antibodies*

The antibody was dialyzed into carbonate buffer. It is important to have no primary amines present in the buffer, that would otherwise react with the succinimide moiety of the dye inactivating it for the labeling reaction. The antibody solution was diluted with carbonate buffer to a concentration of 1 mg/ml. The procedure also works in PBS buffer although the labeling reaction is about 2-fold less efficient. The dye comes as a dry solid (IC5-OSu, MW= 630.23 g/Mol, Dojindo) (Cy5 by Amersham or any other succinimide activated dye with the right fluorescence work as well). Succinimides are sensitive to moisture, since they can hydrolyze and thus lose their coupling activity. Therefore it is best to keep the dye as a solid under dry conditions. Avoid storage in aqueous or organic solvent. To save material, small portions of dye with a known amount should be prepared. 1 mg of IC5-OSu (Dojindo, 135 US$) was dissolved in 1 ml of chloroform and divided into 10 aliquots of 100 µl each in 1.5 ml Eppendorf vials. The chloroform is evaporated in a Speed Vac (5-10 minutes) and the vials are stored in sealed light-excluding bag at -20°C (a desiccation bag is helpful to keep moisture from inactivating the functional group of the dye, both come with the dye from the

manufacturer). These aliquots can be further divided to give 10 µg per aliquot, which is enough to label about 0.5 mg of antibody. One aliquot of 10 µg IC5-OSu was dissolved in 10 µl DMSO (1.6 nmol/µl) and 2 µl (3.3 nmol) was added to 100 µl of NT73 MAb solution (1 mg/ml; 0.66 nmol) to give a 5-fold ratio of IC5-OSu to antibody. While the reaction was incubated for 15 minutes at room temperature (24°C), a Pharmacia G-25 Sequencing column was equilibrated with the PBS buffer. Since carbonate buffered solutions are not stable, PBS buffer is preferred for later storage of the protein. This was done by washing the column twice with 500 µl of PBS buffer. The buffer was mixed with the resin in the spin columns and spun at 4000 rpm (do not exceed 2000 g !) for 30 seconds in the microfuge. The 100-µl labeling reaction was then loaded onto the resin and eluted by spinning at 4000 rpm for 30 seconds. This was done to separate the labeled MAbs from the unbound IC5 label which can be seen as a blue residue left on the columns after the last spin. Spin columns with a G-25 gel-filtration resin is used in place of dialysis, since it is more suitable for the small volumes down to 20 µl. We also observed very poor dialysis efficiency for the cyanine dyes.

### *6.3.4 Evaluation of labeling efficiency (optional)*

10 µl of the resulting 100 µl blue solution, eluted from the column was diluted 20-fold with PBS buffer and the amount of protein and dye is determined spectrophotometrically (molar absorptivity of IgG: 206,000 $M^{-1}$ $cm^{-1}$ at 280 nm, molar absorptivity of IC5: 90,000 $M^{-1}$ $cm^{-1}$ at 650 nm). From the measurements we calculated the molar ratio of the label with respect to the protein as well as amount of total label incorporated by the labeling reaction.

### *6.3.5 Use of IC5-labeled NT73 in a Western blot*

For SDS PAGE and subsequent wet blotting we used pre-casted 4-12% NuPAGE gels according to the manufacturers protocol. This involves a running time of 35 minutes for the SDS-PAGE at constant 200 V and a transfer for 1 hour at constant 200 A. The labeled antibody was used in the appropriate dilution (routinely 1:2000; up to 1:10,000 for testing) in 20 ml Blotto. The blotted membrane (blocked with 1% Blotto for a minimum of 15 min and up to 2 days) was incubated for 5 to 60 minutes with the labeled primary antibody at room temperature. The blot was rinsed three times with 25 ml PBS buffer and scanned with a Storm Imager (Molecular Dynamics) in the red fluorescence scanning mode. For better background, longer washes (5 min each) can be made.

### *6.3.6 Use of IC5-labeled anti-σE and anti-σFecI antibodies in quantitative Western blots*

We loaded known quantities of purified σE and σFecI on a pre-casted 4-12% NuPAGE gel. On the same gel we loaded a sample of crude extract from *E. coli* that was spiked with a known amount of the sigma factor, to determine whether we can verify its amount in our procedure. After wet blotting we labeled the antibodies 1RE53 (anti-σE) and 1FE16 (anti-σFecI) and used them at a 1 to 4000 dilution in 20 ml 1% Blotto. The blotted membrane was blocked with 1% Blotto for 15 min and incubated with labeled primary antibody for 60 minutes at room temperature. The blot was rinsed three times with 25 ml PBS buffer for 5 min each and scanned with a Storm Imager (Molecular Dynamics) in the red fluorescence mode.

## 6.4 Results

### *6.4.1 Labeling efficiency for IC5-labeling of antibodies*

We tested the labeled sample for labeling efficiency by measuring the absorbance of the labeled antibodies (according to manufacturer's information, the molar absorptivity of IC5 at 650 nm is approximately 90,000 $mol^{-1}cm^{-1}$). The measured concentration was compared with the protein concentration measured by absorbance molar absorptivity of an IgG antibody at 280 nm is approximately 206,000 $mol^{-1}cm^{-1}$. Labeling efficiencies were roughly 2.5 mole dye per mole protein using a 5-fold molar ratio of IC5 dye to protein for the reaction. We have compared labeling efficiencies for 5 to 15-fold ratios of dye to protein and the results are shown in Figure 6.1. We observed maximum labeling efficiencies with up to 7-fold ratio of dye to antibody. Above that, decreased solubility led to a loss of protein (data not shown). Judging from the emission of the labeled protein and the free dye applying densitometry to the SDS gel, the remaining content of free dye was less than 5%.

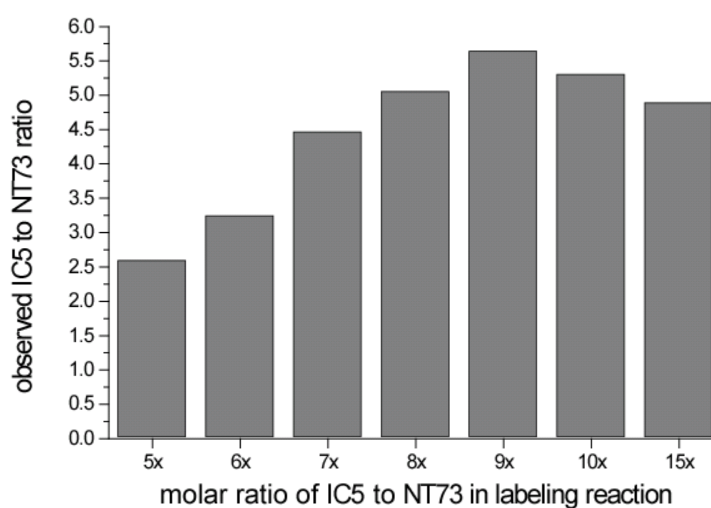


**Figure 6.1: Labeling efficiency of MAb NT73 with IC5-OSu. 100 µg of NT73 in 100 µl was labeled with different ratios of IC5-OSu and the final ratio of dye-to-protein was measured by absorption at 280 and 645 nm. Final dye-to-protein ratio increases to 5.5 up to a ratio of 9 of dye to NT73. From the total yield of protein, however, we saw a decrease of protein solubility starting at a a ratio of more than 7 (data not shown).**

### *6.4.2 Performance of labeled antibodies in Western blot*

We tested the limit of detection for IC5-labeled NT73 in a Western blot. A pre-casted 4-12% NuPAGE gel was loaded with a dilution series of 1 ng to 1 µg of purified RNA polymerase (0.4 to 400 ng of the β'-subunit). We probed the blot with 1-to-2000 diluted IC5-labeled NT73 for 60 min and scanned the blot as described. We were able to detect a minimum of 0.4 ng of the β'-subunit (1 ng of RNA polymerase loaded). We also tested the linearity of the signal with respect to the loaded amount of protein and the signal intensity. A linear correlation between the loaded amount of protein and the obtained signal intensity in the Western blot was obtained from 30 to 400 ng. The results are summarized in Figure 6.2.

