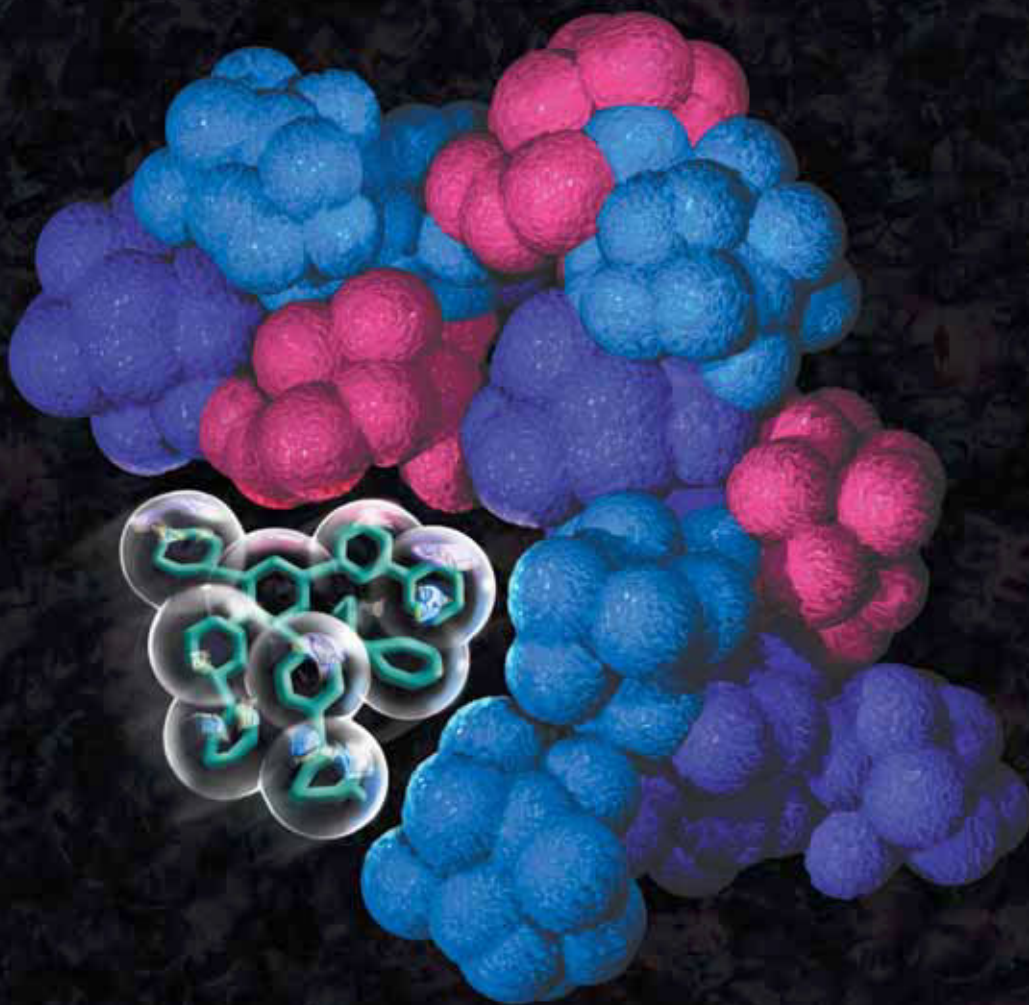


CHAPTER

# 5

**About the Image:**

*This illustration represents a small organic molecule, for instance one produced by combinatorial chemistry techniques, that is being screened for its fit to the larger protein molecule. The inhibition of function of the protein molecule upon attachment by the organic molecule would signify the identification of a successful drug candidate or target.*



**Chapter Five: Screening Applications**

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**Introduction**

Pioneering work leading to the elucidation of the genetic code and the characterization of the protein synthesis machinery relied heavily upon in vitro transcription and translation systems. Such systems allow the use of defined templates to direct the synthesis of proteins and permit the direct analysis of the transcription and translation machinery. The emergence of combinatorial chemistry techniques and the need for new antibiotics to overcome increasing bacterial resistance has brought the basic tool of in vitro translation systems back into the spotlight. Both prokaryotic and eukaryotic in vitro translation systems have found great utility in efforts to screen organic compounds for inhibition of the basic cellular functions of transcription and translation, common targets for antibiotic compounds (Figure 1).

