BIOAGENT DETECTOR PROPOSAL EXCERPT
(TO ACT AS TEMPORARY WHITE PAPER)

We propose to demonstrate a comprehensive environmental pathogen detection system which involves the crude chemical treatment of sample, the specific stabilization of the pathogen nucleic acid, the degradation of non-pathogen nucleic acids in the background and the fail-safe readout of stabilized pathogen nucleic acid copies at low copy number. We will accomplish this by utilizing an early stage detection technology, molecular locks, to provide sequence-specific pathogen nucleic acid detection. The proposed pathogen detection technology utilizes separate, specific, binding assemblies to nucleic acid targets, target binding assemblies (TBAs or molecular locks) which independently directly stabilize, immobilize and carry the readout molecules. The TBAs will be made to bind to adjacent double-stranded nucleic acid sequences of the pathogen under conditions which degrade non-pathogen nucleic acids. A signal amplification polymer provides for fail-safe detection of pathogen nucleic acid immobilization by containing a large number of fluorophores which can be effectively read as a bar-code. Each bar-code is specific for a particular pathogen. If it is not necessary to count copies but simply to know if the agent is present, a simple color change indicator can be placed on one or more molecular locks, eliminating the need for a laser read-out. The focus of the proposed work is to demonstrate this detection system for the identification of [redacted] and other targets of interest from crude samples. The system described above is depicted in attached figures 1 and 2.

Technical POC: Susan A. Weininger

EXECUTIVE SUMMARY

Bioagent Detector System Utilizing Molecular Locks and Multiple (Color, Booster) Readout

We propose to develop and demonstrate a comprehensive environmental pathogen detection system which involves the crude chemical treatment of sample, the specific stabilization of the pathogen nucleic acid, the degradation of background, non-pathogen nucleic acids and the fail-safe readout of stabilized pathogen nucleic acid copies at low copy number. The proposed pathogen nucleic acid stabilization and immobilization technology utilizes separate, specific, nucleic acid binding assemblies, target binding assemblies (TBAs or molecular locks), which independently directly stabilize, immobilize and carry the readout molecules. The TBAs will be made to bind to adjacent double-stranded nucleic acid sequences of the pathogen under conditions which degrade non-pathogen nucleic acids. A signal amplification polymer provides for fail-safe detection of pathogen nucleic acid immobilization by containing a large number of fluorophores which can be effectively read as a bar-code which is specific for the particular pathogen. The focus of the proposed work is to demonstrate this detection system for the identification of [redacted] from environmental samples. The proposed system will have the following features:

- fast, direct pathogen engagement (no hybridization of nucleic probes in system),
- immediate stabilization of the pathogen nucleic acid in a crudely lysed sample,
- degradation of the non-pathogen background nucleic acids,
- high fidelity and sensitivity of molecular locks,
- scaleable, inexpensive components,
- readily adaptable to new pathogens,
- ability to simultaneously read multiple pathogens,
- format independence,
- chemically and thermally hardened components, and
- easily diversifiable into a small number of stabilization, recognition and readout molecules leading to a “covering” set for new pathogen detection.

Pathogen stabilization and immobilization is accomplished by utilizing molecular locks. The use of the molecular lock does not require that the double-stranded pathogen is denatured in order to engage a nucleic acid probe or that a nucleic acid probe engage the pathogen. The following locks will be made: 1) two capping and stabilizing molecules which bind on either side of the pathogen immobilization and readout sequences, 2) a lock which secures the pathogen and immobilizes it to a solid support or bead, and 3) a lock which carries a bar-coded signal amplification polymer which can be read out. Each molecular lock has at least two protein binding domains which recognize adjacent areas of the pathogen nucleic acid. The molecular locks have high fidelity for their pathogens sequences. Unanchored, unattached lock components will be introduced early in sample preparation to stabilize and prevent nuclease degradation of the pathogen immobilization and readout nucleic acid specific

sequences. Nucleases are added which are pre-screened so that they do not cut within the immobilization or readout binding sequences but do degrade non-pathogen nucleic acids. Molecular locks can be also be used to monitor the pathogen changes or modifications and to provide new test development information.