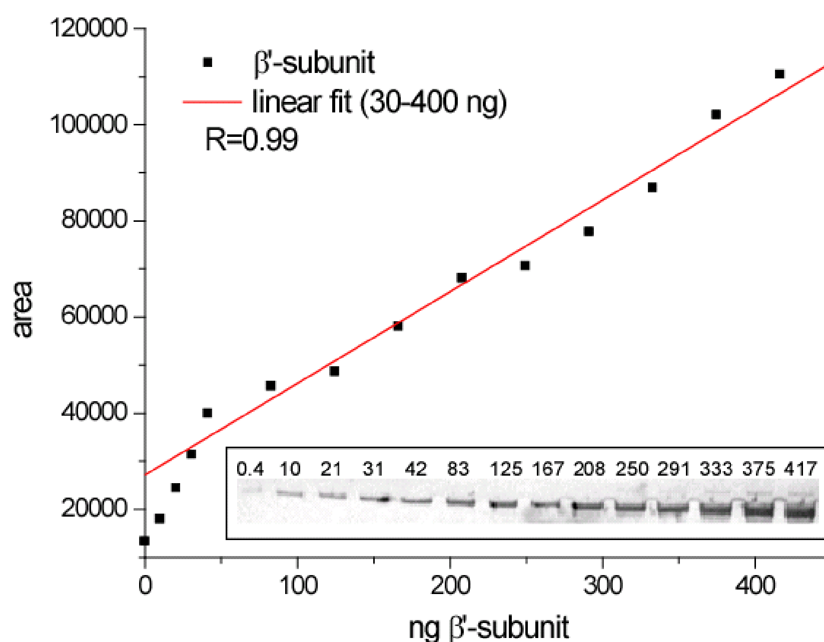


**Figure 6.2: Linearity and limit of detection of the β'-subunit of RNA polymerase in a Western blot with IC5-labeled NT73. The graphic shows the evaluation of a Western blot obtained after loading 1 ng to 1 µg of purified RNAP on a 4-12% NuPAGE gel, transfer onto a nitrocellulose membrane and incubation with IC5-labeled NT73 (2.5 final ratio of dye to protein, 1:2,000 dilution, 60 min incubation). The signal for the β'-subunit of RNA polymerase is visible down to 0.4 ng protein loaded on the SDS-PAGE gel (bottom right). Above each lane of the gel the amount of loaded β'-subunit is indicated in ng. However the signal increases linearly with respect to the amount of protein loaded from 30 ng up to the maximum of 400 ng loaded β'-subunit. A linear regression of the results in this interval gave a R-value of 0.99.**

The procedure took about three hours from the time the SDS-gel was started to the time the image of the Western blot was taken and over half of that time was involved in electrophoresis and the transfer of the protein from gel to nitrocellulose membrane.

### *6.4.3 Effect of incubation time and dilution of labeled antibody on quantification*

We repeated the experiment described above but this time we incubated the blot for 5, 15, 30 and 60 min with 1-to-2000 diluted IC5-labeled NT73 and scanned the blot as described. We again tested the linearity of the signal with respect to the loaded amount of protein as well as the signal intensity. The signals increase roughly 10-fold as the incubation time is increased from 5 to 60 min although linearity doesn't seem to be compromised for shorter incubation times (see Figure 6.3).

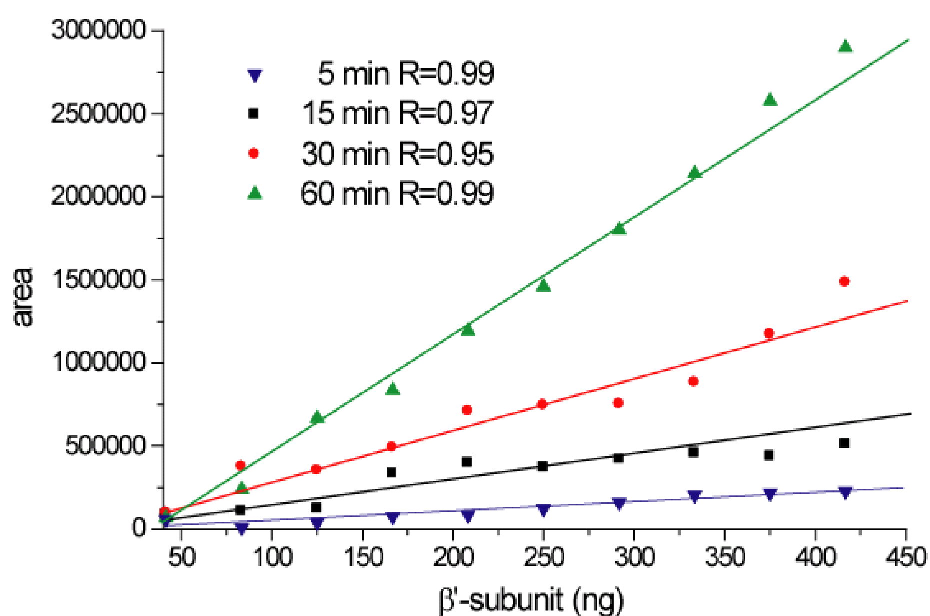


**Figure 6.3: Effect of incubation time on the signal from IC5 in Western blots. The diagram shows the effect of incubation times at 1 to 2,000 dilution of with a final ratio of IC5 to NT73 of 2.5 which were varied at 5, 15, 30 and 60 minutes. The gel was loaded with purified RNA polymerase containing 31 to 417 ng of β'-subunit. Regression of the results gave slightly different R-values for each different measurement. A significant increase in intensity can be observed with an increase of incubation time.**

We also tested different dilutions (from 4000 to 10,000-fold) of labeled antibody with an incubation time of 60 min. Again there was no change in linearity but a change in intensity was observed (see Figure 6.4). The limit of detection was only slightly raised (from 0.5 ng to 10 ng detectable β'-subunit) for a 10,000-fold dilution. 1 ng β'-subunit was still detectable with a 6000-fold dilution. It is important to note that 1 ng of the 156 kDa β'-subunit would be equivalent to 0.2 ng of a 30 kDa typically sized protein.

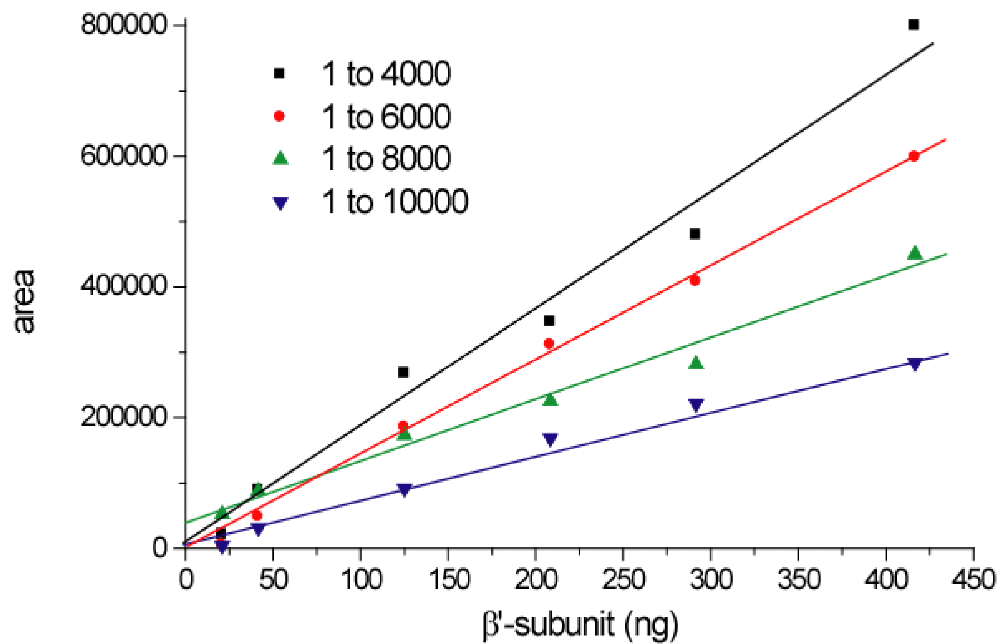


**Figure 6.4: Effect of dilution of IC5-labeled NT73 on the signal in Western blots. The diagram shows the effect of dilution factor at 60 minutes incubation with a final ratio of IC5 to NT73 of 2.5 which were varied at 1 to 4,000, 6,000, 8,000 and 10,000 dilution. The gel was loaded with purified RNA polymerase containing 31 to 400 ng of β'-subunit. Regression of the results gave a R-value of 0.99 for each dilution. A significant increase in intensity can be observed with an decrease of dilution of labeled antibody.**

### *6.4.4 Quantitative analysis of whole cell extracts*

We tested quantitative analysis of crude extracts with a known amount of sigma factor (σE and σFecI) added. We tested σE in the range of 10 to 100 ng and σFecI in the range of 100 to 1000 ng of loaded protein. In both cases we obtained signal response linear over the

tested range of loaded protein as well as accuracy for the measured standards within the range (error of < 10%). The linearity and accuracy of the measurements with a whole cell extract matrix confirmed that we can detect and quantify the Western blots within the linear range of the assay. These results are summarized in Figure 6.5 and Figure 6.6.

