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**Abstract:**

**Title of Thesis:** A sensitive non-radioactive assay for Mammalian RNA polymerase II activity.

Kathleen Elizabeth Foreman, Master of Science, 2012.

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The development of a highly-sensitive nonradioactive activity assay for RNA polymerase II (RNAPII) would offer a substantial benefit for both replacing standard radiolabeling assays used by the research community and for the potential screening of compounds for research and clinical applications. RNAPII is the machine that drives transcription - the fundamental process enabling the "reading" of the human genome. Ultimately, RNAPII is responsible for RNA synthesis for all protein encoding genes. I developed a "nonspecific" RNAPII assay to enable the study of RNAPII with its associated transcription factors. The assay may prove helpful for disclosure of inhibitors of TFIIS and possibly other RNAPII associated transcription factors for the treatment of breast and other cancers. The assay is also effective for detection of bacterial RNA polymerase activity; therefore, the assay holds potential for high throughput screening for inhibitors against bacterial polymerases, while ruling out effects upon the mammalian enzyme.

A sensitive non-radioactive assay for mammalian RNA polymerase II activity.

By

Kathleen Elizabeth Foreman

Thesis submitted to the Faculty of the Graduate School of the  
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## **List of Abbreviations**

bRNAP: bacterial RNA Polymerase

BSA: Bovine Serum Albumin

ELISA: Enzyme-linked Immunosorbant assay

ER: Estrogen Receptor

GTF: General Transcription Factor

HTS: High throughput screening

mRNA: messenger RNA

NELF: Negative elongation factor

NTP: nucleotide triphosphate

PIC: Pre-initiation Complex

P-TEFb: positive transcription elongation factor b

RFU: Relative fluorescence unit

Rif: Rifampicin

RNAPII: RNA Polymerase II

rRNA: ribosomal RNA

TAFs: TATA-binding protein Associated Factors

TBP: TATA-Binding Proteins

TF: Transcription Factor

## **I. Introduction**

### *Background*

Transcription is the essential and fundamental process enabling the “reading” of the human genome. Of the three eukaryotic RNA polymerase enzymes (RNAPI, II, and III), RNA polymerase II (RNAPII) is responsible for transcribing all protein-encoding genes. Regulation of transcription is a highly complex mechanism that involves hundreds of polypeptides acting in concord at a promotor sequence. The regulation of gene expression is the direct result of the processing of input from transcription factors by the core transcription machinery. After the core machinery receives input, it integrates the information and generates an output signal, which can include low to high gene expression or silencing of expression. As a fundamental cellular function, misregulation of transcription has critical effects on cellular function (Villard, 2004) and underlies many disorders. For example, misregulation of transcription leads to cancer “hallmark capabilities” (Hanahan and Weinberg, 2011).

### *The need to develop an assay for transcription by RNA Polymerases*

The overall goal was to develop a “nonspecific,” non-radioactive RNA polymerase II (RNAPII) assay. A nonspecific RNAPII assay would enable the study of RNAPII activity without the use of a promotor or general transcription factors. This assay would then be used to study RNAPII activity alone and with its associated transcription factors individually and in combination. The assay would be best achievable as a single step that would not require additional steps that could be costly, with regard to finances or time. The assay should also be effective for prokaryotic and eukaryotic RNA

polymerases alike. In its simplest form, the assay would be capable of non-specific transcription; meaning, it is able to transcribe without the need of a promotor sequence or transcription factors.

A non-radioactive bacterial and RNAPII assay would be beneficial for several purposes. First, such an assay would enable the fundamental study of RNAPII and the way in which its directly-associating factors affect transcription. Second, it would permit high throughput screens (HTS) to find inhibitors of transcription factors that directly associate with RNAPII, and whose inhibition may have clinical significance for therapy. Third, it would allow for high throughput screens to disclose inhibitors of bacterial RNA polymerases, which do not inhibit the mammalian RNA polymerase II.

Many assays employed to date were performed using radioactive materials, and/or require multiple steps, and/or have not been shown to be effective in detection of both bacterial and mammalian enzymes. Radioactive techniques have been a more popular method of detecting nucleic acids and offer high-sensitivity applications, such as  $^{32}\text{P}$ -labelled probes capable of detecting as low as 10 femtogram (fg) of target DNA, and allow for reproducible protocols. However, liquid handling of radioactive materials can be difficult to manage especially for HTS and requires proper disposal of all contaminated materials. The use of radioactive labels would therefore not be ideal for a high-throughput application (Osborn 2000).

Enzyme-linked immunosorbant assays (ELISAs) are also an accurate method able to detect relative amounts of nucleic acids by the use of antibodies linked to enzymes that alter substrates, and generate a color that can be measured spectrophotometrically. An unpublished ELISA or colormetric assay was previously designed and used in the Gnatt

lab and could detect RNAPII synthesis; however, the assay required 1-2 $\mu$ g of RNAPII minimum, and, therefore, wasn't highly sensitive. ELISAs also require multiple steps of washing and binding product to a plate before a measurement may be taken. ELISAs do not contain radioactive materials and background interference can be removed by washing.

Non-radioactive methods, such as fluorescent indicators, offer an effective, convenient, and safe alternative. However, using fluorescent dyes for quantification purposes has detection and other limitations. In our assay we use the fluorescent indicator (dye) Ribogreen<sup>®</sup> from Invitrogen that directly binds to RNA and provides a very high product signal (transcribed RNA) with minimum fluorescent background interference. Ribogreen<sup>®</sup>'s detection range extends from 1.0 – 50.0 ng/mL of RNA using 75 nM dye and from 20 ng/mL to 1  $\mu$ g/mL using 750 nM dye. Detection is not as sensitive as using radioactive labels; however, sensitivity is still regarded as high (Osborn 2000). Another issue with non-radioactive assays using fluorescence is that such dyes may generate background since they intercalate in the DNA, which is needed as a template for RNA synthesis. A more complete discussion of the advantages of the assay developed compared to other nonradioactive assays is provided in the discussion.

***Proof of concept: Select studies of the non-radioactive RNA polymerase assay***

Once the general assay, our overall objective, was established, a series of experiments were performed to test a range of applications for which this assay may be used. Assays included testing pure RNAPII with or without pure transcription factors, TFIIS, TFIIF, Gdown1 and combinations thereof to study their effects on transcription. For example, an assay of RNAPII with BromoUTP (BrUTP) instead of UTP and an assay of RNAPII with BrUTP and TFIIS provided insight on the way in which TFIIS affects transcription in the presence of BrUTP's induced effects. The assay with pure RNAPII and TFIIS, enables detection of TFIIS enhanced transcription, may prove helpful for disclosure of inhibitors of TFIIS. Similar studies may be useful for other RNAPII associated transcription factors for the development of treatments for cancer and other diseases.

Finally, assays examining the activity of the bacterial RNA polymerase core enzyme in the new buffers with and without rifampicin, a specific RNA polymerase inhibitor employed for the treatment of tuberculosis, were included. Since the assay is shown to be effective for detecting bacterial RNA polymerase activity, the assay may hold potential for high throughput screening for new antibiotics/drugs against bacterial polymerases, while ruling out effects upon the mammalian enzyme. Altogether, these experiments will shed light on the several applications for which this assay may be used.

This assay could be useful and helpful for enhancing our understanding of transcription regulation by RNAPII associated factors. The assay may also be used for the development of anticancer assays to disclosure inhibitors to transcription factors as a potential treatment of diseases such as breast cancer. TFIIS has been patented as a cancer

target, with additional support from a recent publication by the Oren lab in the Weizmann Institute. Knockdown of TFIIS in triple-negative breast cancer cells has been shown to inhibit cancer cell proliferation and induce apoptosis (Hubbard *et al.*, 2008) This assay could also be used to develop novel antibiotics against pathogenic bacterial polymerases if it successfully tests the specificity of polymerase inhibitors on bacterial and mammalian polymerases.

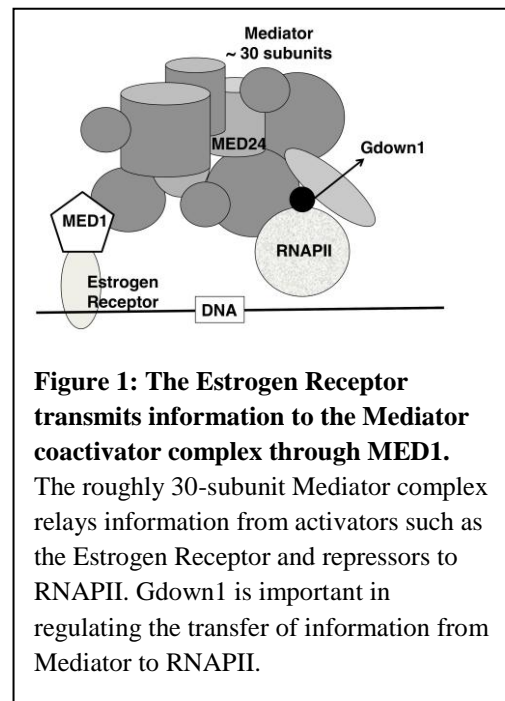
### ***Overview of Transcription***

At the core of the transcription process lays RNA polymerase II (RNAPII), a catalytic enzyme that works like a motor as it “reads” DNA and synthesizes a

complementary RNA copy. Mechanistically, transcription is activated or repressed at the promotor by the binding of trans-acting proteins (DNA binding transcription factors) to cis-acting sequences (binding sites). The transfer of signals from such transcription factors to RNAPII frequently occurs through a third party co-factor. For example, the estrogen receptor (ER), a nuclear receptor, is a trans-acting protein that binds directly to DNA and alters

transcription. The ER transfers signals to RNAPII via direct binding to the MED1 subunit of the large multi-subunit co-activator complex, Mediator (**Fig. 1**).

Output from Mediator to RNAPII is affected by a novel RNAPII subunit, Gdown1, which was discovered in 2006 (Hu *et al.*, 2006). Mediator does not bind

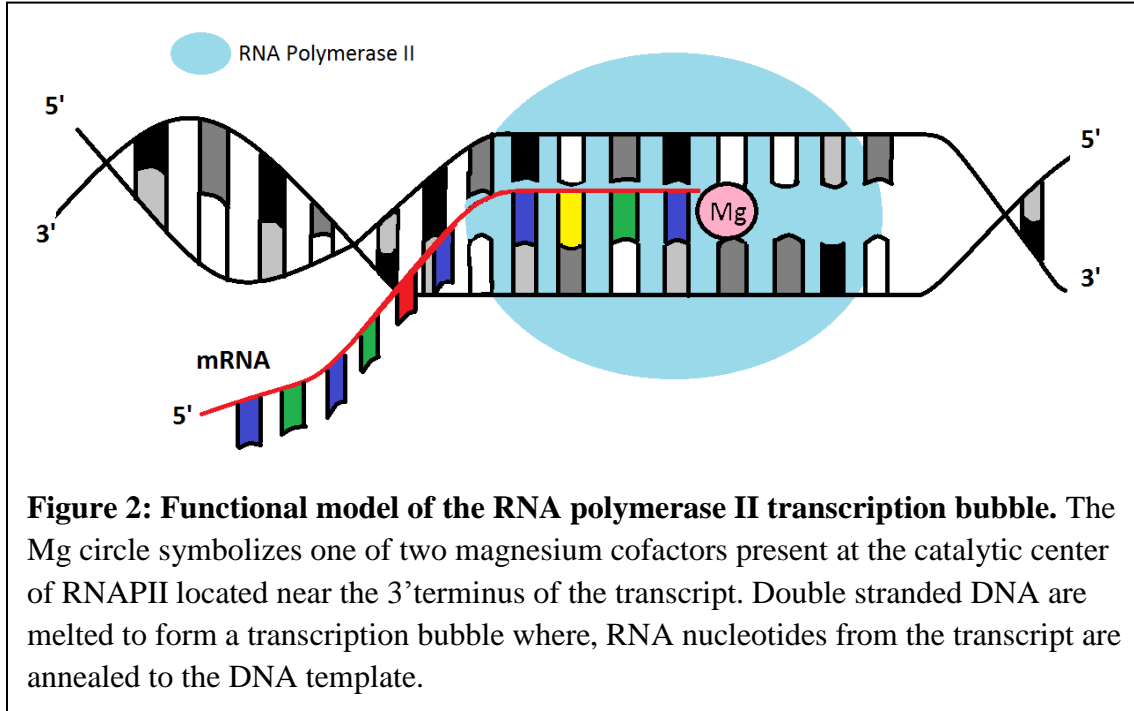


directly to the DNA; instead, it acts as a “bridge” between activators and repressors and RNAPII. Mediator transduces both positive and negative regulatory information by physically interacting with RNAPII and transcription factors (Hampsey and Reinberg, 1999; Myers and Kornberg, 2000; Woychik and Hampsey, 2002).

### ***Transcription has multiple stages***

The synthesis of RNA involves multiple processes by RNAPII. These processes make up what is known as the “transcription cycle.” The primary phases that make up the “transcription cycle” include pre-initiation, initiation, promoter clearance, elongation, and termination (Sims *et al.*, 2004). RNAPII contains twelve highly conserved subunits designated RPB1 to RPB12 (Young, 1991) and a thirteenth subunit, known as Gdown1 was disclosed in Metazoans (Hu *et al.*, 2006). RNAPII undergoes elaborate conformational changes in order to integrate information from transcription factors during each step of the transcription cycle (Borukhov and Nudler, 2008). Although regulation may occur at multiple phases during transcription, it has been demonstrated that a vast amount of regulatory events occur during initiation of gene transcription (Mitchell *et al.*, 1989).