One of the strengths of the proposed approach is that the pathogen can be stabilized immediately after the sample is sonicated or otherwise disrupted to produce a crude preparation. This is desirable as nucleases are present in crude preparation and the pathogen copy number may be extremely low. Most other detection methods for nucleic acids, including branch DNA (bDNA) and PCR, require that the nucleic acid to be detected is single stranded in order that it be engaged by a single stranded probe. For low copy number pathogens, this results in accelerated degradation and loss of target. In our system, the addition of the stabilizing molecular locks provides for the immediate stabilization of the low copy number pathogens. Since the stabilizing locks are smaller than the nucleic acids fragments in the crude preparation, the relatively smaller stabilizing molecular locks can diffuse to their pathogen target sequences faster and in higher concentration than the pathogen can diffuse to the immobilization lock on the solid support or bead. This provides for protection of the pathogen in the short time that before it is immobilized and read. For detection of pathogen RNA for a live /dead assessment, a DNA nucleic acid strand complimentary to the pathogen would need to be introduced and the stabilizing lock added which binds to the DNA/RNA hybrid.

The fail-safe detection of the pathogen engagement will be accomplished utilizing bar-coded signal amplification polymers called boosters. The boosters are assembled from short nucleic acid segments which are labeled and stabilized. The method of assembly results in a specific ratio of incorporated labels which provides the basis for the bar-code. These bar-coded boosters can be chemically ligated to specific molecular locks allowing specific-readout of the pathogens. The process of lengthening the bar-coded molecules is linear and the bar-coded molecules can be readily made which contain several thousand fluorophores. The readout of the boosters to be used in conjunction with this system is a ratio of signals from fluorophores in the booster. This makes it possible to confirm that the signal seen is from the pathogen nucleic acid independent of any noise in the system.

The engagement of the pathogen and the detection of the engagement is a sandwich assay and therefore lends itself to adaptation into a number of formats. The immobilizing lock can be ligated to a bead or solid support, such as a chip. The crudely disrupted sample can be passed as a thin film over a surface containing immobilized molecular lock. The surface molecular lock immobilizes the pathogen. High sensitivity can be achieved by devices with a large surface to volume ratio. This strategy is responsible in vivo for the high transfer rates of molecules in the nose, gut and lungs. The booster molecular lock which is carrying a bar-coded booster allows the readout of the immobilized pathogen. This immobilization and readout technology may be adapted so that it runs on a solid support, in a dip stick device, in a microfluidics device and in the magnetic resistance cantilever device developed at

NRL. A further refinement would be to create a cycling system to reduce the number of consumable reagents used and the majority of the reagents are recycled rather than replenished for a period of time.

A schematic of the environmental pathogen detection system is shown in figure 1 and 2. The sample is crudely lysed and the pathogen target [redacted] is released. The DNA pathogen is immediately stabilized by proteins which bind specifically to sequences within the target pathogen and stabilize it against degradation. If the RNA of a pathogen is to be detected, RNA of the pathogen must be immediately picked up by a bead and stabilized by adding a DNA fragment which is complimentary to the RNA and stabilizing TBAs for the complex. Mounted on a bead is a dimer comprised of two TBA monomers, which are made to bind tightly and specifically to sequences within the pathogen duplex or the DNA/RNA hybrid and to compete off the stabilizing molecules. Once immobilized, the pathogen DNA or DNA/RNA hybrid can be engaged by a third TBA domain which carries either a bead or a bar-coded signal amplification polymer. The bead can be sensed by the NRL cantilever device and the bar-coded signal amplification polymer or color change indicator can be read by a number of machines in a number of formats.