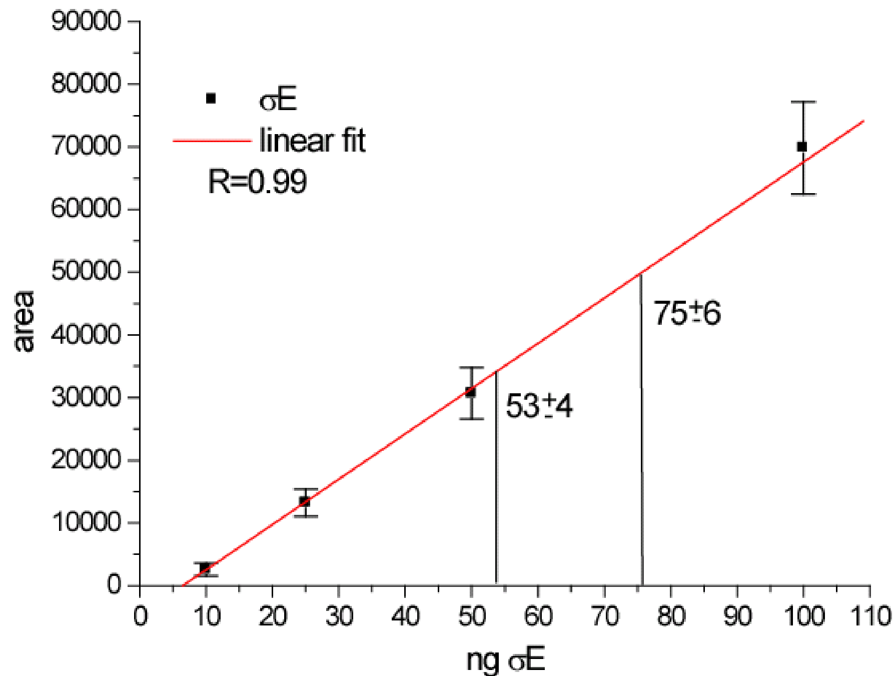


**Figure 6.5: Use of IC5-labeled anti-σE antibody in a quantitative Western blot. The diagram shows the evaluation of a Western blot from a 4-12% polyacrylamide NuPAGE gel loaded with increasing amounts of σE (10, 25, 50 and 100 ng) as standards using the σE specific antibody 1RE53 labeled with IC5-OSu (1:1000 dilution, 60 min incubation). As samples for quantification, 50 and 75 ng σE were added two times each to a crude *E. coli* cell extract and loaded on the gel. This was done to validate the quantification in the presence of a typical sample matrix. The amounts protein obtained by the calibration line were 53 +/- 4 ng for the 50 ng sample loaded and 75 +/- 6 ng for the 75 ng sample loaded (vertical line down from the area reading obtained from the imaging indicate the sample data point). Thus these results were accurate within a margin of error of less than 10%.**

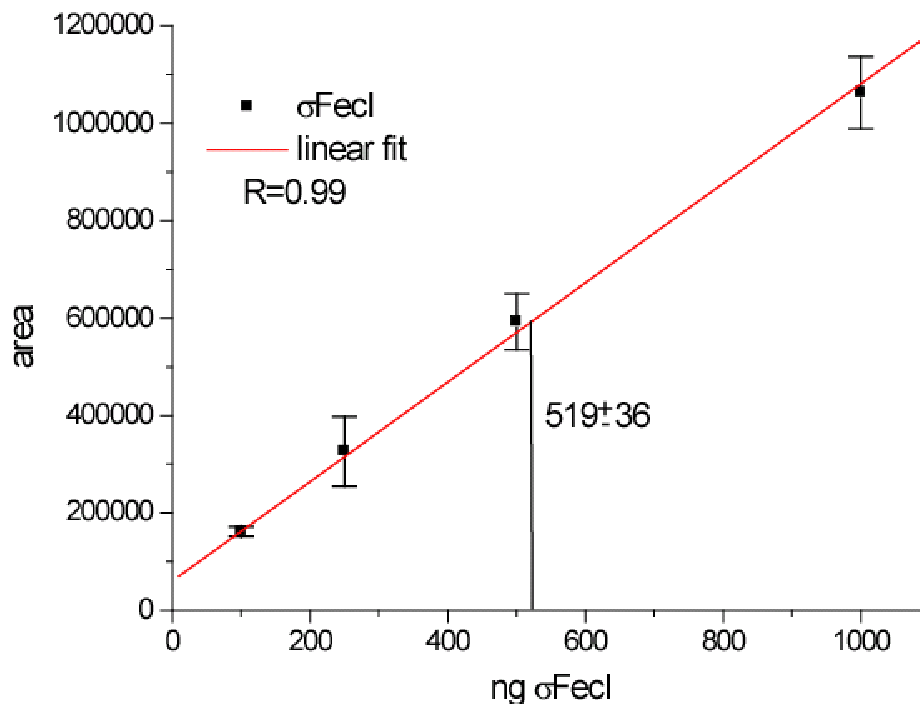


**Figure 6.6: Use of IC5-labeled anti-σFecI antibodies in a quantitative Western blot. The diagram shows the evaluation of a Western blot from a 4-12% polyacrylamide NuPAGE gel loaded with increasing amounts of σFecI (100, 250, 500 and 1000 ng) as standards using the σFecI specific antibody 1FE16 labeled with IC5-OSu (1:1000 dilution, 60 min incubation). As samples for quantification, 500 ng σE were added four times each to a crude *E. coli* cell extract and loaded on the gel. The amount of protein obtained by the calibration line was 519 +/- 36 ng for the 500 ng sample loaded (vertical line down from the area reading obtained from the imaging indicate the sample data point) and was also accurate within a margin of error of less than 10%.**

## 6.5 Discussion

The current methods for Western blots are well developed and highly sensitive non-radioactive kits are available, that create signals with very high intensity that are easy to detect and evaluate. However it is still a challenge to get reliable quantitative data with these methods. The source for errors and loss of reproducibility can be found in many aspects of the classical procedure. The fact that a ternary complex of the blotted protein with primary and secondary antibody is necessary for detection is one aspect. Therein lies the intrinsic

problem, that the antibody has to recognize its antigen which was separated in a SDS PAGE under denaturing conditions and was bound to a membrane surface. Both processes can influence significantly the shape of the recognized epitope and therefore the efficiency of recognition by the antibody. Probably the greatest problem for quantification of Western blots is the amplification step achieved by a color or luminescence producing reaction. This is catalyzed by an enzyme which is linked to a secondary antibody that recognizes the primary antibody. The rate of this reaction is heavily dependent on the local concentration of the enzyme and the reactants, which can change during development and thus may cause a non-linear response to varying amounts of antigen bound to the membrane.

In order to circumvent the need for a secondary antibody and an amplification reaction, we decided to avail ourselves of very common fluorescence labeling procedures and detection systems in combination with the known Western blotting techniques. In contrast to common perception, fluorescence labeling of an antibody is a very simple and rapid procedure. Necessary reagents and methods are readily available and can be easily performed by anyone in a straight-forward procedure. Apart from the fact that the necessary labeling procedure can be performed within 15 minutes while the proteins are blotted to the membrane, it is also very cheap to label enough protein for 10 Western blots without the need for a conjugated secondary antibody and its substrates. The amount of 1 mg of dye can be used to label enough antibody for 500 to 5000 western blots depending on the dilution and volumes used for the incubation. Storage of labeled antibody was possible (after 1:1 dilution with 100% glycerol) over several months at -20°C without any observed loss of performance. However the use of different dyes, the amount of label per antibody or different antibodies may result in reduced solubility. We studied the labeling efficiency of NT73 with IC5-OSu in carbonate buffer at pH 9 and found similar results to those described in commercially available kits (CyDye, Ammersham). A 7-fold molar ratio of dye to antibody gave optimal

amounts of up to a final dye to antibody ratio of 5.5 without loss of protein due to decreased solubility. Incubation times with primary antibody were varied from 5 to 60 minute and although signal were linear over a similar range of antigen at lower incubation times, we observed a 10-fold increased signals for 60 minutes compared to 5 minutes incubation times. The degree of dilution had a similar effect of labeled antibody in the incubation step. Lower dilution led to increased signal without a significant effect on linearity. However optimization of the dilution factor may result in better specificity of the antibody and reduced the observed background.