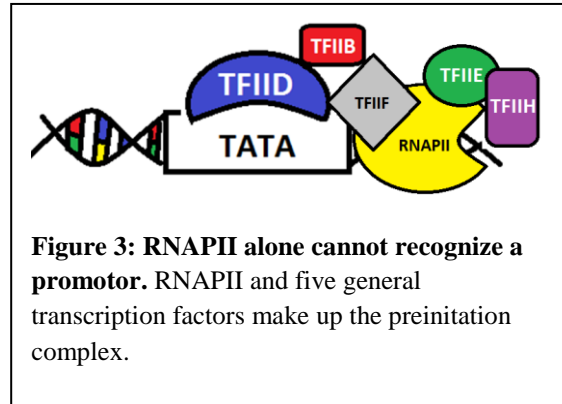
RNAPII alone is not capable of effectively initiating on a double stranded DNA. For normal transcription initiation to occur, double stranded DNA is unwound at the promoter, forming a DNA bubble (Nudler *et al.*, 1997). RNAPII protects approximately 30bp of DNA while ~12 bp of DNA are melted to form a “transcription bubble” (**Fig. 2**).



Artificial bubbles therefore mimic the native state of transcript initiation and RNAPII has been shown to effectively recognize and initiate at such templates (Nudler, 1999). A mismatch bubble in double-stranded DNA suffices to direct precise transcription initiation by *Escherichia coli* RNA polymerase (Aiyar *et al.*, 1994).

As previously mentioned, the production of mRNA involving specific initiation at a promoter requires a number of additional protein accessory factors, also known as transcription factors (TFs) (Roeder, 1976; Weil *et al.*, 1979; Matsui *et al.*, 1980). In eukaryotes, General Transcription Factors (GTFs) assemble into what is known as the pre-initiation complex (PIC) that arrange themselves on the core promoter to form stable nucleoprotein complexes that recruit RNAPII and specify the transcription start site – the site where RNAPII will bind and initiate transcription.

The GTFs that make up the PIC include TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (**Fig. 3**). Other transcription factors regulate the assembly of GTFs to the promotor by acting as activators. Activators enhance production of mRNA by stimulating various steps along the pathway – such as accelerating recruitment of GTFs and the assembly of the PIC (Orphanides *et al.*, 1996). Similarly, transcription factors may also act as repressors and inhibit GTF recruitment or block PIC assembly.



The pre-initiation complex assembly appears to have a hierarchical nature at the promotor region – a stepwise manner known as the sequential assembly pathway (Buratowski and colleagues, 1989). First, the TATA box, a core promotor element and a cis-acting sequence, is recognized by TFIID. TFIID consists of a TATA-binding protein (TBP) and TBP associated factors (TAFs) which helps TFIID bind directly to the TATA box. Then, TFIIB follows and helps to stabilize the promotor-bound TFIID. Next, RNAPII is recruited with TFIIF. TFIIE and TFIIH follow in a stepwise manner to finalize the formation of the PIC. Studies also suggest there may be an alternative pathway for PIC assembly, as purified preassembled RNAPII holoenzyme complexes have been isolated from many different laboratories (Kim *et al.*, 1994., Koleske and Young, 1994). Elongation factors can also regulate transcription during RNA synthesis. In order for transcript elongation to be efficient, it must overcome several blocks that are intrinsic to RNAPII catalytic activity. These intrinsic blocks cause transcription to be “arrested” or “paused” – with blocks that cannot be overcome by RNAPII alone and thus require

accessory factors for the production of full length RNA (Sims *et al.*, 2004). For example, TFIIS reinitiates transcription if it becomes arrested and is unable to complete RNA synthesis (Reines *et al.*, 1989).

Pausing and arrest sites of transcription appear to be a natural process for RNAPII and transcriptional regulation. In addition to TFIIS, which reinitiates transcription after “arrested” transcription, other factors modulate and regulate transcriptional pause and arrest. For example, NELF (negative elongation factor) promotes RNAPII pausing near the promotor, also known as “promotor proximal pausing,” to allow 5’-capping of RNA transcripts (Sims *et al.* 2004; Yamaguchi *et al.* 1999). P-TEFb (positive transcription elongation factor b) relieves NELF-mediated pausing by phosphorylating a serine amino acid on the c-terminal domain of RNAPII (Shim *et al.* 2002). Recently Gdown1 was also suggested to play a role in promotor proximal pausing (Cheng *et al.*, 2012).

### ***Breast Cancer and Synthetic Lethality***

Despite our best clinical efforts, there remain approximately 40,000 breast cancer mortalities per year in the United States alone (American Cancer Society, 2011-2012). The most challenging type of breast cancer to treat is triple negative breast cancer, which lack expression of the estrogen receptor, the progesterone receptor, and ErbB2. (Glueck *et al.*, 2009). It is well- established, that mutations in DNA drive the cancer phenotype. Such mutations induce misregulated transcription, which underlies cancer due to the effects of altered states of transcription factors upon transcriptional regulation. Misregulated transcription results in altered gene expression , leading to cancer hallmark capabilities. For example, cancer involves the overexpression of oncogenes or

suppression of tumor suppressors. Cancer hallmarks consist of six or seven biological capabilities acquired during the multistep development of human tumors (Hanahan and Weinberg, 2011). Therefore, it is obvious cancer cells do not exist within the context of normal transcription.

Cancer cells maintain improper DNA repair mechanisms; and so, they also maintain the ability to mutate rapidly and evolve additional survival pathways to compensate for specific pathway knockdown – leading to multiple drug resistances. But, genetic instability may render breast cancer vulnerable to select drug targets. In the case of a drug, “synthetic lethality” is a strategy that describes a lethal combination wherein the drug and the cancer mutation would induce tumor cell death – turning the cancer’s greatest strength into a weakness (Garber, 2012). However, the drug in a normal cell lacking the mutation, would have limited or no effects. Considering that cancer cells maintain aberrant transcription, it was proposed that targeting the core transcription components may add an additional insult to the already improper transcription within cancer cells and thus be synthetically lethal. Therefore, an assay to screen for inhibitors of transcription factors associated with RNAPII that could induce synthetic lethality would have potential as a clinical tool for treatment.

There is a degree of caution however, that comes with targeting the core transcription machinery in that only those core transcription elements not necessary for normal cell survival can be targeted. For example, Triptolide is a bioactive ingredient used in traditional Chinese medicine and is under study for its anticancer capabilities. Triptolide has recently been discovered to bind to TFIIF and trigger a pathway leading to degradation of the largest subunit of RNA polymerase II: Rpb1. Triptolide was shown to

decrease Rpb1 levels in cancer cells (Manzo *et al.*, 2012). Targeting RNAPII subunit 1 (RPB1) would be extremely risky; however, the targeting of core transcription factors and inhibition of their activity for as long as normal cells can tolerate, may enable the selective elimination of cancer cells by adding an insult to their already aberrant transcription machinery.

Therefore, one may argue that a therapy achieved by altering the processing of improper information by targeting the core transcription machinery would be more successful than targeting the specific pathways that contain mutated and/or overexpressed proteins. Some success in modifying core transcription for cancer therapy is available. For example, Dactomycin (actinomycin D) is a chemotherapeutic DNA intercalating agent that inhibits transcription and has been used for many years in the treatment of various cancers (Perry and Kelley, 1970; Dubois *et al.*, 1994). Other transcription regulation inhibitors inhibit modifiers, such as HDAC's (histone deacetylase's, ie. SAHA), which modify histones and are also clinically approved.

Previously, it was mentioned that the elongation factor, TFIIS, also regulates transcription during RNA synthesis by promoting "readthrough" of RNAPII at transcriptional arrest sites. Three isoforms of TFIIS have been discovered. The most common and ubiquitously expressed TFIIS isoform is encoded by the TCEA1 gene, which is lacking introns. The other two isoforms are encoded by separate intron-containing genes, TCEA2 and TCEA3, and were described as tissue specific: TCEA2 as testes specific (Weaver *et al.*, 1997) and TCEA3 as kidney specific (Labhart and Morgan, 1998).

TFIIS may fit our requirements as a transcriptionally synthetic lethal cancer target and has indeed been implicated as a potential target for cancer therapy (Hubard *et al.*, 2008; Shema *et al.*, 2011). In addition both TFIIS and Gdown1 have been patented as cancer targets (US7704967). Therefore, the designing and implementation of an assay that will effectively detect the activities of TFIIS and Gdown1 with RNAPII may be useful to disclose parent small molecule inhibitors for potential cancer therapy. A successful assay can serve as the basis for high throughput screening of drugs and inhibitors for TFIIS, Gdown1 as well as other RNAPII interacting proteins.

### ***Bacterial RNA polymerase***

Over the past 60 years increasing amounts of antibiotics have been discovered and widely used; yet, bacteria have responded by producing drug-resistant progeny. This, along with misuse, has led to the existence of drug-resistant bacterial strains (Levy, S. 2002). Bacterial resistance poses itself as a major threat and critical health hazard in the case of a serious outbreak because of lack of therapeutic options to control the spread. Multidrug resistance (MDR) is a term that is used to describe multiple drug resistances in microorganisms such as bacteria (and other microorganisms including parasites and fungi). As more antibiotics are discovered and coming into use, bacteria with MDR are also being discovered. It would seem that we are living in an “arms race” between our technology and bacteria’s ability to evolve and adapt.

Bacteria contribute to a large list of pathogenic biological agents that can cause major infectious diseases such as mycobacterium tuberculosis, meningitis, and pneumonia. It is crucial that we have antimicrobial drugs to target these microorganisms;

however, what appears to be an inevitable problem is their ability to acquire resistance to antibiotics.

One of the first modern antibiotics, penicillin, was discovered in 1928 by Sir Alexander Fleming, who observed growth inhibition of the microbe, *Staphylococcus aureus*, caused by an accidental contamination of the mold: *Penicillium notatum* (Hare, 1982). It appeared that the mold produced biological compounds capable of killing and suppressing the growth of the bacteria. Penicillin was very effective in preventing infection during World War II and afterwards was widely available without a prescription. However, in 1947 *Staphylococcus aureus* was the first bacterium discovered to have developed penicillin resistance.

At the time of penicillin's discovery the mechanism of action was unknown. Today we know penicillin works by specifically inhibiting transpeptidase – an enzyme necessary in the final step of cell wall biosynthesis (Yocum *et al.*, 1980). Bacteria susceptible to penicillin developed resistance by spontaneous mutations that altered the target protein, penicillin-binding protein 2b, to which penicillin binds (Tenover F. 2006). The bacteria propagated rapidly and passed this genetic mutation onto their progeny.

Similar to the mechanism in mammals, gene expression in bacteria is linked to RNA transcription and requires RNA polymerase as the core machinery. In humans, there are three RNA polymerases (RNA pol I-III) that have been found to be involved in transcription (Clancy, S. 2008); however, in bacteria, all transcription is performed by a single RNA polymerase. The bacterial polymerase contains only four catalytic subunits:  $\beta'$ ,  $\beta$ ,  $\alpha$ , and  $\omega$  with a regulatory subunit known as sigma ( $\sigma$ ). Sigma factors are

weaklybound subunits that provide an extra level of control to bacterial transcription – several sigma factors have been identified and each bind to a distinct promoter sequence (Cooper, 2000).

Bacterial resistance to an antimicrobial drug can be gained through several mechanisms. In response to drugs, stress, or changes in the environment, spontaneous mutations may occur and lead to the expression of acquired new genes that may inactivate or alter the effects of anti-microbial drugs. Bacteria may also respond to antibiotics by upregulating proteins that inhibit or dispose of the drug (McManus, 1997). These acquired genes are then selected for and passed down to their progeny – passing on genes encoding drug resistance. The selected mutation may also be acquired by other bacteria through various genetic mechanisms (e.g. transformation, transduction, and conjugation) (Tenover, 2006). The transfer of genetic material encoding drug-resistance is often a major problem observed because of the improper use of antibiotics.

The development of our assay may prove effective in delivering new antibiotics specific for bacterial RNA polymerase. An assay that allows the detection of activity in both mammalian and bacterial polymerases would be ideal for high throughput screening of inhibitors. It would allow us to screen for drugs and/or antibiotics against bacterial polymerases and rule out effects on the mammalian enzyme.

There is prior art regarding both the targeting of bacterial RNAP for antibiotic use. Rifampicin (Rif) specifically targets bacterial RNA polymerase and has proven to be an effective and extremely potent antibiotic. Rif was introduced in 1968 and was extremely effective against *M. tuberculosis* (Campbell *et al.*, 2001). However, today, *M.*

*tuberculosis* still leads in the number of adult deaths by a pathogenic biological agent (Shinnick, 1996; Raviglione *et al.*, 1995). Despite Rif's success, bacteria have developed resistance to it– which is why the use of Rif is restricted to treating only tuberculosis or in the case of an emergency. However, considering the large surface area involved in RNA synthesis, and DNA and RNA binding, it is likely that many pockets on the protein can be targeted for antibiotic development, and so additional RNAP antibiotics are likely plausible.