In vitro transcription and translation systems can provide some advantages over in vivo systems for screening purposes. In vitro systems allow exact manipulation of compound concentrations. This is an important parameter when evaluating the potential potency of the lead compound. There is no need for cellular uptake to evaluate the effect of the compounds. While uptake evaluation is important for determining the eventual efficacy of the drug, it can unnecessarily eliminate valuable lead compounds in an initial screen. The interpretation of results in living cells is complicated by the large number of intertwined biochemical pathways and the ever-changing landscape of the growing cell. In vitro systems allow the dissection of effects in a static system for simpler interpretation of results and the ability to specifically monitor individual processes such as transcription or translation. Individual targets not normally present, or found at low concentrations, can be added in controlled amounts. Beyond the basic transcription and translation machinery found in the extracts themselves, target proteins for screening can be generated from genetic material by simple in vitro expression from added DNA templates. In short, in vitro transcription and translation systems provide a flexible and consistent tool for screening.

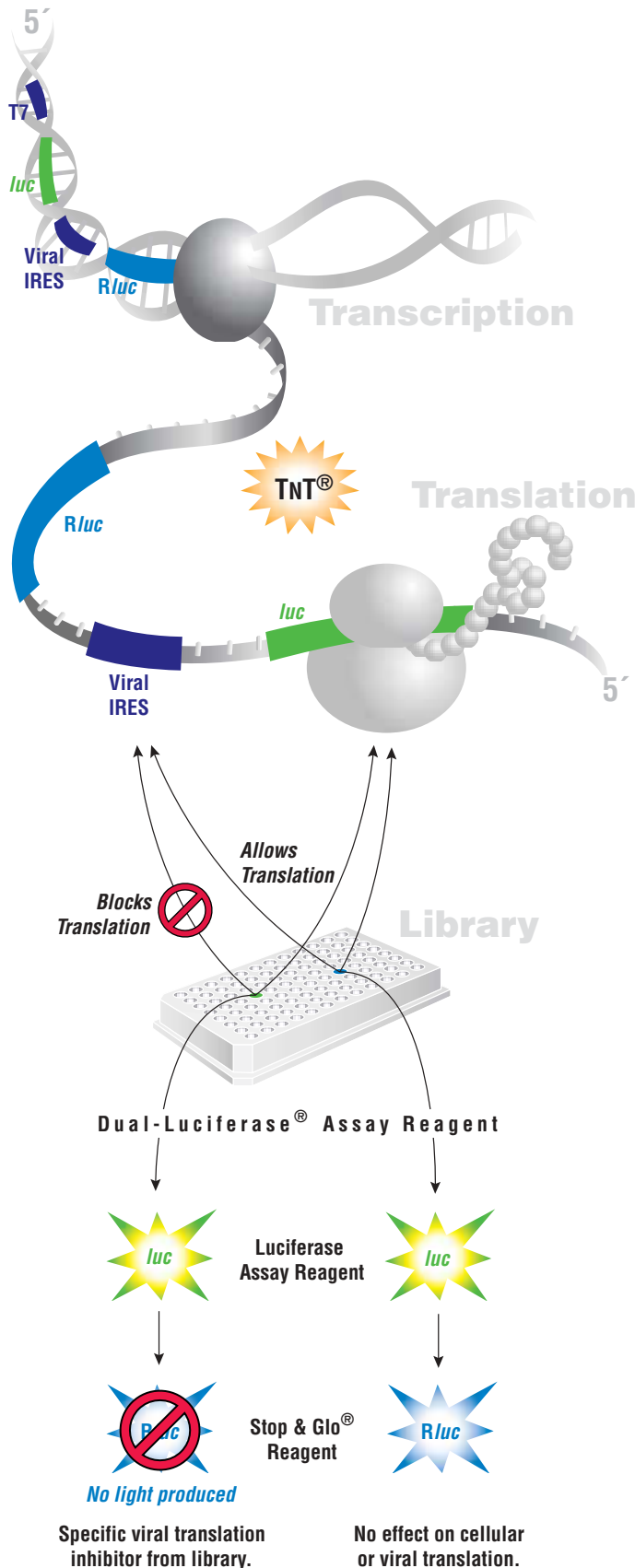
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**Figure 1. In vitro expression screening of a small molecule library for compounds that specifically inhibit virus-directed translation, using a Dual-Luciferase® readout assay.**