The fact that one can omit a wash step, an incubation step with a secondary antibody and the reaction for the development of the signal contributes to a significantly shortened procedure time as compared to most common procedures. Remarkably the procedure takes less than two hours from the point of time the gel was run, which takes only 35 minutes in case of the gels used, until the image of the Western blot is taken. In contrast to the most common procedures which allow the recording of the image by film exposure or simple photography, this procedure requires a fluorescence scanner like the one used in this study. It also has to be equipped with the right excitation source and acquisition filter. However this method of detection is superior in sensitivity and linearity over photography (5) and the equipment should be accessible in most biological institutes since it is used as a fluoroimager. It is also superior in workable range over film exposure, since film can be saturated easier than the detector of a fluorescence scanner.

An important aspect in this study was the use of this procedure in quantitative analysis of Western blots. We tried to get a more linear response in the measured signal by a fluorescence labeled primary antibody, thus avoiding the use of a secondary antibody and the amplification reaction by its conjugated enzyme. We were able to show that the described procedure can be used for quantitative experiments with a large workable linear range (up to

3 orders of magnitude) within a margin of error of less than 10%. However, we observed that it is necessary to run the standard samples used for calibration on the same gel and blot in order to obtain reliable results, probably since transfer efficiency, sample conditions and backgrounds can vary significantly from one preparation to another. Obviously this limits the number of samples that can be analyzed in one blot. It is also important to note that different antibodies vary in their binding efficiency, so it is necessary to check each antibody for its performance with purified antigen in the assay first. Still the simplification of the procedure to just one incubation step with only one antibody with one less washing step should increase the performance of antibodies with weaker affinities. Since we tested the described procedure with three different antibodies, we are confident that this method is not limited to just a few antibodies. Overall we believe that due to its increased simplicity, increased performance, reduction of procedure time to less than three hours for quantitative procedures and lower than 2 hours for qualitative measurements including the SDS PAGE, and less material expenses, this work describes a valid improvement of the common procedure.

## 6.6 Acknowledgments

We would like to thank Larry Anthony for providing with purified sigma factors. We thank Kit Foley and Dr. Nancy Thompson for their support and for helpful advice throughout this work. This work was supported by NIH Grant GM28575 to RRB. Preliminary experiments were done in the Cold Spring Harbor course "Protein Purification and Characterization" in April 2002. We appreciate the involvement of the course participants.

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# Chapter 7

# Conclusion and Future Directions

## 7.1 Initial proposal

The primary goal of this thesis was to develop an assay for sigma-core binding of bacterial RNA polymerase based on LRET and demonstrate its use for biochemical characterization and drug discovery. The investigations started on the basis of the results published by T. Arthur (1-3) from this lab who characterized the key binding region between $\sigma^{70}$ and core RNA polymerase from *E. coli*. The project was inspired by the work from T. Heyduk (4) who established the use of LRET to study protein-DNA interactions in the transcription machinery of *E. coli*. The prospect of a homogeneous binding assay for sigma and core that could be performed in microwell plates raised the possibility of its application in both basic biochemical research and drug discovery. The latter was supported by *in vivo* studies that showed an inhibitory effect of the coiled-coil from the β'-fragment that binds to $\sigma^{70}$ on cell growth of *E. coli* (1).

## 7.2 Chapter 2

In order to convert the available LRET-procedures into the assay presented in this work the major challenges encountered were connected to fluorescence labeling techniques. Since LRET can occur at fluorophore concentrations above 200 nM by diffusion it is crucial for the performance of the assay to purify the labeled proteins from free dye. A new on-column fluorescence labeling procedure described in Chapter 2 using Ni-NTA and anion exchange resins (5) was a key breakthrough in the development of the LRET assay.

**7.3 Chapter 3**

The reduced solubility of some labeled proteins tested in the beginning led to the use of the more stable β'-fragment(100-309). Further improvements with respect to intrinsic requirements for assays used in high-throughput screening were developed and are subject of Chapter 3. For example storage conditions, that can sustain the stability of the labeled proteins up to 24 hours, depending on the size of the screened compound library. Therefore storage under denaturing conditions and refolding through dilution directly into the assay were established for the assay. Finally a very small library of 100 natural product extracts were used to test the assay under conditions encountered in screening. The undefined nature of the library challenged the performance of the LRET assay through a high intrinsic background. However an active sample was identified in the screen and confirmed for its inhibition of enzyme activity by *in vitro* transcription. The purification of the active component has recently started in collaboration with Prof. Tanaka, who provided the library. This hit encourages future efforts for an extensive screening for an inhibitor of RNA polymerase and possibly a new class of antibiotics. The use of this assay in an automated high-throughput screen of two libraries containing 16,000 and 65,000 purified compounds has not been performed because the completion of a screening facility here at the University of Wisconsin was significantly delayed.

**7.4 Chapter 4**

This part of the thesis extends the applications of the LRET assay by the use of randomly labeled full-length core RNA polymerase and other sigma factors and a new pair of dyes (IC3 and cs124-DTPA-Phe *Tb). In this study we gained preliminary results for the determination of binding constants for the interaction of sigma factors and core RNA

polymerase. The obtained values for $K_{diss}$ of $\sigma^{70}$ and $\sigma^{32}$ were in good agreement with the best of the previously published data and can serve as a good basis for more comprehensive binding studies of all sigma factors and other binding partners of RNA polymerase from *E. coli*.

### 7.5 Chapter 5

In addition to the binding studies focused on in this thesis, the expertise of this lab in making MAbs and using polyol responsive MAbs for rapid and gentle immunoaffinity purification of proteins was taken as an opportunity to learn these techniques and apply them in a broader context of this thesis. The isolation of the MAb 8RB13 and its use to purify core RNA polymerase from different species is presented in Chapter 5. The antibody will prove to be very useful for the purification of RNA polymerase from other prokaryotes, especially from problematic pathogens, to test the activity of inhibitors identified by the LRET assay.

### 7.6 Chapter 6

The experience with fluorescence labeling techniques that was gained through the LRET studies led to the development of an improved quantitative Western blot procedure described in Chapter 6.

### 7.7 Appendix

The methods developed for the studies described in Chapter 2 and 3 were summarized for an invited publication in Methods in Enzymology (6) and are attached to this thesis as an Appendix.

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6. Bergendahl, V., and Burgess, R. R. (publication expected in 2003) Studying Sigma-Core Interactions in *Escherichia coli* RNA Polymerase by Electrophoretic Mobility Shift Assays and LRET. *Methods Enzymol*

# Appendix

## Studying Sigma-Core Interactions in *Escherichia coli* RNA Polymerase by Electrophoretic Mobility Shift Assays and LRET

The manuscript presented in the Appendix has been solicited for publication as:

Bergendahl, V., and Burgess, R. R. (publication expected in 2003), Studying Sigma-Core Interactions in *Escherichia coli* RNA Polymerase by Electrophoretic Mobility Shift Assays and LRET. *Methods Enzymol*

My contributions are that I performed all the experiments and wrote the manuscript.

R. R. Burgess is the principal investigator.

**A.1 Introduction to protein binding studies**

There is great interest in identifying and characterizing protein-protein interactions. Many biological processes such as cell signaling, metabolism, enzymatic activity and specificity are controlled by modulation of protein structure and function upon binding to another protein. Transcriptional regulation, for example, is mediated significantly by protein-protein interaction because binding of transcription factors to RNA polymerase confers its specificity for promoters of transcribed genes. We would like to use the transcription machinery from *E. coli* in particular, to illustrate two very useful strategies to investigate protein binding. Our work focuses on the interaction of the main transcription factor σ70 with core RNA polymerase (RNAP). Their major interaction interface is part of region 2.1 to 2.2 (residues 360-421) in σ70 and a coiled-coil at residues 260-309 in the β'-subunit of RNAP (1-3).