Highly conserved features in RNA polymerase are shared between prokaryotes and eukaryotes (Cramer *et al.*, 2001). Rifampicin binds to the bacterial RNA polymerase but does not interfere with substrate binding, catalytic activity, or the intrinsic translocation mechanism of RNAP. Structural studies of Rif bound to bacterial RNAP, shows binding at a specific non-conserved site not shared between prokaryotes and eukaryotes (Campbell *et al.*, 2001). Rifampicin was suggested to inhibit bacterial RNAP during the initiation phase – as it has no effect on bacteria already in the elongation phase of transcription. Others have argued that Rif decreases affinity of RNAP for short RNA transcripts (2-3 nucleotides long – still in the initiation phase) (Schulz and Zillig, 1981). Either way, it appears Rif inhibits RNAP by blocking transcription elongation (Campbell *et al.*, 2001).

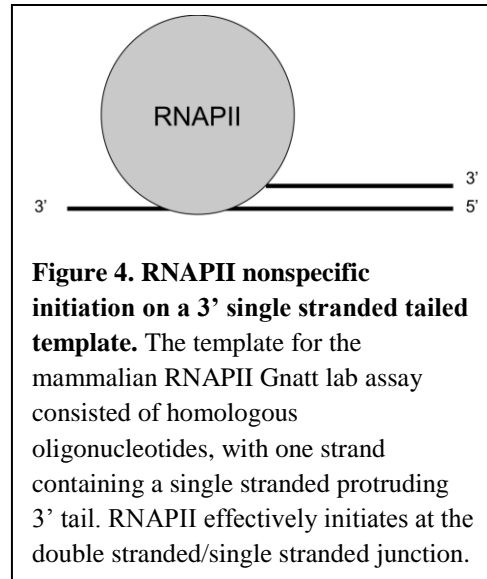
Since the assay as described herein was shown to successfully detect both bacterial and mammalian RNA polymerase activity under the same conditions, and is simple to execute, it should prove to be an aid in finding multiple drugs or antibiotics that work just as effectively as Rifampicin.

## II. Materials & Methods

### *Development of a Fluorescent-Based Nonspecific Initiation RNAPII Assay*

The starting points and basis for the bacterial and mammalian RNA polymerase assay were two previously developed assays and are summarized in **Table 1**. We

developed a hybrid of the two assays with a novel template that was eventually successful in finalizing our assay. The first assay was a radioactive assay for yeast RNAPII in the Gnatt lab. The assay consisted of a double stranded template with a 3' single stranded protruding region of nine bases or more. Initiation was non-specific in that it did not require a promotor, nor, did it require transcription



factors (Kane and Chamberlin, 1985). The Gnatt lab assay is herein termed the tailed template assay (Gnatt *et al.* 1997) as it employed a “tailed” DNA template as illustrated in **Figure 4**. One or more of the four ribonucleotides added were radioactive, enabling detection of RNA synthesis. The same assay was optimized for buffer and employed with calf thymus RNAPII in the Gnatt lab (Hu *et al.*, 2006). Calf thymus RNAPII was purified as previously described (Hu *et. al.*, 2006). Those buffers were adopted in this fluorescent assay and proved to work for both mammalian RNAPII and bacterial RNAP. Key biochemical modifications that were made relative to the tailed template assay included using single stranded M13 DNA template, which replaced the tailed template. In

addition, fluorescent detection of products was used as opposed to radioactive detection.

<b>Kool Assay</b>	<b>Tailed Template Assay</b>
<b>Fluorescent Dye:</b> Ribogreen	Radiolabeling nucleotide
<b>Nonspecific Initiation</b> Rolling Circle Transcription	<b>Nonspecific Initiation</b> Single stranded-ds junction
Bacterial RNAP buffers	<b>Mammalian RNAPII buffers</b>
<b>Simple fluorescent assay protocol</b>	Multi-step radioactive protocol
<b>Single stranded DNA template</b>	“Tailed Template” – Protruding 3’ single stranded end.

**Table 1: Combining the best attributes of two assays.** Combined characteristics (light gray) from the Kool assay (assays bacterial polymerase activity using fluorescent detection) and from the tailed template assay (radioactive assay for RNAPII) were employed for the new assay.

The second assay, employed as a “starter” or “template” to frame our assay, was the commercially available *E. coli* Kool™ NC-45™ RNAP Activity & Inhibitor Screening Kit (Illumina Cat. #KNK49025, see below: *The Kool Assay*). Development of the new “hybrid” assay employed the bacterial enzyme provided by the manufacturer, and began by replacing the major component of the Kool kit - the signature Kool NC-45 template, an expensive 45-base single stranded circular DNA template, with the less expensive single stranded M13 phage single-stranded DNA as template (BioLabs, Cat. #N4040S), and replacing the buffers with those employed in the mammalian tailed template assay (Hu *et. al*, 2006). The signal from the initial “hybrid assay” was then improved upon by refining parameters (see below). In this manner, the efficiency and effectiveness of the new hybrid assay was compared with that of the Kool assay.

### ***The Kool Assay***

The Kool™ NC-45™ RNAP Activity & Inhibitor Screening Kit (Illumina Cat. # KNK49025) contains a unique Kool NC-45 Template (45 nucleotide circular ssDNA), which serves as a universal RNA polymerase template to screen for inhibitors of “rolling-circle” transcription by bacterial polymerases (Liu *et. al.*, 1996). The kit contains *E. coli* RNAP for screening of inhibitory compounds and uses a fluorescent dye (Ribogreen®) that binds the RNA for real-time detection of RNA activity. Though there exists a background signal from the template which interacts with the Ribogreen® the results are considered significant if a signal-to-noise ratio is calculated to be above ~4 and more preferably above 5.0. The signal to noise ratio is the overall signal divided by the overall background fluorescence (found by assaying all reaction components together without substrate). This indicates that a sufficient amount of excess RNA is generated (overall signal) compared to template (which would contribute quite a bit to background fluorescence). The Kool assay is a way to measure bacterial RNA polymerase activity in a fairly rapid and simple fashion using nonspecific initiation, similar to the tailed templates, as opposed to specific initiation requiring a promotor or additional transcription factors. However, the Kool template did not provide for an effective signal with mammalian calf thymus RNAPII. If one were to screen for inhibitors for bacterial RNAP, the calf thy enzyme could not be effectively ruled out employing the Kool assay.

### ***Fluorescent dye for RNA detection***

To select a fluorescent dye, we compared Ribogreen<sup>®</sup> (Molecular Probes, #R11490) and SYBRGold (Invitrogen, #S11494) for detection of RNA synthesis emissions using 480/520nm light (emission/detection). We used various concentrations of ribosomal RNA (rRNA) to examine how accurate each dye would be in detecting 1.0 ng/ $\mu$ l-50.0 ng/ $\mu$ l rRNA.

### ***Refining parameters of the initial “hybrid” assay***

Buffers for my initial hybrid assay contained 30mM HEPES with 3mM magnesium chloride ( $MgCl_2$ ) and salt, based on the tailed template assay for mammalian RNA polymerase II (Hu *et al.*, 2006). Through months of trial-and-error, the buffers were eventually replaced with TRIS buffer and manganese chloride ( $MnCl_2$ ). Buffers, template, enzyme, and substrate NTPs were assayed for their contribution to background fluorescence to create optimal conditions with as low a background signal as possible. Bacterial *E. coli* RNA polymerase (core enzyme) was replaced with calf thymus RNAPII.

My calf thymus RNAPII assay was initially executed in a 12.5  $\mu$ l volume reaction in 0.5 ml test tubes, with incubation for one hour at 37°C in a water bath. 7.5 $\mu$ l of reaction samples were taken from test tubes and added to 200 $\mu$ l of 1/400 Ribogreen<sup>®</sup> and measured in a black 96 well plate from Fisher Scientific. Optimal conditions later introduced the use of Corning<sup>®</sup> Low Volume 384 Well Black Flat Bottom Polystyrene NBS<sup>™</sup> Microplates (Cat. #3820).

These initial conditions were again improved upon for the mammalian enzyme. Customizing and developing the assay required numerous titrations over a time-span of eight months to determine the optimal concentrations for nucleoside triphosphates (NTPs), salt (ammonium sulfate and manganese chloride), DNA template and enzyme (optimal DNA-to-enzyme ratio), Dithiothreitol (DTT), and Ribogreen<sup>®</sup> fluorescent dye. Time of incubation at 37°C and labeling with Ribogreen<sup>®</sup> were also optimized. Assays were repeated to calculate a mean, standard deviation, and standard error. As a control, the specific RNAPII inhibitor,  $\alpha$ -amanitin (SIGMA, #SLBD2979V), was employed at 10 $\mu$ g/mL to inhibit RNAPII and indicated that transcription was performed solely by RNAPII.

The final assay reaction buffer was effective for both mammalian and bacterial polymerases and contained: 30mM TRIS pH 7.8, 3mM Manganese chloride (or 3mM Magnesium where indicated), 50-60mM ammonium sulfate, 1mM DTT, 50ng/ $\mu$ l RNAPII, 2.5ng/ $\mu$ l single stranded M13 template (BioLabs, Cat# N4040S) and 500 $\mu$ M NTP's each (Fermentas # R0481). Reactions were incubated at 37°C for 2 hours. 5.0  $\mu$ l reactions were placed into 15.0  $\mu$ l of 1/300 Ribogreen<sup>®</sup> (final concentration 1/400, Invitrogen Cat. #R11490) in a Corning<sup>®</sup> Low Volume 384 Well Black Flat Bottom Polystyrene NBS<sup>™</sup> Microplate (Cat. #3820). Plates were mixed and incubated undisturbed for an additional hour in the dark at room temperature. After 2 hours, the fluorescence was measured with excitation at 485nm and emissions at 520nm.

### ***Fluorescent Microplate Readers***

Initially, the assays were performed in a black 96-well plate and were measured using a Biotek Fluorescent Microplate reader owned by the Translational Shared Services Laboratory at the University of Maryland Marlene and Stewart Greenebaum Cancer Center. The Biotek micoplate reader was effective in detecting changes in fluorescent for initial adjustments of assay parameters; however, the machine was not capable of reading 384-well plates. A nearby alternative was then found, the VICTOR plate reader (manufacturer: PerkinElmer), owned by the Division of Endocrinology, Diabetes and Nutrition at the University of Maryland. During the final development of my assay and the initial process of testing my assay with various other applications, my department acquired a new fluorescent plate reader: the Tecan infinite M1000Pro (Serial #1109005812).

### ***The final assay was effective for RNAPII alone and in complex with transcription factors***

The optimized conditions were also effective in detecting effects of mammalian transcription factors upon mammalian RNAPII. Human TFIIS/TCEA1, both wild type and an inactive mutant D290A, were titrated and later employed at a 10-fold molar excess. Human TFIIS/TCEA1, both wild type and mutant D290A were expressed from the pET16b n-terminal 6Xhistidine tagged vector (Invitrogen) in BL21(DE3) *E. Coli* and purified by IMAC and MonoS ion exchange chromatography.

### *Statistical Analysis*

All experimental data was statistically verified. Experiments were repeated 2-4 times with similar results unless otherwise indicated. Each data points in each experiment (sample size) was repeated 3-5 times. Bar graphs represented the calculated mean from the sample values. The standard errors of all samples were calculated from the mean and standard deviations and are represented as error bars on graphs. Significance was determined by a two-tailed t-test, where data was defined as significantly different when  $P < 0.05$  and data defined as similar, with no significant difference, when  $P > 0.9$ . All assays were compared by their signal-to-noise ratio for optimal activity. The signal was measured as relative fluorescent unit (RFU) values and were determined by the difference between average overall fluorescence and controls containing reaction buffers and enzyme without NTP substrate (NTP substrate alone did not contribute at all to the signal).

Graphs and tables were made using Microsoft Excel 2010 for Windows 7. Figures, unless otherwise indicated, were made using Microsoft paint and powerpoint 2010 for Windows 7.

### III. Results & Discussion

Acceptable ranges for parameters at which the “hybrid” assay may be performed were studied by assessing results obtained from varying multiple components of Dr. Gnatt’s tailed-template assay and the various components of the Kool Assay.

#### *Defining the DNA Template for the assay*

RNAPII alone is not capable of effectively initiating on a double stranded DNA. In the tailed template assay, RNAPII recognizes the tailed template as half of a transcription bubble due to the protruding 3’ end; therefore, it results in an effective initiation site (Kane and Chamberlin, 1985; Gnatt *et. al.*, 1997). With both templates, a single stranded/double stranded region is sufficient for initiation. With effective RNAPII initiation on the tailed template or an “artificial bubble” template, pronounced elongation ensues in the presence of magnesium. Salmon sperm DNA that has been sonicated produces “sheared” DNA strands that are similar to the tailed template or bubble DNA. Sheared DNA will randomly contain 3’ single stranded DNA tails and other single stranded/double stranded regions, which are allows for effective RNAPII initiation.