The diagram depicts transcription and translation of the firefly luciferase gene (*luc*) containing normal cellular translation signals (5' untranslated region and Kozak initiation sequence) followed by the *Renilla* luciferase gene (*Rluc*) directed by a viral translation initiating sequence (IRES, internal ribosome entry site). Potential small molecule inhibitors are tested for the ability to specifically inhibit the viral-based translation of the *Rluc*, while not affecting the normal cellular translation of *luc*. The Luciferase Assay Reagent specifically produces light from the firefly *luc*, followed by the addition of Stop & Glo® Reagent, which immediately turns off enzymatic light production from *luc* and activates expression from *Rluc*. This dual-reporter assay is very powerful for detecting viral specific inhibitors that do not affect normal cellular translation.

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## Monitoring and Detection

By far the most common means of monitoring protein synthesis in the standard analysis of translation reactions is by the incorporation of radiolabeled amino acids. Typically, <sup>35</sup>S-labeled methionine or <sup>14</sup>C-labeled leucine is added to the reaction. Only newly synthesized proteins incorporate the radiolabel, allowing specific detection of the template-encoded polypeptides. The radiolabeled proteins can be visualized by autoradiography after electrophoresis or isolated by immunoprecipitation. While such detection methods are valuable for analysis of gene products, they are not readily adaptable to high-throughput screening methods. Capture methods using antibodies or other receptor proteins can be employed, but the difficulties in working with and disposing of radioactivity still remain. Reporter systems widely used in gene expression offer a more tenable solution for screening efforts.

The in vitro transcription/translation systems usually generate proteins that are correctly folded and retain their enzymatic activity. This enzymatic activity can be directly assayed as long as the extract proteins and buffers do not interfere. The level of synthesis of a reporter protein is directly dependent on the activity of the transcription and translation system in the extract. Therefore, by simply determining the increased levels of these easily assayed proteins one can determine the relative protein expression activity of the extract. The individual transcription and translation components can be assayed through the choice of template. For evaluating the effect on translation, mRNA encoding the reporter gene can be added directly to the reaction. An example of this

method of screening is using RRL and luciferase to screen environmental samples for trichothecene mycotoxins, potent translation inhibitors (1). This reaction differs from transcription, which uses a DNA template encoding the reporter gene. Transcription can be evaluated further by comparison of expression from phage RNA polymerase promoters versus the endogenous RNA polymerase found in the S30 extract.

## Model Enzyme Systems

A number of simple enzymatic assays have been used in transcription/translation reactions including  $\beta$ -galactosidase,  $\beta$ -lactamase, galactokinase, chloramphenicol acetyltransferase, luciferase, and alkaline phosphatase.  $\beta$ -galactosidase is an excellent reporter gene for screening of in vitro transcription/translation extracts. Enzyme activity is typically determined by addition of ONPG, a colorimetric substrate, which gives measurable readings after a short incubation.

Luciferase is another common reporter for in vitro systems. Even very small amounts of functional luciferase can be measured in a luminescent assay, allowing sensitive detection of protein synthesis. Both firefly and *Renilla* luciferase enzymes can be expressed simultaneously in the in vitro transcription/translation reactions and measured using different substrates (Dual-Luciferase<sup>®</sup> Reporter Assay Systems<sup>(a,i)</sup>, Cat.# E1910, E1960, E1980). In this way, both proteins can be expressed and measured in the same reaction to allow for internal standardization and controls. This simultaneous expression can be exploited to immediately determine whether a compound is acting as an inhibitor of transcription or translation.

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Transcriptional activity can also be measured directly through the use of a nucleotide analog that releases a fluorophore upon incorporation into RNA.

Individual reactions require 0.1–1 µg of DNA template and typically produce 50–200 ng of protein from that template in a 30–60-minute reaction. This represents only a small percentage of the total protein present, and the potential for interfering contaminating activities is always a concern. Controls should always be employed to avoid potential problems. The S30 extract is tolerant of reasonably high levels of organic solvents such as DMF and DMSO, which are commonly used to dissolve organic compounds. They are, however, sensitive to alcohols and these should be avoided as solvents.

In vitro systems are valuable tools for studying transcriptional and translational machinery. They are easy to use, and both reactions and assays are readily adaptable to automation. The reactions and assays are rapid, allowing thousands of compounds to be evaluated in a screen. Their in vitro nature makes them more easily reproducible than in vivo systems. They are also flexible and allow exact additions and combinations of compounds. These properties make them important additions to the screening repertoire.