There are many approaches to studying protein-protein interactions. Here we have chosen to describe electrophoretic mobility shift (EMS) assays and fluorescence resonance energy transfer (FRET) for several reasons. EMS assays are fairly simple and quick to perform with the equipment present in most biologically oriented labs. At the same time they can give useful initial information about a protein binding to another protein, DNA or RNA. EMS assays are based on the change of mobility of a protein during polyacrylamide gel electrophoresis (PAGE) upon binding to DNA, RNA or another protein. Since binding of a protein to its binding partner predominantly requires that its higher order structure is intact, non-denaturing or native gels are used. Crucial to EMS assays is the fact that the procedure involves separating the complex from the unbound binding partner by size and charge differences. This changes the equilibrium conditions at which initial binding occurs and thus weak interactions, i.e. in complexes that have a half life shorter than the time scale of the

separation step, are underrepresented or cannot be detected since the interaction doesn't persist throughout the procedure. This is true for all non-homogenous techniques like surface plasmon resonance (SPR, used in BIAcore instruments), co-immunoprecipitation or "pull-down" assays, ELISA assays, Far-Western blotting, size exclusion and affinity chromatography, as well as sucrose gradient centrifugation.

In a homogeneous binding assay, i.e. all components of the assay are free in solution and no separation steps are used. A key benefit of such an assay is that the binding measurement is done without perturbing the binding equilibrium. This often allows more accurate assessment of the binding forces and mechanisms, thus resulting in most cases in the superior although more technically more demanding way of investigating binding. The most common techniques involve spectroscopic methods because the use of light as a probe doesn't change the conditions in most systems. These methods use NMR, light scattering, and very often fluorescent probes like in fluorescence polarization (FP) and FRET assays. Recent developments of FRET also allow analysis of protein-protein interactions *in vivo* (4). Together with two-hybrid screens and functional assays these are currently the only established methods to obtain reliable *in vivo* data.

In a FRET-based assay, the binding partners have to be labeled with two different fluorophores. In order to allow resonance energy transfer between the two, the emission spectrum of the fluorophore serving as a donor has to overlap sufficiently with the excitation spectrum of the other fluorophore serving as the acceptor. Resonance (i.e. acceptor frequency equals donor frequency) facilitates a radiationsless energy transfer through dipole-dipole interactions. As described by the Förster Theory (5), the amount of transferred energy decays with the inverse sixth power of the distance between the dyes. The intensities of the donor or the acceptor emission can therefore give information about the distance of the two dyes and ultimately tell whether the labeled proteins are bound to each other.

We decided to focus on LRET (Luminescence Resonance Energy Transfer)-based assays for a homogenous assay to measure the formation of the σ70-β'-complex. LRET is a recent modification of FRET (6-9) that uses a lanthanide-based donor fluorophore. The more general term luminescence instead of fluorescence (as in FRET) indicates that lanthanide emission is technically not fluorescence (i.e., arising from a singlet to singlet transition). Its most characteristic property is a very long fluorescent lifetime of up to several milliseconds, compared to the short lifetime of most organic-based fluorophores in FRET (picoseconds up to a microsecond). The conventional dipole-dipole theory of Förster is still applicable for LRET, so that energy transfer from a lanthanide donor to an generally short lived (nanoseconds) acceptor fluorophore results in its prolonged emission (milliseconds).

We labeled σ70 with a Europium-DTPA-AMCA complex as a donor and the β'-fragment with IC5-PE-maleimide. Since fluorescence detection is usually very sensitive, the labeled proteins can also be used very efficiently for their detection in gels resulting from EMS assays, avoiding the need for other labeling methods like $^{32}P$-labeling.

### A.2 Introduction to fluorescence labeling of proteins

For uniform labeling of proteins with only one fluorophore we exploited the specific reaction of maleimide-linked dyes with cysteine residues in proteins with only one cysteine residue. The maleimide moiety of the dye reacts readily in sulfhydryl-free buffers and is highly specific to cysteine residues at pH-values between 6.5 and 8. If uniform labeling is not critical, succinimide derivatives of the dyes can be used to randomly label lysine residues in amine-free buffers at pH 7.5 to 9.0. In our studies we have addressed a few challenges in fluorescence labeling of proteins. For example oligomerization and oxidation of the sample can lead to a significant loss of labeling, so that a reduction step before derivatization can increase yields. Unfortunately reducing agents such as 2-mercaptoethanol (2-ME) and Tris(2-carboxyethyl)phosphine (TCEP) have to be removed before the labeling reaction, since they inhibit maleimide labeling (10). Another challenge when labeling proteins is the separation of labeled product from the excess dye. This is especially important for samples used in FRET and LRET assays, since too much free dye can cause additional signal by diffusion-controlled resonance energy transfer when working at free dye concentrations above 200 nM. For cyanine dyes like IC5 it is very difficult to remove excess dye by dialysis and it usually requires an extra gel filtration step.

Here we describe a procedure for purification and labeling where reduction, labeling and washing away excess dye is carried out, while the protein is bound to the column. Additional information about the labeling procedures presented here can be found in Bergendahl et al. (11). In case of the hexahistidine-tagged beta prime (RpoC) fragment (residues 100-309), that has a single naturally-occurring cysteine at residue 198, we worked on a Ni-NTA column. We purified and labeled a single cysteine σ70 mutant (C132S, C291S, C295S, S442C)(12) on a DE-52 anion exchange column. To circumvent reducing the Ni-

resin by 2-ME we used TCEP instead. After reduction, TCEP was washed away and the on-column derivatization was carried out immediately to avoid oxidation. Excess dye could be washed from the column while the labeled product remained bound to the resin.

Below we present the following protocols:

1. Ni-NTA Purification and Labeling of HMK-$His_6$-β'(100-309)

2. DE52 Purification and DTPA-AMCA-Europium Derivatization of σ70(S442C)

3. Electrophoretic Mobility Shift (EMS) Assay for Complex Formation of Labeled σ70 and β'-Fragment

4. LRET Assay for Protein-Protein Interaction of Labeled σ70 and β'-Fragment

All chromatographic procedures were carried out by gravity flow at room temperature. The assays described in 3. and 4. can be performed with native labeled proteins or with denatured proteins directly added into the reaction mixture, which by dilution of GuHCl in the final assay allowed instant refolding. This can be done to prevent precipitation of the refolded labeled protein upon storage before the assay. In order to assure that refolding occurs and to confirm the results from the experiments with denatured protein being added, controls with native or refolded proteins should be carried out.

**A.3 Materials and chemicals**

IC5-PE-maleimide was purchased from Dojindo Molecular Technologies, Inc. (Gaithersburg, MD). DTPA-AMCA-maleimide was produced as described by T. Heyduk. (12) (CAS numbers of the used starting materials: DTPA-anhydride: 23911-26-4, Maleimido-

propionic acid, NHS ester: 55750-62-4, AMCA: 106562-32-7). These so-called Selvin-chelates for Europium are being made commercially available by PanVera (Madison, WI). Other Europium-labels are sold by Packard. Any of these labels should work in these protocols as long as they contain the maleimide group for derivatization. Disposable columns (10 ml PolyPrep, 0.8x4 cm) were purchased from BioRad. Ni-NTA resin was purchased from Qiagen (Hilden, Germany). TCEP and TritonX-100 were purchased from Pierce. $EuCl_3$ and all other chemicals were purchased from Sigma unless otherwise indicated in the text. Pre-cast NuPAGE PAGE-gels were purchased from Invitrogen. A multi-plate reader (Wallac, Victor$V^2$ 1420) was used to perform the LRET assays.

### A.4 Buffers and solutions

Native PAGE buffer: 200 mM Tris-HCl pH 7.5, 20% glycerol, 0.005% bromphenol blue; NTG buffer: 50 mM NaCl, 50 mM Tris, 5% glycerol; pH 7.9; TGE buffer: 50 mM Tris-HCl, pH 7.5, 5 % glycerol, 0.1 mM EDTA; NTTw buffer: 500 mM NaCl, 50 mM Tris-HCl pH 7.9, 0.1 % (v/v) Tween20; NTGED buffer: 50 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 % glycerol, 0.1 mM EDTA, 0.1 mM DTT; storage buffer: 0.2 M NaCl, 50 mM Tris-HCl pH 7.5, 50% glycerol, 0.1 mM EDTA, 0.1 mM DTT

## A.5 Ni-NTA purification and labeling of HMK-His$_6$-β'(100-309)

### *A.5.1 Overproduction of HMK-His$_6$-β'(100-309) and Purification of Inclusion Bodies.*

HMK-His$_6$-β'(100-309) was overproduced in *E. coli* BL21(DE3) from pTA133 (10) and is a chimera (25 kDa) with the amino acid sequence MARRASVHHHHHHM N-terminally fused to β'(100-309). The underlined sequence represents a heart muscle kinase (HMK) recognition site and was used in the original construct to allow $^{32}$P-labeling of the protein, but we did not make use of it in this paper. The cells were grown in Luria Broth (LB) at 37°C, induced for 2 hours with 0.5 mM IPTG, harvested and lysed. Inclusion bodies were isolated and washed in 10 ml NTGED-buffer + 0.1% (v/v) TritonX-100. Washed inclusion bodies were stored in aliquots at -20 °C until use.