Tailed templates were not used in our assay since the small oligonucleotides would result in small RNA transcripts. Radiolabeling provides for a very high signal to noise ratio and the signal from short transcripts would be detectable in gel electrophoresis. However, radiolabeling is not best suited for high throughput screening, in that only minimal samples can be safely handled.

Since the commercial fluorescent-based Kool assay was effective for the bacterial enzyme, I tested the “Kool template,” a single stranded circular 45-base template, with mammalian RNAPII. However, calf thymus RNAPII was extremely ineffective in transcribing the signature Kool NC-45 template in the presence of magnesium or manganese chloride (**data not shown**) – most likely because the precise geometry of the small circular 45-base pair template is structurally suited to the bacterial enzyme. Optimizing the size of the Kool template for the mammalian enzyme might be successful, however, it would require complex and expensive synthesis of many small circular templates as well as a considerable amount of time. Furthermore, these customized circular single stranded templates are expensive to generate and would contribute considerably to the cost of any high throughput screen.

In keeping with single stranded DNA however, we found that the presence of  $MnCl_2$  in our assay allowed calf thymus RNAPII to recognize and transcribe on the single stranded M13 DNA template without the need of a promotor or associated transcription factors. M13 template is easily generated, and is commercially available as well.

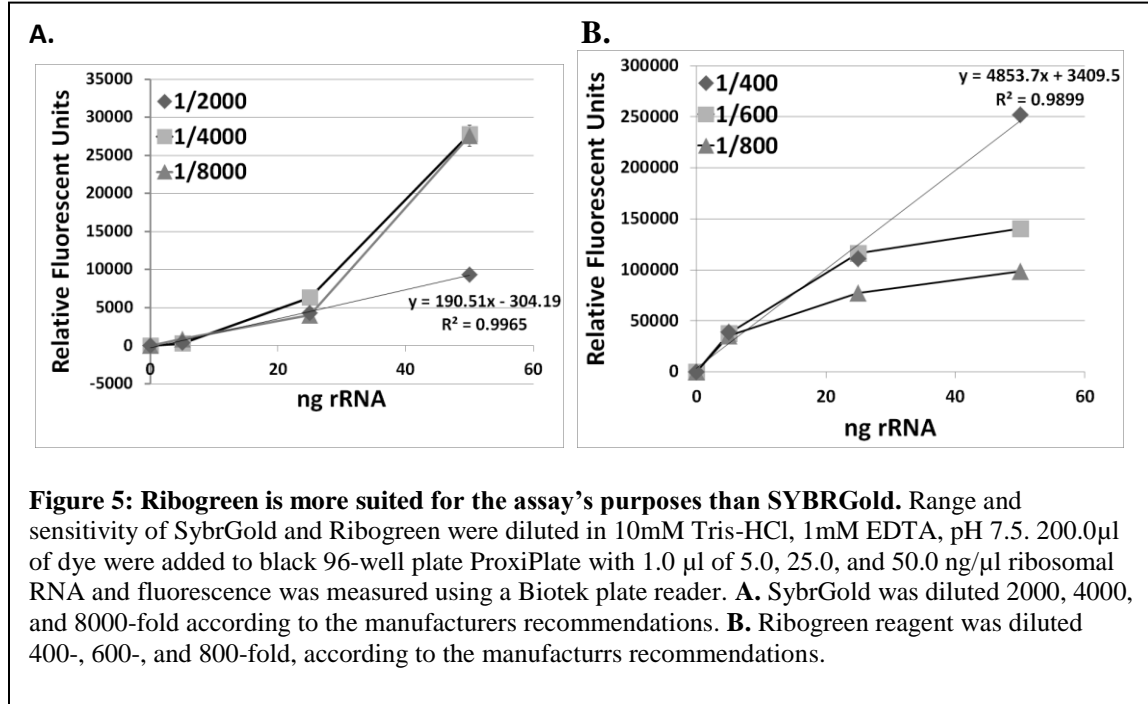
In several experiments employing buffer conditions of the tailed template assay, I found that salmon sperm DNA is also an effective template and can be detected using magnesium or manganese. However, the signal to noise ratio of preliminary studies with magnesium and salmon sperm DNA were considerably poorer than signals measured with manganese as metal cofactor (**data not shown**). Furthermore, salmon sperm DNA and M13 template provided for a similar fluorescent signal when using manganese (**data not shown**). Therefore, I continued optimizing conditions for the M13 ssDNA (BioLabs, Cat# N4040S) and focused my attention on increasing the signal to noise ratio.

***Fluorescent dye: Ribogreen<sup>®</sup> proved more effective than SYBRGold***

Since radioactive labeling is not optimum for high throughput screening, an optimal fluorescent dye for RNA detection was determined. Two well-known fluorescent dyes employed for the detection of DNA or RNA, Ribogreen<sup>®</sup> from Invitrogen (catalogue #R11490) and SYBRGold from Invitrogen (catalogue #S11494) were therefore tested for both sensitivity and linearity across a range of ribosomal RNA (rRNA) concentrations. Regarding detection of RNA, the 1/2000 dilution of SYBRGold dye provided a signal that was linear across the range of rRNA tested (**Fig.5, panel A**); however, signal intensity was considerably weaker than the Ribogreen<sup>®</sup> signal at 1/400 dilution (**Fig. 5, panel B**). Interestingly, diluting the SYBRGold increased the signal, though to no avail, as the signal was not linear across the range of rRNA tested, that between 25 and 50ng (**Fig. 5, panel A**). According to a study on photophysical properties of fluorescent DNA-dyes (Cosa *et al*, 2001), dimers of SYBRGold that are formed by the dye are responsible for energy transfer and self-quenching. Dimers are also more frequent in single stranded DNA than double stranded DNA, and, therefore, more prone to undergo deactivation. Therefore, higher concentrations of SYBRGold may lead to a decreasing signal due to self-quenching.

Ribogreen<sup>®</sup> diluted 1/400 was found to be the most dependable dye in terms of sensitivity and accuracy across all concentrations tested with an  $R^2$  of 0.9899 as opposed to greater dilutions of Ribogreen<sup>®</sup>, which were not linear past 25ng ribosomal RNA (rRNA) (**Fig. 5, panel B**). In conclusion, SYBRGold diluted 1/2000 showed a strong linear relationship with an  $R^2 = 0.996$ ; however, the greatly reduced sensitivity compared to Ribogreen<sup>®</sup> diluted 1/400, indicated Ribogreen<sup>®</sup> as more effective for our assay.

Ribogreen<sup>®</sup> at 1/400 presented a linear relationship between RFU (relative fluorescent



units) and concentration of rRNA and a relatively high fluorescent signal and was thereafter employed as the reagent of choice for our assays. There may be other fluorescent dyes with better qualities, though time and financial constraints did not allow for an exhaustive search, and Ribogreen<sup>®</sup> is quite effective as is, in our assay.

### ***Optimal Assay Buffer Determination***

#### ***Buffer and Metal cofactor***

Due to the large number of assays performed to define the assay buffer, a summary of assays and outcomes are shown below. The initial buffers for my assay were based on the tailed template assay, which employed calf thymus RNAPII, and consisted of 30mM HEPES pH 7.8, 3mM magnesium chloride (MgCl<sub>2</sub>, for catalysis), 10mM Dithiothreitol (protein stability), and salt, based on Dr. Gnatt's previous assay for mammalian RNA polymerase II (Hu *et al.*, 2006). Buffer conditions were then modified

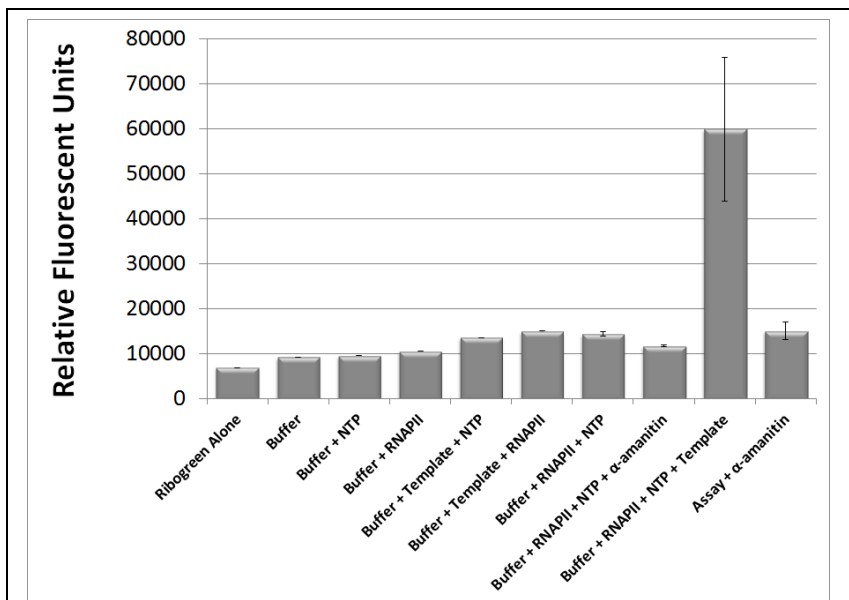
while testing various DNA templates for efficacy including: sonicated salmon sperm DNA from Invitrogen (catalogue # 15632-011), the Kool 45 base single stranded circular DNA template developed for the bacterial assay, or M13 single stranded bacteriophage DNA (6407 bases) from Biolabs (catalogue #N4040S) as a template. TRIS buffer with a pH of 7.8 replaced HEPES buffer with a pH of 7.8 without affecting the assay outcome.

I also replaced magnesium chloride with manganese chloride ( $\text{MnCl}_2$ ) because the latter has been shown to enhance RNA polymerase II activity. Although the mechanism is not completely understood, it is believed that manganese enhances RNAPII synthesis because it is a more reactive metal than magnesium. In this regard  $\text{MnCl}_2$  was previously shown to enable RNAPII nonspecific initiation on RNA, or single stranded DNA, dramatically enhancing the amount of RNA product (Sekimizu, *et al.* 1984). On the other hand, magnesium as co-factor, minimizes RNAPII nonspecific initiation from double stranded DNA, single stranded DNA or RNA.

### ***Contributing factors to the background noise of our assay***

Background fluorescent signals can dramatically reduce the signal to noise ratio and reliability of an assay. A two-fold increase in background likewise reduces the specific signal two-fold. A study of the contribution of reagents to our background appears in **figure 6**. Some items such as the nucleotides (NTP) did not contribute significantly to the background. The highest contribution to the background was the Ribogreen<sup>®</sup> fluorescent dye, as expected. Beyond the dye, the single stranded M13 DNA template and the RNAPII enzyme had the highest contribution to background

fluorescence. Relative Fluorescent Units (RFU) of the Ribogreen<sup>®</sup> alone and Ribogreen<sup>®</sup> with Buffer were approximately 6,934 and 9,832 respectively, out of a total of 60,000 RFU in the executed activity assay with all components including RNAPII. The M13 ssDNA template contributed approximately 3,993 additional RFUs to the background and RNAPII contributed approximately 3,670 (Fig. 6).



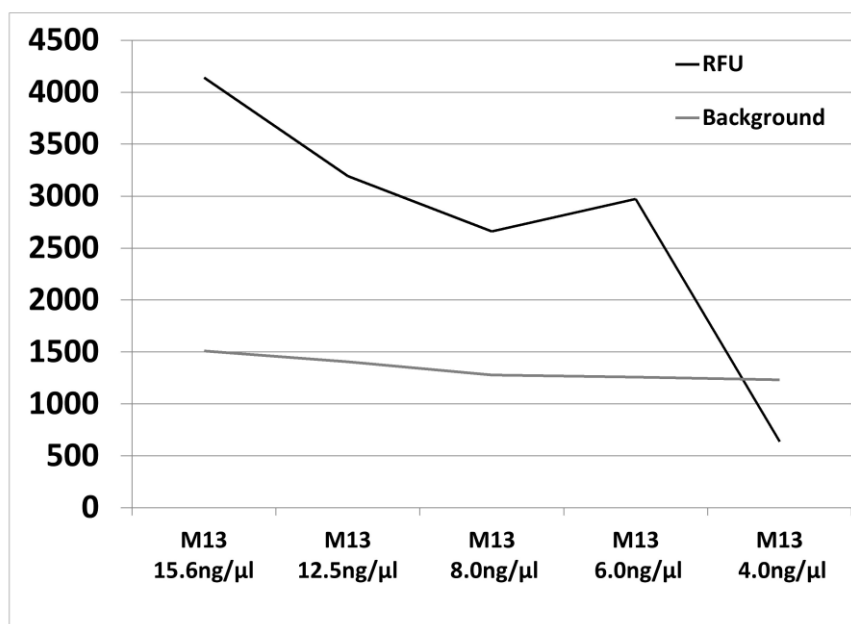
**Figure 6: Contribution of assay components to background fluorescence.** Graphs represent the mean calculated from 3 samples per assay. Error bars represent the standard error calculated from the mean and standard deviation. Experiment was repeated three times for accuracy. Incomplete reaction samples were made containing components of the assay mix separate or in combination. Buffers for RNAPII reaction were 30mM HEPES pH 7.7, 54mM Ammonium Sulfate, 3mM Mn with RNAPII 1.0  $\mu\text{g}/\mu\text{l}$  reaction. Samples were incubated for one hour at 37°C in a water bath. 7.5 $\mu\text{l}$  of each sample were added to a black 96-well plate and mixed with 200 $\mu\text{l}$  of 1/400 Ribogreen<sup>®</sup> and fluorescence was measured using the Biotek microplate reader. Assay was initially unstable with a p-value for reactions  $\pm\text{NTPs} = 0.09$ .