### Development of Antiviral Agents

The rapid reticulocyte coupled transcription/translation screening system has been applied to the identification of antisense oligodeoxynucleotides capable of inhibiting hepatic D viral replication (2). Other groups developing specific viral inhibitors have utilized in vitro expression to test different target regions with modified

oligonucleotides (peptide nucleic acid and phosphorothioate oligonucleotides; 3,4). Additional studies have shown the circular 2'-deoxyribo-oligonucleotides to be potent inhibitors of luciferase expression in an in vitro coupled transcription/translation system (5).

### Antisense Regulation

Antisense oligonucleotides have been shown to inhibit gene expression at either the transcriptional or translational level (6). The mechanism for translational inhibition is thought to involve activation of RNase and is currently a target of intense investigation. Attempts to rationally design effective antisense RNAs have yet to yield consistent results, giving rise to the use of an empirical approach using randomized oligonucleotide arrays to identify ideal candidate antisense oligonucleotides (7,8). An in vitro reticulocyte lysate coupled transcription/translation system has been established for rapid screening of antisense oligodeoxyribonucleotides to determine which are the most effective in arresting mRNA translation (2). The potential for heteroduplex formation of an oligonucleotide has been shown to correlate closely with inhibition of translation in vitro.

### Non-Isotopic Detection

Charged tRNA complexes containing epsilon-modified lysine, such as biotinylated lysines, can be added to the translation reactions and the modified lysines will be incorporated into the synthesized protein. Biotinylated proteins can be detected non-isotopically using streptavidin-alkaline phosphatase or horseradish peroxidase conjugates (9,10); these detection systems became commercially available in 1993 (11).

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Recently, a method of sensitive nonisotopic fluorescence-based detection of nascent proteins directly in polyacrylamide gels has been reported (12). Nanogram levels of nascent proteins with incorporated BODIPY<sup>®</sup>-FL labeled methionine can be detected by laser-based fluorescent gel scanner or even by a conventional UV transilluminator (12). In addition, Promega recently introduced a non-isotopic fluorescence-based detection product, the FluoroTect<sup>™</sup> Green<sub>Lys</sub> in vitro Translation Labeling System (Cat.# L5001).

## Ultra-Sensitive DNA Detection and Immunoassays

A highly sensitive immunoassay, known as the ultrasensitive expression immunoassay (USEI) was developed based on a "solid-phase" coupled transcription/translation system described by Christopoulos and Chiu (13). A DNA fragment encoding firefly luciferase is biotinylated and complexed with streptavidin. Biotinylated specific antibodies are used to quantify antigen immobilized on microtiter wells. After completion of the immunoreaction, streptavidin-DNA complex is bound to the immunocomplex. Subsequent expression of the solid phase-bound DNA by a coupled transcription/translation reaction produces luciferase. As few as 3,000 molecules of DNA label can detect a minimum of 50,000 antigen molecules. The luminescence is a linear function of the number of antigen molecules in a range extending over 3 orders of magnitude. The high sensitivity achieved results from the combined amplification due to transcription/ translation and the substrate turnover.

The same investigators developed a similar approach for DNA detection (14). This system is also very sensitive due to the coupled transcription/translation system. The resulting luminescence is linearly related to target DNA levels for between 5 and 5,000amol. Because the assay is performed in microtiter wells and avoids membrane hybridization and blotting, it can be adapted to automatable HTS detection. Recently this approach has been expanded to allow simultaneous detection of the target sequences using co-expression/determination of firefly and *Renilla* luciferases (15).

## Expression Immunoassay

Most recently work by Chiu and Christopoulos (16) has extended the use of the coupled in vitro transcription/translation systems to a "sandwich-type" expression immunoassay, using as a label an expressible DNA fragment encoding firefly luciferase. The DNA label contains a T7 RNA polymerase promoter, a firefly luciferase-encoding sequence and a poly(dA/dT) tail. The 3'-end of the DNA label is biotinylated and complexed with streptavidin. A sandwich immunoassay can then be generated using a coating antibody to a specific antigen, biotinylated polyclonal Ab to the same antigen and the streptavidin-luciferase coding DNA complex. The bound DNA complex label is expressed in vitro by coupled transcription and translation.

The luciferase generated is measured using a standard bioluminescent luciferase assay. The authors used this technique to successfully detect prostate-specific antigen (PSA) in human serum.

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