### *A.5.2 Procedure*

1. One aliquot of about 20 mg (wet weight, from 100 ml of culture) of β' inclusion bodies is solubilized in 1.5 ml NTTw-buffer + 6 M GuHCl + 5 mM imidazole.
2. The precipitate from the solubilized inclusion bodies is spun down in a microfuge at 18,000 g (14,000 rpm) for 5 min.
3. The supernatant is loaded on a BioRad column (10 ml PolyPrep, 0.8x4 cm) containing 0.5 ml Ni-NTA matrix (Qiagen) that has been equilibrated with 5 ml NTTw-buffer + 6 M GuHCl + 5 mM imidazole. That way the column is saturated with approximately 5 mg of the target protein.
4. The column is washed with 5 ml NTTw-buffer + 6 M GuHCl + 20 mM imidazole to remove unbound protein.

5. To reduce any disulfide bonds, the column is washed with 5 ml of freshly prepared NTTw-buffer + 6 M GuHCl + 20 mM imidazole and 2 mM TCEP.

6. Excess TCEP is removed by washing with 3 ml NTTw-buffer + 6 M GuHCl + 20 mM imidazole.

7. The bound protein is derivatized with IC5-maleimide by loading 2 ml freshly prepared NTTw-buffer + 6 M GuHCl + 20 mM imidazole and 0.1 mM IC5-PE-maleimide.

8. The flow-through is reloaded onto the column twice before excess dye is removed by washing with 3 ml NTTw-buffer + 6 M GuHCl + 20 mM imidazole.

9. The derivatized protein is refolded while column-bound by washing with 3 ml NTTw-buffer + 20 mM imidazole to wash away the denaturant GuHCl.

10. Derivatized protein is eluted with NTTw-buffer + 200 mM imidazole, dialyzed into storage buffer and stored at -20°C at concentrations of around 0.5 mg/ml.

### *A.5.3 Comments*

The procedure can be done under denaturing conditions throughout, eluting the labeled protein denatured. Another option is to load the protein onto the Ni-NTA-resin after refolding the β'-fragment into a 65-fold excess of TGE-buffer. A third variation is to refold the β'-fragment on the column just before labeling with the dye. TCEP is used as a reducing agent because it doesn't interfere with the Ni-NTA resin like 2-ME. TCEP is an odorless white powder that can be added as a solid just before use. It dissolves readily in aqueous solutions and is very stable unless chelators like EDTA are present. IC5-PE-maleimide is dissolved in chloroform and divided into aliquots of 0.1 micromole. The aliquots were then dried down in a SpeedVac (SpeedVac SVC100H, Savant Instruments, Farmingdale, NY).

The use of water is not recommended as it may cause hydrolysis of the maleimide moiety over time, especially upon storage.

### A.6 DE52 purification and DTPA-AMCA-europium derivatization of σ70(S442C)

Purification and derivatization of σ70 is done after refolding and in a similar fashion as the β'-procedure, this time using Whatman DE52 resin instead of the Ni-NTA agarose and using TGE-buffer instead of NTTw-buffer:

#### *A.6.1 Overproduction of σ70 (S442C) and purification of inclusion bodies*

Sigma70(442C) is overproduced from a plasmid derived from the σ70-expression system pGEMD (13, 14) that had a HindIII fragment containing the *rpoD* gene from *E. coli* cloned into a pGEMX-1 (Promega) vector. The cells were grown, induced, harvested and lysed. Inclusion bodies were isolated and washed in 10 ml NTGED-buffer + 0.1% (v/v) TritonX-100. Washed inclusion bodies were frozen in aliquots at -20 °C until use.

#### *A.6.2 Procedure*

1. One aliquot of about 20 mg (wet weight, from 100 ml of culture) of σ70 inclusion bodies are solubilized in 5 ml TGE-buffer + 6 M GuHCl.
2. To refold proteins, the denaturant is diluted 100-fold by dripping into 500 ml of chilled TGE-buffer + 0.01% TritonX-100 slowly stirring on ice. If precipitation occurred, the precipitate is removed by centrifugation at 25,000 g (15,000 rpm, SS-34 rotor) for 15 min at 4°C.

3. The soluble refolded protein is then bound to an anion exchange resin by adding 1 g DE52 (Whatman) dry resin as a suspension in 5 ml TGE-buffer + 0.01% TritonX-100 directly into the 500 ml of diluted, renatured protein.

4. After stirring slowly for 15 min, the suspension is poured into an empty 25 ml Econo-Pack column (BioRad) and washed with 5 ml NTG-buffer + 0.01% TritonX-100.

5. 5 ml NTG-buffer + 2 mM TCEP + 0.01% TritonX-100 is loaded onto the column to reduce any disulfides formed by dimerization of sigma.

6. The column is washed with 5 ml NTG-buffer + 0.01% TritonX-100 to remove TCEP.

7. To label σ70, 1 ml NTG-buffer + 0.01% TritonX-100 + 1 mM DTPA-AMCA is loaded onto the resin and flow-through is reloaded onto the column 2 times to ensure maximum labeling.

8. The Europium-complex is formed by loading 5 ml NTG-buffer + 0.01% TritonX-100 + 1 mM $EuCl_3$ onto the resin containing the derivatized protein. (Phosphate buffers should be avoided when working with lanthanides since insoluble complexes can lead to undesired precipitation.)

9. The column is washed with 5 ml NTG-buffer + 0.01% TritonX-100.

10. Labeled protein is eluted with TGE-buffer + 500 mM NaCl. The eluted fractions are analyzed by SDS-PAGE and pooled according to their purity and protein content.

11. The fractions of labeled σ70 are pooled and brought up to 50% glycerol or dialyzed against storage buffer (final protein concentration is about 2 mg/ml). The labeled protein is stored at -20°C until use.

### *A.6.3 Comments*

Labeling can also be done after purification by adding the purified protein to an aliquot of the dye (same buffers and conditions). That way samples of 10-100 µl can be labeled. Excess label and Eu-ions are then removed either by dialysis or by using a Pharmacia G50 spin-column. A SDS-gel of a typical purification is shown in Figure A7.1.