The contribution of RNAPII to the background, though slight, may indicate that some DNA may be associated with the enzyme throughout its purification, or that the RNAPII binds the dye. The final buffer concentrations after numerous trial-and-error optimization assays for high fluorescent signal with the lowest background signal were

finalized at 30mM TRIS pH 7.8; 3mM MnCl<sub>2</sub>; 50-60mM ammonium sulfate; and 1mM DTT. The signal-to-noise ratio for the commercial Kool template based assay with bacterial RNAP was 6.0 and our assay employing calf thymus RNAPII, our signal to noise ratio at this point was 3.6.

*DNA Template concentration was optimal at 12.5 ng M13 ssDNA/ $\mu$ l reaction.*

The background study indicated that M13 ssDNA contributed a degree of fluorescence to the background (**Fig. 6**). Optimizing the template concentration in our assay could increase signal and/or decrease background and was therefore pursued. Results indicated that by decreasing concentrations of M13 ssDNA the assay's overall signal was severely reduced with only a minimal decrease on the background noise (**Fig. 7**). However, increasing the M13 ssDNA concentration significantly increased activity. That said, the initial background fluorescence remained too high to yield an improved signal-to-noise ratio.



**Figure 7: Decreasing the M13 ssDNA template concentration decreased overall signal.**

Experiment was repeated three times for similar results. M13 concentrations of 15.6 ng/μl, 12.5 ng/μl, 8.0 ng/μl, 6.0 ng/μl, and 4.0 ng/μl were assayed. Each assay was performed  $\pm 500 \mu\text{M}$  NTPs. Buffers: 30mM HEPES pH 7.7, 54mM Ammonium Sulfate, 3mM Mn with RNAPII 1.0 μg/μl reaction. Samples were incubated for one hour at 37°C in a water bath. 2.5 μl were taken from each reaction sample and mixed with 200.0 μl of 1/400 Ribogreen in a black 96-well plate and read 5 minutes after labeling. Relative fluorescent units (RFUs) were determined by subtracting background fluorescence from the overall signal (reaction samples without NTP). Fluorescence was measured using the Biotek microplate reader.

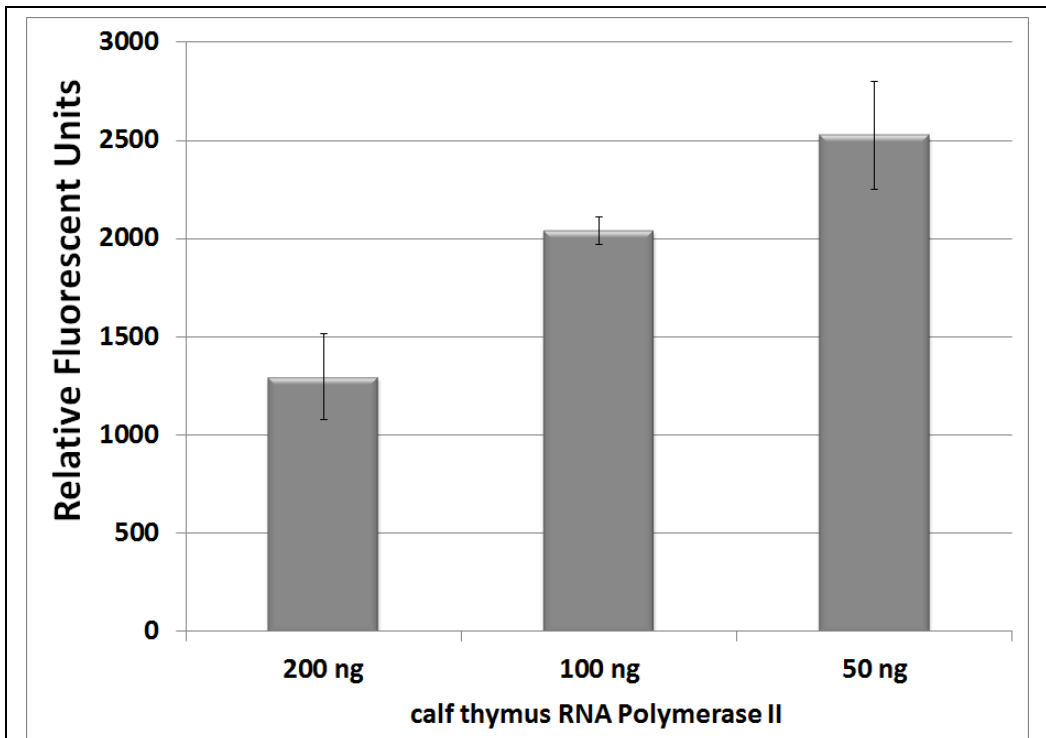
***The enzyme-to-template ratio significantly affects the fluorescent signal***

**Table 2** shows the molecular weight of the single stranded M13 DNA template and of the mammalian RNAPII enzyme. When comparing the molecular weights of the enzyme using an initial concentration of 1 µg/reaction, derived from the tailed template assay, and 15.6 ng/reaction M13ssDNA, it was found that there was 2,900-fold molar excess of RNAPII to M13 DNA. This suggests that 2,900 molecules of RNAPII would compete for a single M13 molecule. It would be unreasonable to assume that all 2,900 RNAPII molecules could bind to a single M13 molecule. Assuming about 120 Å of DNA will be in contact with each RNAPII (Gnatt *et al.*, 2001) and a rough length of each single stranded base as 3.7Å (Tinland *et al.*, 1997), 32 bases of DNA could be accommodated per RNAPII molecule with 2,900 RNAPII molecules accommodating 92,800 DNA bases, an equivalent of 14.4 M13 templates!

<b>Protein/DNA Template</b>	<b>Molecular Weight</b>	<b>Ratio of RNAP II:compound</b>	<b>Stock Concentration in 10µl reaction</b>
Mammalian RNA Polymerase II	55,000 D	---	500ng RNAPII = 50ng/ µl
M13mp18 Single-stranded DNA	21,000,000 D	1,820:1	12.5ng M13 ssDNA = 1.25ng/ µl
TFIIS	33,970 D	1:10	310 ng = 31 ng/ µl
TFIIF	78,000 D	1:10	710 ng = 71ng/µl

**Table 2: The molecular weights and ratios of RNAP II assay components.** The molecular weights of mammalian RNA polymerase II, single stranded M13 DNA template, TFIIS/TCEA1 and Gdown1 are shown (Flores et al., 1989; Hu et al., 2006; Cell Signaling Technology, Inc.). Also depicted is the ratio of RNAPII to ssDNA or transcription factor, and the concentration of stock solutions employed.

Surprisingly, decreasing the initial concentration of RNAPII from 200 ng to 50 ng increased the overall signal and therefore likely increasing effective RNAPII synthesis (**Fig. 8**). Therefore, enzyme-to-template ratio has critical effects on the reaction. The increase in activity observed with the decreased RNAPII/M13 DNA ratio may be due to the decreasing of contact between RNAPII molecules, freeing more molecules for transcription. Another possibility is that RNAPII overcrowding on the circular M13 template might prevent transcription as the enzymes mutually disrupt one another's movement



**Figure 8: Enzyme-to-template ratio significantly affects the fluorescent signal.** 200ng, 100ng, and 50ng of calf thymus RNAPII were measured in 5.0  $\mu$ l sample sizes. Graphs represent the mean calculated from 5 samples per assay. Error bars represent the standard error calculated from the mean and standard deviation. Experiment was repeated 5 times for accuracy. 12.5ng/ $\mu$ l of M13 ssDNA template concentration remained constant. Buffers :30mM HEPES pH 7.7, 54mM Ammonium Sulfate, and 3mM MnCl<sub>2</sub>. Each assay was performed  $\pm$ 500  $\mu$ M NTPs. Samples were incubated for two hours at 37°C. 5.0  $\mu$ l of reaction sample were added to a black 96-well plate and mixed with 200.0  $\mu$ l of 1/400 Ribogreen<sup>®</sup> relative fluorescent units (RFUs) were determined 5 minutes later with the VICTOR<sup>™</sup> microplate reader.

### *Determining the salt concentration*

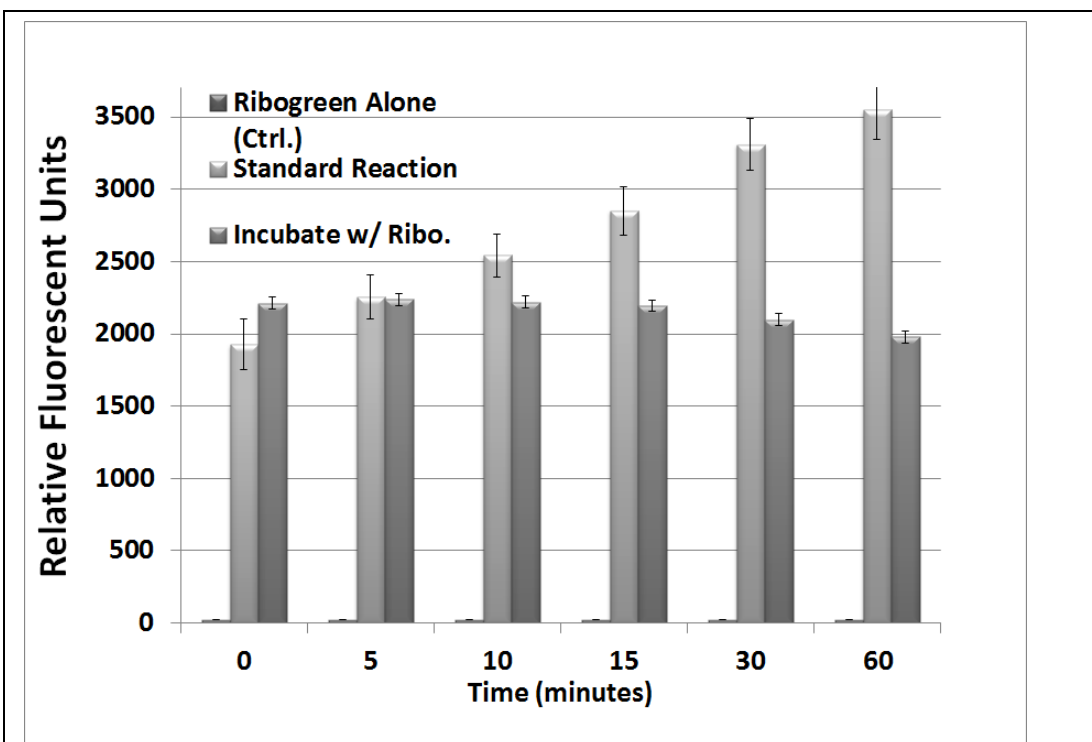
RNA polymerase II's optimal salt concentration was previously determined for the tailed template assay employing calf thymus RNAPII (Hu *et al.*, 2006) and ranges from 50mM to 60mM ammonium sulfate. Our fluorescent assay proved to have similar optimal ranges of ammonium sulfate, so that ammonium sulfate was added to each reaction to maintain this salt range. After changing the concentration of RNAPII and the buffers, the amount of ammonium sulfate added the reactions had to be re-calculated

ensure a 50-60mM overall salt concentration. Calculations were required after every change to enzyme or buffer and provided a slight improvement to the assay's signal-to-noise ratio, as opposed to assays whose salt concentration fell out of this range. (**data not shown**).

### ***Ribogreen<sup>®</sup> Time Curve***

Initially, calf thymus RNAPII assay employed a 12.5  $\mu$ l reaction volume incubated for an hour at 37°C in a water bath. After an hour, 7.5  $\mu$ l of reaction were added to a black 96 well plate and labeled with 200  $\mu$ l of 1/400 Ribogreen<sup>®</sup>. The mixture sat in the dark, undisturbed, for five minutes as suggested by the manufacturer, before measuring. An improved signal to noise ratio was achieved by both increasing the reaction time, and another increase in the signal to noise ratio was afforded by increasing the incubation time with Ribogreen. I found that the signal-to-noise ratio improved by extending the assay an additional hour for a total incubation period of two hours (**data not shown**). Since our assay is an endpoint assay, overall signal and the relative signal to noise rather than kinetics was our prime concern, especially if the assay will be used for high throughput screening of inhibitors of bacterial RNAP and mammalian RNAPII.

Another improvement of the signal to noise ratio was afforded by an increased incubation time of the dye with the reaction mix. Ribogren provided a signal that was linear across the range of time (**Fig. 9**), with a positive directly proportional relationship between relative fluorescent units and time after labeling. In essence, as the time of incubation with Ribogreen increased from five minutes to sixty minuts, so too did the signal, without affecting the background Ribogreen fluorescence at all.