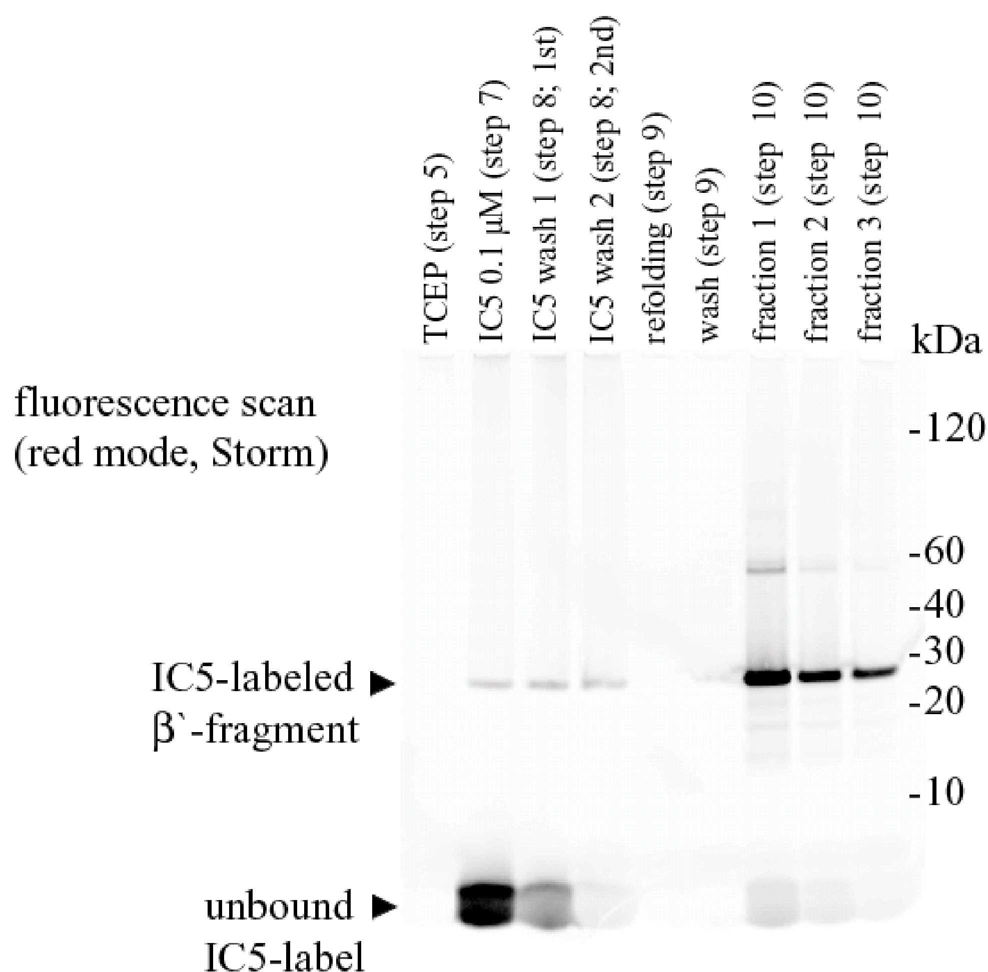


**Figure A7.1: SDS-PAGE gel of the β'-purification and derivatization steps. The fluorescence scan of a gel with samples taken after each step throughout the procedure illustrates the purification of the β'-fragment (refolding after fluorescence labeling). The labeling above the lanes represent the step numbers in the procedure at which the samples were taken. Purification from minor impurities can be observed as well as very low elution of the β'-fragment at any time except for the fractions 1 to 3 eluted with 200 mM imidazole. The IC5-scan was performed with a Molecular Dynamics Storm system in the red fluorescence**

**mode and thus shows the IC5-labeled proteins. The gel was scanned before staining with Coomassie blue. Note the low level of free label in the eluted fractions.**

## A.7 Electrophoretic Mobility Shift (EMS) assay with labeled σ70 and β'-fragment

For the performance of an EMS assay it is important to use the appropriate buffer conditions. Salt concentration has to be chosen wisely, since some protein-protein interactions are sensitive to high salt. The pH of the buffer has to be chosen according to the pI of the proteins. Since the sample buffer and the native gel contain no SDS, which confers the proteins with the negative charges, the proteins themselves have to have a negative charge in order to migrate into the gel during electrophoresis. Most sequence analysis software allows an estimate of the pI of a protein and even the net charge at a given pH. The EMS assays were performed in a total volume of 20 µl. The buffer conditions are 5% glycerol, 50 mM Tris-HCl, pH 8.8, 50 mM NaCl, bromphenol blue 0.005% (w/v). Standard final protein concentrations were 250 nM of σ70 (Eu-chelate labeled protein) and 0.1 to 2 µM β'-fragment (IC5-labeled protein). Concentrations can be lowered to 10 nM. While the detection limit of the Coomassie blue stain is reached at these concentrations, the fluorescence scan still gives good readouts.

### *A.7.1 Procedure*

1. Labeled σ70 is added to the appropriate amount of water and 5 µL of Native PAGE buffer (to result in a final volume of 20 µL after all components are added).

2. For competition experiments unlabeled β'-fragment is added.

3. Labeled β'-fragment is added last.

4. The mixture is incubated for 5 min at room temperature.

5. 15 μl is loaded on a pre-cast native PAGE-gel (12-well, 12% polyacrylamide, Tris/glycine).

6. The gel is run at constant 120 V in the cold room at 4°C for 4 hours.

### *A.7.2 Comments*

The concentration of the added components should be adjusted by dilution, so that the added volume does not exceed 2 μl. That way mixing and final concentrations are not varied much by the addition of each component. The solutions have to be mixed well after addition of each component by pipetting. This is especially important to allow good refolding when denatured proteins are added. The electrophoresis should be run with pre-chilled buffers, gels and apparatus in the cold room (4°C) at constant voltage of 120 V (5-20 mA, variable). In some cases running the gel at 4°C might not be crucial but in our case it increased observable complex significantly. Running times may vary according to the charge and therefore the mobility of the proteins. In our case running at 2-4 hours gave best results in terms of separation and sensitivity. The IC5-emission was scanned on a Storm system (Molecular Dynamics) in the red fluorescence mode. The Europium emission can be scanned by using an Fotodyne UV light box ($\lambda_{excit.}$= 312 nm) with 6 seconds acquisition time and the common filter for scanning ethidium bromide-stained gels. Total protein was stained with Coomassie blue stain using the Gel Code staining solution (Pierce) according to the procedure of the manufacturer and scanned with a Hewlett Packard flat bed scanner (ScanJet 6200C). For a typical gel from a EMS assay see Figure A7.2. We have also successfully used EMS assays with core RNA polymerase to investigate sigma binding. Since in the native gel multiple bands arise from core due to oligomerization, it is recommended to use only the σ70 in the labeled form and core RNA polymerase unlabeled.

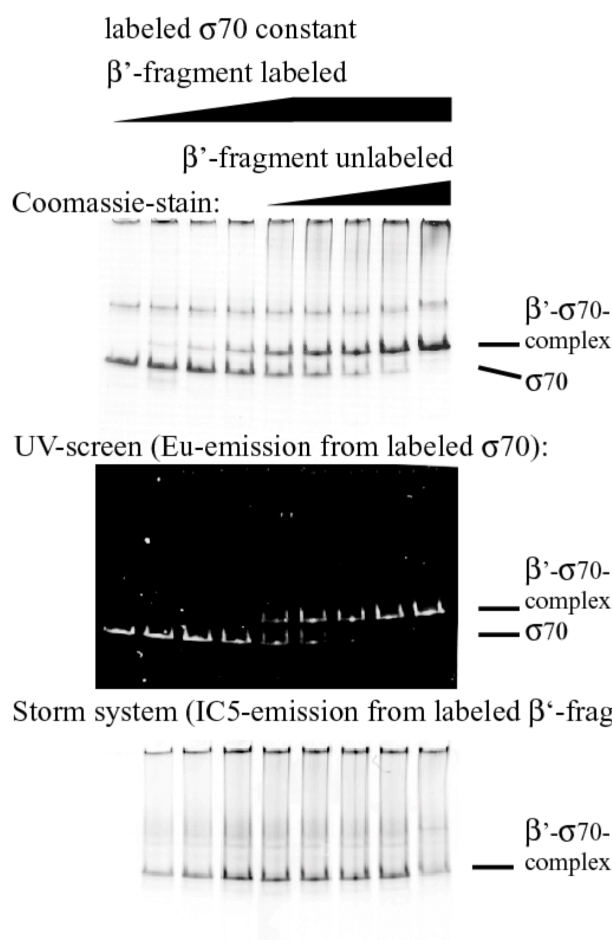


**Figure A7.2: The result of an EMS assay. The three pictures of the same gel taken by different staining and scanning techniques show increasing amounts of labeled β'-fragment (0, 50, 100, 250 nM) that can shift the labeled σ70 (250 nM) into the upper band. In the five lanes to the right unlabeled β'-fragment (100, 250, 500, 750, 1000 nM) was added to compete with the IC5-labeled β'-fragment for Eu-labeled σ70 which were kept at a constant concentration in a ratio of 1:1 (250 nM). The upper frame shows the Coomassie-stained gel. Labeled σ70 shows up in the lower band and the complex in the top band. The extra band above the complex arises from σ70 dimers linked by a disulfide bond which prevented their fluorescence labeling. The middle frame represents the same gel, but was acquired before Coomassie staining with a UV-box and an orange filter on the camera that can only visualize the Eu-emission due to the excitation wavelength (312 nm). It confirms that both bands contain Eu-labeled σ70. The bottom frame of the same gel, was taken with a Storm imager (Molecular Dynamics) that can only visualize the IC5-label. It confirms that only the upper band contains labeled β'-fragment. The free β'-fragment runs as a diffuse band barely migrating into the gel. The unlabeled β'-fragment can clearly compete for the labeled σ.**

**A.8 LRET assay for protein-protein interaction of labeled σ70 and β'-fragment**

The format of the assay presented here was first designed to be suitable for high throughput screening for inhibitors of the protein-protein interaction. Thus it is robust, quick to perform and can give useful initial data about binding, although the use of a screening device like the multiwell plate reader does not fully exploit the potential of LRET. Much higher accuracy and sensitivity can be achieved by the use of a suitably equipped fluorimeter like the one described in Heyduk et. al. (15). Furthermore such a setup allows accurate measurement of the distance between the dyes, which in case of allosteric binding can give valuable information about mechanistic details. Nevertheless the cost, operation and the access to such instruments is much more demanding compared to plate readers.