**Figure 9: Ribogreen® Time Curve Assay.** Graphs represent the mean calculated from triplicate points. Error bars represent the standard error of the mean. Ribogreen alone - Ribogreen alone in buffer without reaction components. Standard reactions - consisted of 15.0  $\mu$ l of Ribogreen diluted 1/400 added to a black 384 well plate with 5.0  $\mu$ l of assay sample **after** a two-hour incubation period. Incubation with ribogreen proceeded for the duration listed in minutes listed in the graph. Incubate with Ribogreen - ribogreen was added before the two-hour assay incubation. Essentially, the assay took place in the presence of ribogreen with 5-10 minutes of incubation prior to reading at time 0 as listed in the image. Essentially, 10 minutes were permitted for RNA synthesis before the first reading at time 0. Fluorescence of all reactions was measured using the VICTOR™ microplate reader. Reaction samples contained 50ng/ $\mu$ l of RNAPII and 12.5ng/ $\mu$ l of single stranded M13 DNA template  $\pm$ 500  $\mu$ M NTPs. Relative fluorescent units (RFUs) were determined by subtracting background fluorescence (reaction samples without NTP) from the overall signal.

Additionally, **Figure 9** shows that Ribogreen can be added to the transcription reaction on its onset, so that RNAPII synthesis is not inhibited, or at least not fully inhibited by the Ribogreen. However, both the signal and signal to noise values decreased. Such an assay though, may have value for RNAPII kinetic assays and those that determine the effects of inhibitors or various substrates. In conclusion, the assay showed a significant increase in fluorescent signal with little added background fluorescence after two hours of incubation at 37°C and with an hour of incubation with the fluorescent dye.

#### ***Transfer to a Black-384 Well plate***

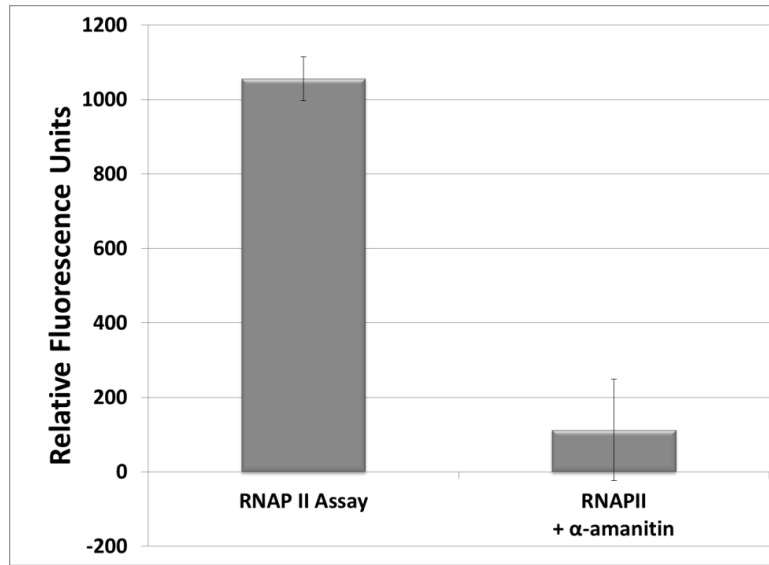
If the RNAPII fluorescent assay were to be used for screening compounds, the smaller wells and reaction volumes would allow for more reactions that could be performed at an overall decreased cost. Detection of activity in a 384 well should be preferred over the 96-well plates initially employed. To this aim the first attempt at transfer from a 96-well to a 384-well plate assay began with a Proxiplate, Black 384-well microplate by PerkinElmer (catalogue #6008260). The transfer was successful; however, repeated assays resulted in inconsistent signal-to-noise ratios – approximately one in four assays would result in a highly variable n signal-to-noise ratio. We attempted to use a white 384-well plate by Roche Diagnostics; however, the white wells increased background fluorescence detrimentally and overwhelmed the signal fluorescence as expected (**data not shown**). The Corning® Low Volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplates proved to be the most successful 384-well plate with little plate interference. An empty well in the Corning® 384 well plate, when measured

by a Tecan infinite M1000Pro (Serial #1109005812), gave an RFU signal of ~4; with 1/400 Ribogreen alone ~24 RFU; Ribogreen and buffers ~232 RFU and, a signal-to-noise ratio of 5.5 for the current optimized assay (**Fig. 10, panel A**). Considering the difference in assay stability for the different plates, it is likely that different plate chemistries likely affect proteins in our assays (**See Table 3**). The Corning® Low Volume 384 Well Black Flat Bottom Polystyrene NBS™ Microplate was used to measure assays after this point. Finally, The addition of 1 µg/µl of bovine serum albumin (BSA) increased the signal-to-noise to ~ 9 (**Fig. 10, panel B**). BSA likely enhanced the signal by enhancing the stability of RNAPII during incubation and RNA synthesis or by preventing nonspecific adsorption of reaction components to the plate.

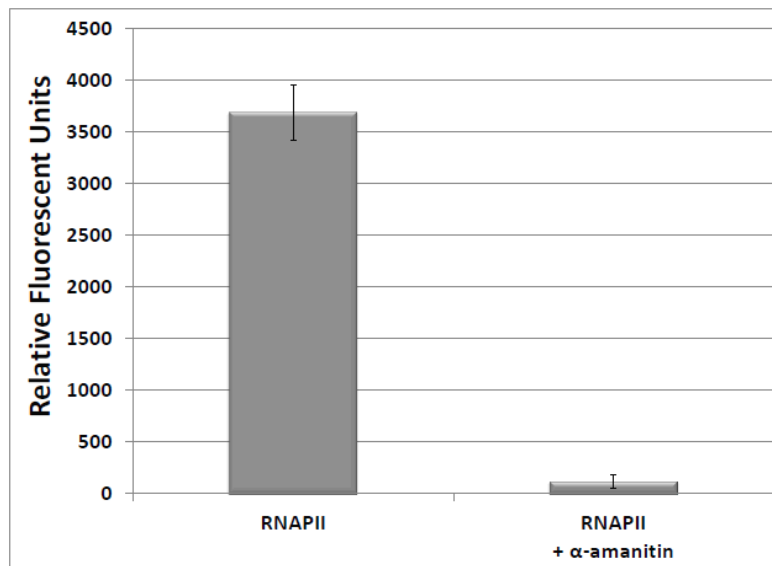
Plate Manufacturer	Signal-to-Noise	Plate Reader
PerkinElmer 96 Well plate	4.0	Biotek
White Corning 384 well plate	Negative	Tecan, Victor
Black Nunc 384 well plate	5.0	Tecan, Victor
Black Corning 384 Well plate	8.0	Tecan, Victor

**Table 3: Transfer from test tubes to 96-well plates to 384- well plates.** Reactions transferred to a Corning F-Bottom 384 Black well plate were the most accurate and had the highest signal-to-noise ratio.

**A.**



**B.**



**Figure 10: Transfer to a Corning® 384 black well plate.** Graphs represent the mean calculated from 4 samples. Error bars represent the standard error calculated from the mean and standard deviation. Both experiments were repeated 3 times for accuracy. RNAPII ± α-amanitin. Buffers: 30mM TRIS Buffer (pH 7.8), 58mM Ammonium Sulfate, 3mM MnCl<sub>2</sub> - with RNAPII 50.0 ng/μl reaction and 12.5μl M13ssDNA. After incubating for two hours, 5.0 μl assay samples ±NTPs were mixed with 15 μl of Ribogreen diluted 1/300 with Tris-EDTA buffer. After an additional hour after labeling samples were measured. Relative fluorescent units (RFUs) were determined by subtracting background fluorescence (reaction samples without NTP) from the overall signal. An hour after labeling, fluorescence was measured using the Tecan infinite M1000Pro microplate reader. **A.** Initial results after transfer to a Corning 384-black well plate. P-value=0.002 for RNAPII and RNAPII with alpha amanitin and a signal-to-noise ratio of 4.0-6.0±2.0 **B.** 1.0 μg/μl BSA was added to all reactions resulting in P-value= 0.000001 and signal-to-noise ratio of 8.0-10.

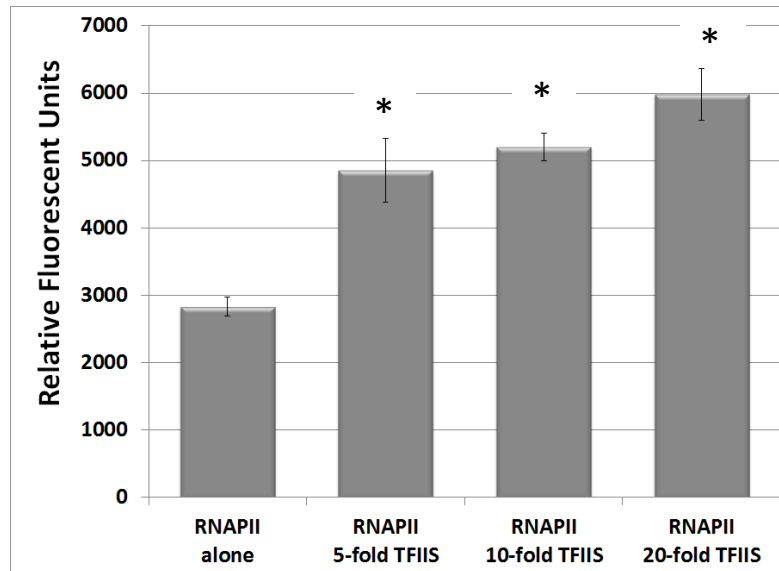
### ***Final optimized assay conditions***

The final assay conditions for optimal activity and minimal background fluorescence were as follows: 30mM TRIS pH 7.8; 3mM Manganese chloride; 50-60mM ammonium sulfate; 1mM DTT; 50ng/μl RNAPII; 12.5 ng/ μl single stranded M13 template (BioLabs, Cat#N4040S); 1.0 μg/μl BSA; 500 μM NTP (Fermentas #R0481). Reactions were incubated for two hours in a 37°C water bath. After two hours, 5.0μl of sample reaction were mixed with 15.0 μl of 1/300 Ribogreen<sup>®</sup> in a Corning<sup>®</sup> Low Volume 384 Well Black Flat Bottom Polystyrene NBS<sup>™</sup> Microplate. Once labeled, samples were left alone in the well, untouched, in a dark room for an hour before measurements were taken by the Tecan infinite M1000Pro for detection of RNA synthesis emissions using 480/520nm light (emission/detection).

### ***Wild-type TFIIS/TCEA1 enhances RNAPII activity***

Increasing concentrations of the human wildtype TFIIS/TCEA1 elongation factor increased the fluorescent signal and therefore RNA synthesis (**Fig. 11**). A 10-fold molar excess of TFIIS appeared to be sufficient to exhibit effects of TFIIS/TCEA1 on RNAPII; thus, a 10-fold molar excess was used for other transcription factors later employed in the assay. An increase in signal suggests wildtype TFIIS/TCEA1 enhances the activity of RNAPII. Studies have shown that TFIIS stimulates RNA polymerase read-through and facilitates the passage of RNAPII through various arrests sites by stimulating RNA transcript cleavage. (Sekimizu *et al.* 1976; Reinberg and Roeder 1987; 1994; Orphanides *et al.* 1996). Although it is evident that TFIIS/TCEA1 enhances transcription (**Fig. 11 & Fig. 12**), it is unclear whether TFIIS enhances the initiation phase, or elongation phase, or

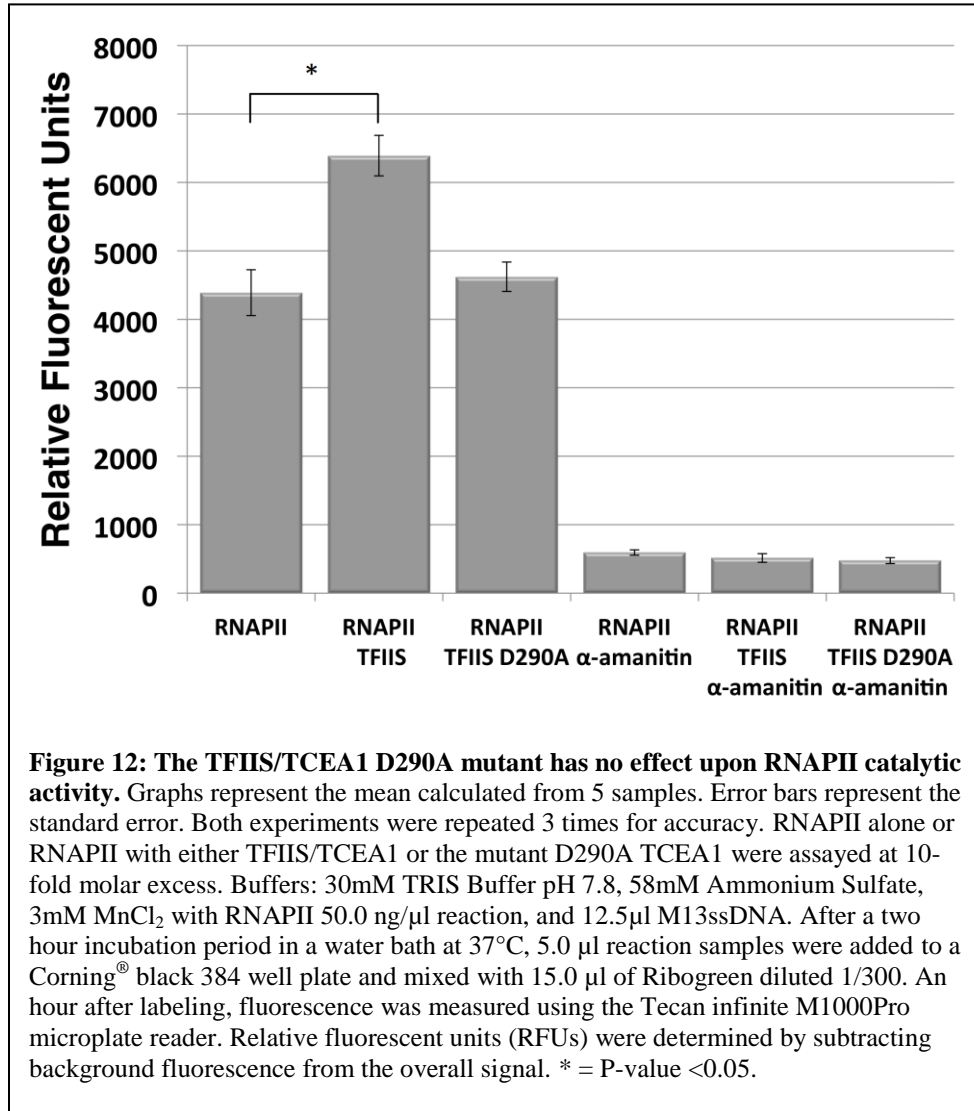
both, because my assay is non-specific (i.e. initiation does not need general transcription factors) and I have not developed a method to differentiate between initiation and elongation phases.