The assay was performed in a total volume of 200 µl in NTG-buffer (plus 2.5% DMSO when library samples were used) with typical concentrations of 10 to 100 nM labeled protein. It is very important to keep salt concentrations constant, since many protein-protein interactions are salt dependent. Therefore amounts of salt added with the proteins and samples should be corrected for when adding NaCl to give the final concentration of 50 mM. Stock solutions (200 nM) of σ70 was prepared prior to the assay by dilution with NTG of labeled protein (40 µM) that was stored at -20°C in storage buffer. Since the labeled β'-fragment shows a tendency to aggregate upon storage, we preferred to store it denatured in 6 M GuHCl and refolded it by dilution into the assay. A stock solution (1.25 µM) of the β'-fragment was prepared by 10-fold dilution of labeled denatured protein (stored at 75 µM in NTTw-buffer containing 6 M GuHCl) with 6 M GuHCl and 6-fold dilution to 1 M GuHCl with NTG. First, 10 µl of the σ70-stock solution was mixed with NTG buffer (amount adjusted to give a final volume of 200 µl), then the potential inhibitor (5 µl in DMSO), salt or solvent was added and finally the 5 µl of denatured labeled β'-stock solution was added.

Mixing (pipetting up and down three times) after addition of each component was very important for reproducible results. The mixture was incubated for 30 min at room temperature and measured in a 96-well plate (Costar 3650) with a multi-plate reader (Wallac, VictorV$^2$ 1420). For this time-resolved fluorescence measurement, the manufacturer's protocol (LANCE high count 615/665) was used (excitation was with 1000 flashes at 325 nm; measurement was delayed by 50 μs and data acquired for 100 μs at 615 and 665 nm). The time for one measurement cycle was set for 1000 μs.

### *A.8.1 Procedure*

1. The appropriate amount of 500 mM NaCl, 500 mM Tris HCl pH 7.9, 50% glycerol and $H_2O$ are mixed to result in the final concentrations 50 mM NaCl, 50 mM Tris HCl pH 7.9, 5% glycerol and 200 μl total volume after samples and proteins have been added.
2. 1-10 picomoles of labeled σ70 (10-100 nM final concentration) is added in a 10 μl volume.
3. For competition or inhibition experiments, unlabeled proteins or samples are added.
4. 5-20 picomoles labeled β'-fragment is added.
5. The mixture is incubated for 30 min at room temperature.
6. Samples are read in the multi-plate reader (Wallac, VictorV$^2$ 1420) with a TR-LRET protocol (here: LANCE high count 615/665).

Typical results from a LRET measurement showing salt dependence of binding are shown in Figure A7.3.

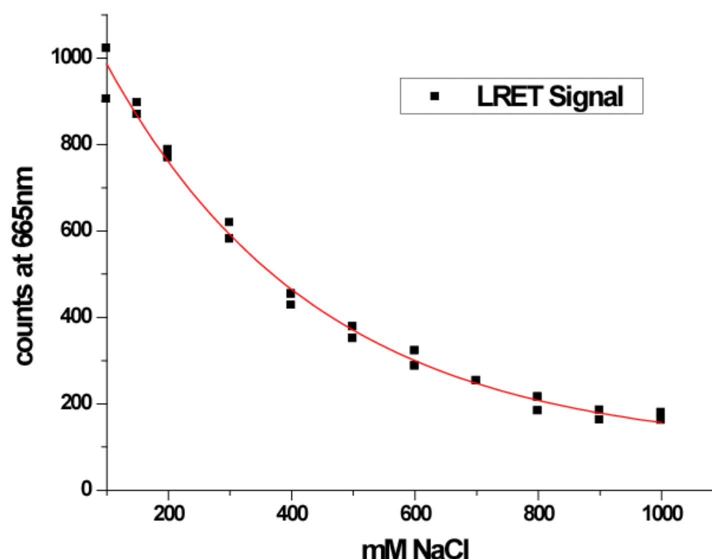


**Figure A7.3: Dependence of binding on NaCl concentration in the LRET-assay. With increasing amounts of salt, the LRET-signal significantly decreases due to the decreased amount of σ70/β'-complex formed. The assay was performed in the fashion described in the text with the following protein concentrations: 20 nM Eu-labeled σ70, 40 nM IC5-labeled β'-fragment.**

### *A.8.2 Comments and Variations*

Without going into detail we would like to point out that for controlled labeling of the target protein, alternative methods can be used. Site-specific protein lableling using biarsenical componds is described in Gaietta et al. (16). The Terbium donor and its compatible biarsenic compound FIAsH-EDT2 are available from PanVera. Site-specific intein-mediated C-terminal 5-(L-cysteinylamido-acetamido)-fluorescein (Cys-F) labeling which can be used in combination with Terbium chelates as donors is described in Mekler et al. (17) and Tolbert et al. (18), respectively. For most of the experiments it is actually not necessary to label the proteins at defined positions. This is only necessary for measurements determining the distance between the fluorescent probes. For general binding studies, random labeling of lysine residues using succinimidyl ester or isothiocyanides of the acceptor is absolutely sufficient. We have successfully labeled core RNA polymerase with IC5-OSu

while it was bound to a DE52 column and used it in LRET assays with this strategy. Since lysine residues are very abundant on most protein surfaces, random labeling with a 3 to 5-fold molar ratio of label to protein resulted in protein mostly active in binding. The molecules that are inactivated through labeling can be neglected since they cannot create signal in the LRET assay. Thus a loss of binding activity has to be corrected for when looking at quantitative results. This approach has the advantage that the target protein doesn't have to be mutated to a single cysteine variant, which would still have to have its activity confirmed. Generally any protein could be studied this way by labeling it in its purified form. Therefore the calculated amount of a 3 to 5-fold molar ratio dye to protein (1 mg protein in 100 µl) is added either dissolved in DMSO or water. The reaction time is usually not more than 15 min at room temperature. Unbound label can be removed afterwards by dialysis. In the case of very "sticky" dyes, like cyanine dyes, spin columns (Pharmacia) are more efficient.

In the same way a specific antibody can be labeled. It can then be used for indirect labeling of its antigen and measured with the fluorescence labeled antigen's binding partner in a time-resolved LRET experiment. Our experiments show comparable results with this approach, although it is critical to keep the concentration of labeled antibodies below the antigen concentration, otherwise the addition of "unlabeled" antigen in competition experiments will increase the signal until the antigen is in excess. It is obvious that in screens for inhibitors of a protein-protein interaction of interest, compounds that interfere with the antibody-antigen interaction would show up as false positives.

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## Abbreviations

| | |
|---|---|
| 2-ME | 2-mercaptoethanol |
| ATCC | american type culture collection |
| $A_\lambda$ | absorption at the wavelength λ [nm] |
| bp | base pairs |
| DMSO | dimethylsulfoxide |
| DTE | 1,4-Dithioerythritol |
| DTPA-AMCA | diethylenetriaminepentateic acid-7-amino-4-methylcoumarin-3-acetic acid |
| DTT | dithiothreitol |
| EDTA | Ethylendiamintetraessigsäure |
| ELISA | enzyme-linked immunoabsorbent assay |
| EMS | electrophoretic mobility shift |
| EtOH | Ethanol |
| FRET | fluorescence resonance energy transfer |
| GuHCl | guanidinium hydrochloride |
| HMK | heart-muscle kinase |
| HTS | high-throughput screening |
| IMAC | immobilized methal affinity chromatography |
| IPTG | isopropyl β-D-thiogalactoside |
| LRET | luminescence resonance energy transfer |
| Ni-NTA | nickel-nitrilotriacetic acid |
| $OD_\lambda$ | Optcal density at the wavelenght λ [nm] |
| PAGE | polyacrylamide gel elektrophoresis |
| RNAP | RNA polymerase |
| SDS | sodium dodecyl sulfate |
| TCEP | Tris(2-carboxyethyl)phosphine |
| v/v | volume per volume |

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