**Figure 11: Titrating TFIS/TCEA1 into the assay.** Graphs represent the mean calculated from 5 samples. Error bars represent the standard error calculated from the mean. Both experiments were repeated 3 times for accuracy. RNAPII alone or RNAPII with TFIS/TCEA1 at a 5-fold, 10-fold, or 20-fold molar excess. Buffers: TRIS Buffer pH 7.8, 58mM Ammonium Sulfate, 3mM MnCl<sub>2</sub> with RNAPII 50.0 ng/μl reaction, and 12.5μl M13ssDNA. After a two hour incubation period in a water bath at 37°C, 5.0 μl reaction samples were added to a Corning® black 384 well plate and mixed with 15.0 μl of Ribogreen diluted 1/300. An hour after labeling, fluorescence was measured using the Tecan infinite M1000Pro microplate reader. Relative fluorescent units (RFUs) were determined by subtracting background fluorescence from the overall signal. \* = p-value <0.05 (compared to RNAPII alone)

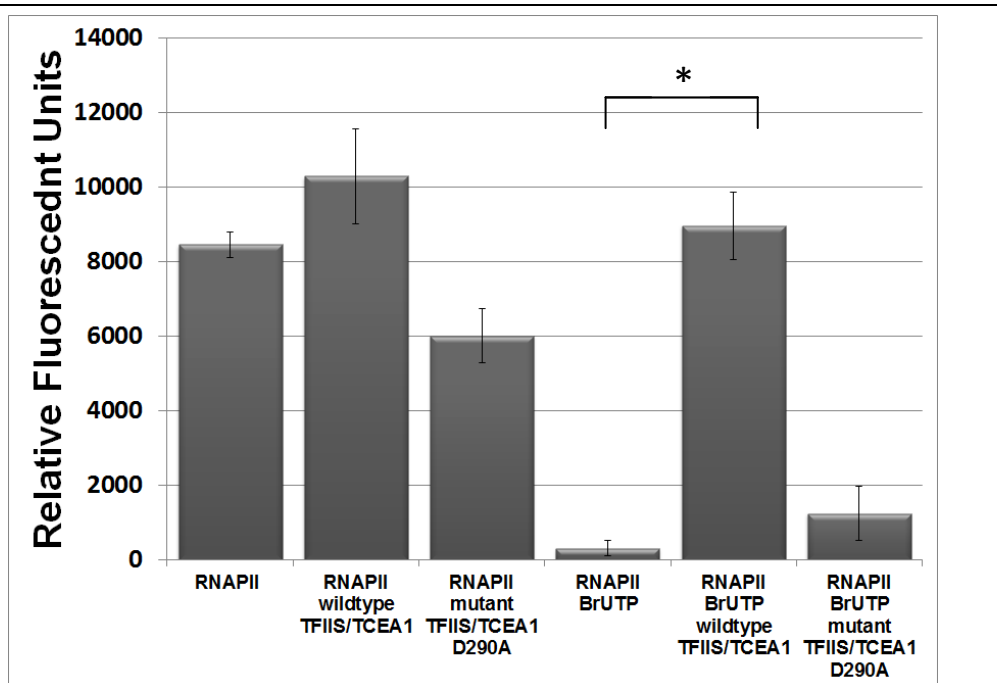
A TFIS/TCEA1 D290A mutant employed at a 10-fold molar excess had no significant effect on RNAPII activity (**Fig. 12**). This mutant contains an alanine in place of an aspartate at amino acid 290. The aspartic acid has been suggested to interact within the active RNAPII active site (Kettenberger *et. al.*, 2003). Controls containing 1.0 μg/μl of α-amanitin indicate that transcription was performed solely by RNAPII. A two-tailed t-test indicated that the activity of RNAPII and RNAPII with wild type TFIS are significantly different (p=0.006), whereas there was no significant difference between the

activities of RNAPII and RNAPII with mutant D290A TFIIS (p=0.94). Signal-to-Noise ratios of RNAPII, RNAPII with wildtype TFIIS/TCEA1, and RNAPII with TFIIS/TCEA1 D290A mutant were 12, 25, and 18 respectively.



### *An assay specific for TFIIS/TCEA1*

Incorporating 2mM 5-Bromouridine 5'-triphosphate sodium salt (BrUTP) into the assay inhibited RNAPII activity (**Figure 13**), by a mechanism which likely induces “arrested” transcription. “Arrest” sites block elongation and stall RNAPII, causing the enzyme to “backtrack” along the template. During arrested transcription, the 3' end of the RNA necessary for the addition of subsequent nucleotides is displaced from the catalytic site - prohibiting the continuation of elongation. The TFIIS elongation factor is known to “save” RNAPII by inducing TFIIS-dependent RNAPII cleavage of the protruding RNA, generating a correctly placed 3' hydroxyl group in the active site, thereby returning RNAPII to the transcriptionally active conformation (Malagon *et al.* 2004; Adelman, 2005). Previous evidence implies that the mutant TFIIS/TCEA1 D290A isoform does not enhance transcription (**Figure 12**), yet the mutant maintains its ability to bind RNAPII, since the binding of TFIIS to RNAPII depends on domain II, and is unaffected by the D290A mutation (Awrey *et al.*, 1998; Kettenberger *et al.*, 2003; Sigurdsson *et al.*, 2010). That said, the TFIIS/TCEA1 mutant is an engineered point mutation at D290A that renders TFIIS inactive (Hengartner *et al.* 1998) (**Figure 13**).

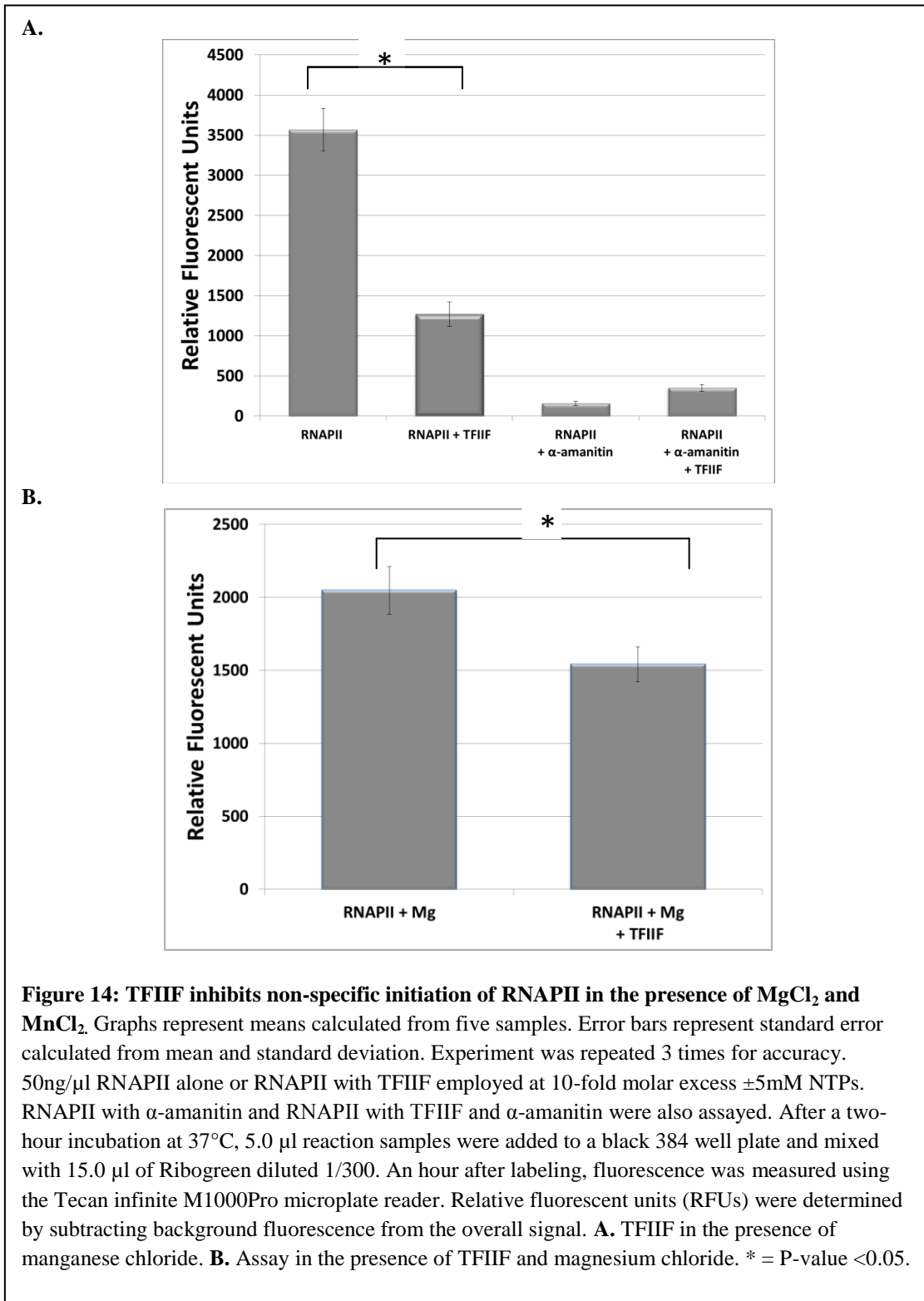


**Figure 13: The TFIIS/TCEA1 transcription elongation factor counteracts BrUTPs inhibitory effects. We now have an assay for TFIIS.** Graphs represent the mean calculated from 5 samples. Error bars represent the standard error calculated from the mean and standard deviation. Both experiments were repeated 3 times for accuracy. Controls: RNAPII alone, and with wildtype and mutant TFIIS/TCEA1. Nucleotide analogue, BrUTP, was incorporated at 500  $\mu$ M in place of 500  $\mu$ M UTPs to create transcription “arrest sites” that would inhibit RNA synthesis. RNAPII with BrUTP incorporated was assayed with wildtype and mutant TFIIS/TCEA1 at 10-fold molar excess. Buffers for RNAPII reaction were 30mM TRIS Buffer pH 7.8, 58mM Ammonium Sulfate, 3mM MnCl<sub>2</sub> with RNAPII 50.0 ng/ $\mu$ l reaction, and 12.5 $\mu$ l M13ssDNA. After a two hour incubation period in a water bath at 37°C, 5.0  $\mu$ l reaction samples were added to a Corning® black 384 well plate and mixed with 15.0  $\mu$ l of Ribogreen diluted 1/300. An hour after labeling, fluorescence was measured using the Tecan infinite M1000Pro microplate reader. Relative fluorescent units (RFUs) were determined by subtracting background fluorescence from the overall signal. \* = P-value <0.05.

### ***Wildtype TFIIF inhibits RNAPII activity***

TFIIF is one of the general transcription factors required for formation of the pre-initiation complex (Orphanides *et al.*, 1996) and TFIIF has been known to stimulate RNAPII elongation (Újvári and Luse, 2011; Cheng and Price, 2007). In our nonspecific initiation assays, specific initiation at a promotor would not come into play. Employing a 10-fold molar excess of heterologous expressed human TFIIF in our assays inhibited RNAPII activity by ~64 % (**Fig. 14, panel A**). This is in full accord with numerous studies wherein TFIIF appears to inhibit nonspecific binding of RNAPII to free DNA (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992; Tan *et al.*, 1995; Chen *et al.*, 2010). In this regard our single-stranded M13 ssDNA template is not normally recognized by mammalian RNAPII and TFIIF suppression of nonspecific initiation could have occurred.

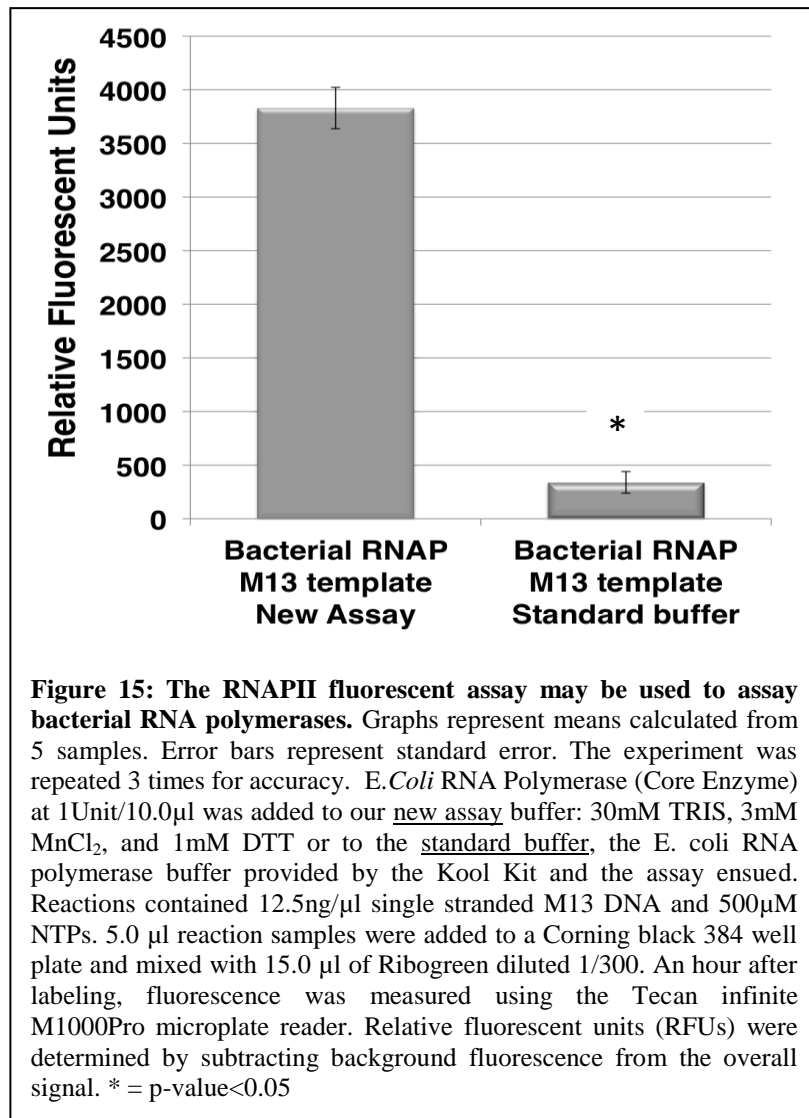
In order to rule out the possibility that manganese may have played a role in TFIIF inhibition of RNAPII activity as opposed to TFIIF enhancement of transcript elongation, TFIIF was assayed in the presence of magnesium chloride as well. Although TFIIF in the presence of magnesium appeared to show lesser effects, the assay continued to show inhibition of RNAPII activity (**Fig. 14, panel B**). However, we must note that RNAPII alone had severely reduced activity in the presence of magnesium because of the M13 ssDNA template. RNAPII in combination with TFIIF and magnesium showed a decrease in activity of ~25 %. In conclusion, my assay shows evidence that TFIIF inhibits transcription by RNAPII when employing nonspecific initiation, which I suggest is the result of TFIIF inhibition of nonspecific initiation.



***The RNAPII nonspecific initiation assay is also effective as is for bacterial RNAP***

Having a single reaction format for both bacterial and mammalian RNA polymerase enzymes would facilitate high throughput screening. Therefore, I employed *E. Coli* bacterial RNA polymerase core enzyme (holoenzyme) instead of mammalian RNA polymerase II in my finalized non-specific fluorescent RNAPII assay. Results indicate that my assay is capable of detecting *E. coli* bacterial RNAP (bRNAP) core holoenzyme activity

(Fig. 15). The data indicates a signal-to-noise ratio for bRNAP in my assay buffers with M13 ssDNA template of 6.3. Most interestingly the *E. coli* bRNAP appears to no longer be able to function with the M13 template using reagents provided by the Kool Kit<sup>TM</sup>. In essence, after many iterations and refinement of buffer,

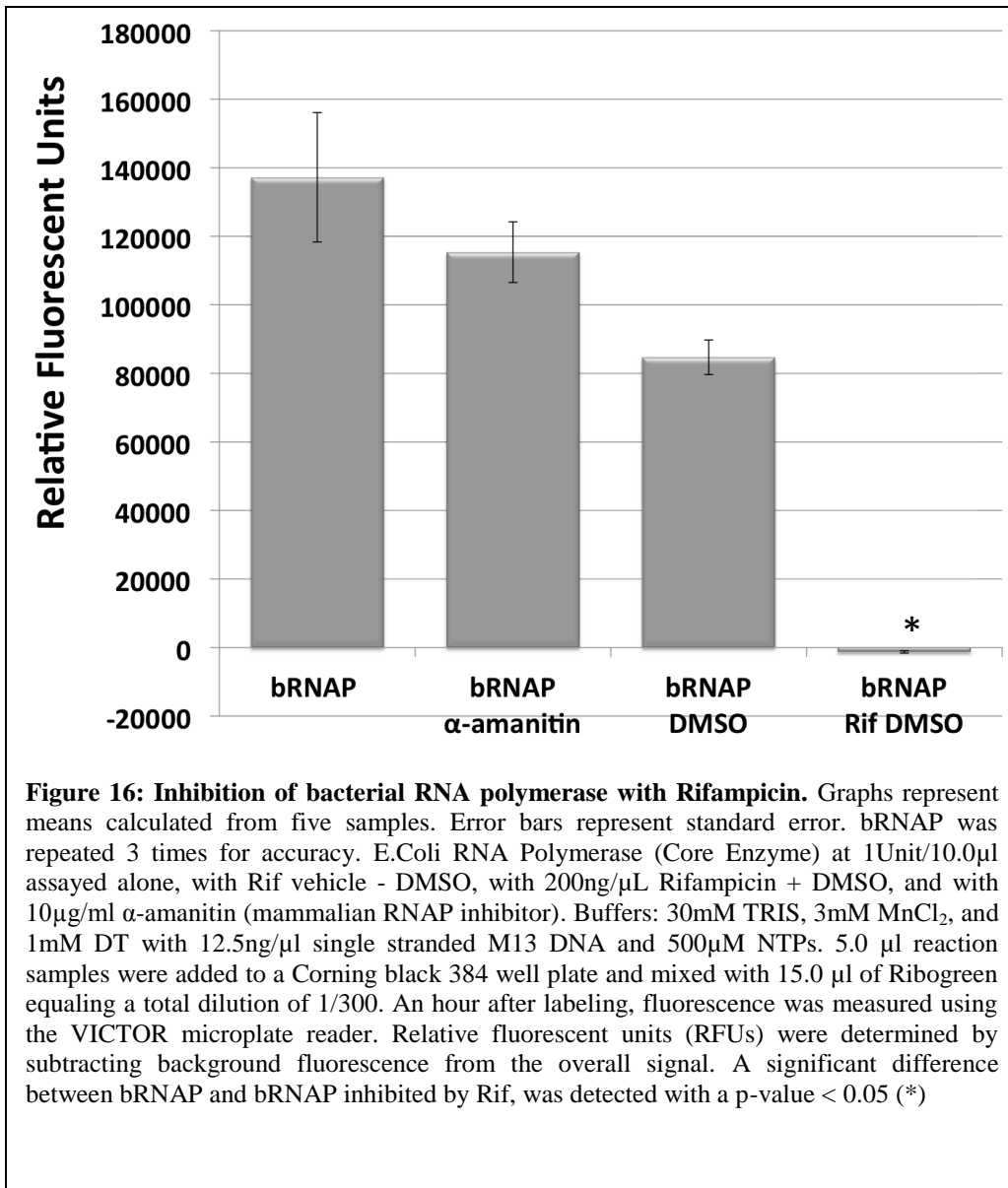


template, reaction time etc., our assay has evolved specifically for our template. The signal-to-noise ratio of *E. coli* bRNAP in the Kool buffers with the M13 ssDNA template

was less than 1.0 using conditions of my assay. In conclusion, bacterial RNA polymerase's activity may be successfully detected in my assay, without the need to change variables.

### ***Inhibition of bacterial RNAP with Rifampicin***

To show the specificity of my reaction and the commercially purchased bRNAP, the bacterial RNA polymerase inhibitor, Rifampicin (Rif) was shown to inhibit *E. coli* bRNAP using the assay buffers developed for the fluorescent mammalian (calf thymus) polymerase (**Fig. 16, panel A**). Signal-to-noise ratio of bRNAP assayed alone was approximately 9.0 and dropped to 0.0 with Rif present. The specific mammalian RNAPII inhibitor,  $\alpha$ -amanitin, had relatively no effect on bRNAP activity (signal to noise ratio ~ 8.0).



#### **IV. Conclusion**

##### *An effective assay for bacterial and mammalian RNA polymerases*

A non-specific RNAPII fluorescent assay for detection of transcribed RNA product was designed, developed, and validated. The developed fluorescent assay has shown to be reliable, effective, and highly sensitive in the detection of calf thymus RNAPII activity alone or in combination with various RNAPII transcription factors. The assay is also effective for the detection of bacterial RNA polymerase activity. The assay is suitable for high throughput screening and is safer to use than radioactive assays. It is more sensitive than colorimetric assays (ELISAs), which require 1.0-2.0 $\mu$ g of RNAPII. This assay only requires 10-50ng of RNAPII/ $\mu$ l. Reaction volumes can be as small as 2.5  $\mu$ l/reaction and the overall procedure requires 5-6 hours. Activity can be detected in 384 well plates.

A disadvantage for the assay is the inability to detect specific transcripts. In the future, this could be possible by using molecular beacons specific for M13 ssDNA transcripts. The assay is also unable to detect specific initiation at a promoter. To use this assay in future studies to detect specific initiation would require a new DNA template with a promoter, all General Transcription Factors and detection of specific transcripts. Again, detection of specific transcripts might be achievable with use of molecular beacons.

## ***Multiple applications for RNAPII fluorescent assay***

### ***1. As a tool to study the biochemistry of RNAPII associated factors***

This assay is simple to use, easy to replicate, more economical than other RNAPII assays, and a safer alternative to radioactive assays. I provide evidence on the effects of the transcription factors TFIIF and TFIIS, on RNAPII alone or in various combinations that are supported by recent publications. Although it is unclear which phase of transcription is being affected by the addition of transcription factors, based on the literature, TFIIF is likely to inhibit initiation (Conaway and Conaway, 1990; Killeen and Greenblatt, 1992; Tan *et al.*, 1995; Chen *et al.*, 2010), and TFIIS likely affects elongation (Sekimizu *et al.* 1976; Reinberg and Roeder 1987; 1994; Orphanides *et al.* 1996). All transcription assays must entail a stage of initiation. It is apparent that the developed RNAPII fluorescent assay exhibits activity in both the initiation and elongation phase. The assay may be adapted and capable of differentiating between the initiation and elongation phase in the future.

### ***2. Potential uses in discovering new cancer therapeutics***

This assay may be used for the development of anticancer agents. Previous studies have shown that the transcription elongation factor, TFIIS may be a viable target for cancer therapy (Hubbard *et al.*, 2008; Shema *et al.*, 2011). Knockdown of TFIIS by RNA silencing has been shown to inhibit cell proliferation and induce apoptosis in breast cancer cell lines. (Hubbard *et al.*, 2008). With little modification, this assay would likely be capable of high throughput screening using the developed TFIIS specific assay with RNAPII and BrUTP. This assay could potentially be used to screen other associated

transcription factors for the disclosure of drugs and/or inhibitors for the treatment of cancer.

### ***3. Potential to discover new anti-bacterial polymerase drugs***

The assay also has potential for use in high throughput screening (HTS) with bacterial RNAP for disclosure of inhibitors against specific strains of bacteria. Indeed, the assay effectively detects effects of rifampicin, an approved drug for the use of tuberculosis, on bRNAP. HTS for antibiotic development would involve an initial screening of chemical libraries for inhibition of pathogen RNAP, and a second screen to rule out inhibition of mammalian RNAPII. Toxicological follow-up on mammalian cells in culture, and animal models would then be called for, to determine values for lethal dose and maximal tolerated dose. Development of inhibitors to any pathogen would be possible as long as sufficient amounts of its RNAP can be purified.

### ***4. Future Directions: RNAPII fluorescent assay as a tool***

There are many more possibilities for future studies involving this assay that would be beneficial for the study of transcription regulation, cancer therapeutics, and exploring new antibiotics. One could use this assay to examine the influence of other transcription elongation factors such as ELL, Elongin pTEFb, and NELF. Another use of the assay could involve testing of nucleotide analogues for effects upon RNAPII. Some such analogues are used to inhibit viral polymerases or for cancer therapy. Toxicological information could thereby be generated. The assay would be capable of studying effects

of transcription that initiates at a promoter (normal cell regulation). That would require the use of templates that contain promoter sequences and the addition of all the general initiation factors that make up the pre-initiation complex (PIC) – assaying each GTF with RNAPII and assaying effects of the sequential addition of each GTF to further the understanding of RNAPII dependence on the GTFs and their direct effects on the regulation of transcription.

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