Description of TBA Binding Competition and Displacement

The biophysical basis of multiple domain protein binding to DNA is somewhat counter-intuitive. Consider a wild-type protein (WT in figure 3) which has a 10^{-8} binding affinity. Experience has shown that almost any change in the non-structural and non-functional portion of the wild-type protein will result in a decrease in its binding affinity. This process of decreasing the binding affinity of a protein is called down-shifting. A typical down-shifted version of a protein (WTD in figure 3) may have a binding affinity of 10^{-6}. When two down-shifted protein domains are combined together by a molecular chaperon, such as the cro protein, their combined binding affinity is increased to 10^{-12}. The increased binding affinity of the construct is higher because as long as one domain holds the construct close to the DNA then the probability that the second domain will bind to the DNA is increased. The time that the lock is in contact with the DNA is longer and the binding affinity is therefore higher. If the underlying DNA sequence specificity of the second domain is not correct then only the first domain will bind with a relatively weak binding affinity. If a wild-type protein domain is already bound specifically to the DNA and the sequence specificity of the adjacent DNA does not match the sequence specificity of the second domain then the two-domain construct formed, the target binding assembly (TBA), will not displace the wild-type protein bound to the first DNA sequence. If the underlying DNA sequence specificity for both domains is correct then both down-shifted domains in the construct will together bind with an affinity that is approximately the product of the individual binding affinities. As the TBA binds with high affinity, the individual wild-type domains are displaced. The binding competition, TBA and the stabilizing molecules is shown in figure 4. The design of the TBA allows specific targets to be selected which contain

components found elsewhere in the genome (e.g. control regions in pathogens are comprised of subsets of sequences found elsewhere). The TBA targets the whole promoter and has tight binding only to the promoter and not to the subsets of sequences found in other contexts elsewhere in the genome.

The cro protein in its native form is a 67 amino acid protein monomer from the bacteriophage lambda which has been cloned into many plasmids including pBR322. The cro protein dimer under biological conditions exists as a dimer and is an ideal chaperone for the nucleic acid binding domains as it is highly stable and there is little structural or organizational similarity between most nucleic acid recognition domains and the cro monomer. The structure of the cro protein dimer is shown in figure 5. It is the simplest and easiest to produce of all of the chaperones.

Most dimers and other multimers such as DNA binding proteins utilize the enhanced binding affinity of the combined, positioned binding domains. The binding affinity of each binding domain is relatively weak. As one domain specifically binds to a target, it brings the adjacent locking domains into close proximity to the adjacent DNA. If there is a match between the domain specificity and the DNA then the adjacent domains will bind and the affinity of the binding of the TBA will be the product of the binding affinities of the individual recognition domains.

Manipulation of the relationships between the binding affinities of the wild-type proteins and the down-shifted proteins used in constructing a complex is necessary in order to determine the optimal relationships between sample concentration and volume. These parameters are very important when relatively low copy-numbers of the sample DNA are involved. More advanced chaperones may be used for specialized functions including idiotyping.

Signal Amplification Boosters

The signal amplification booster ("booster") is a stable nucleic acid polymer that is resistant to chemical and thermal degradation. The booster is a defined-length, defined-sequence polymer which is mainly comprised of nucleic acid. A small number of oligonucleotides (6) are synthesized with more than one type of fluorescent labels. These are then assembled and stabilized in a controlled and systematic fashion. This results in a polymer, called a booster, which has a specific length length and a specified ratio of labels incorporated. The booster carries a specific number of different labels in a specific order and we refer to it as being "bar-coded". A schematic of the booster assembly is shown in figure 6. Multiple boosters can be used simultaneously each having their own unique signal.

Introduction to System Construction

The components of the [redacted] will be made, purified and tested in phases I, II and III. This consists of 1) making, purifying and testing locking proteins and the booster, 2) ligating the booster one of the locks, and 3) ligating one of the locks (dimer) to a magnetic bead.

Immobilized Multivalent TBAs

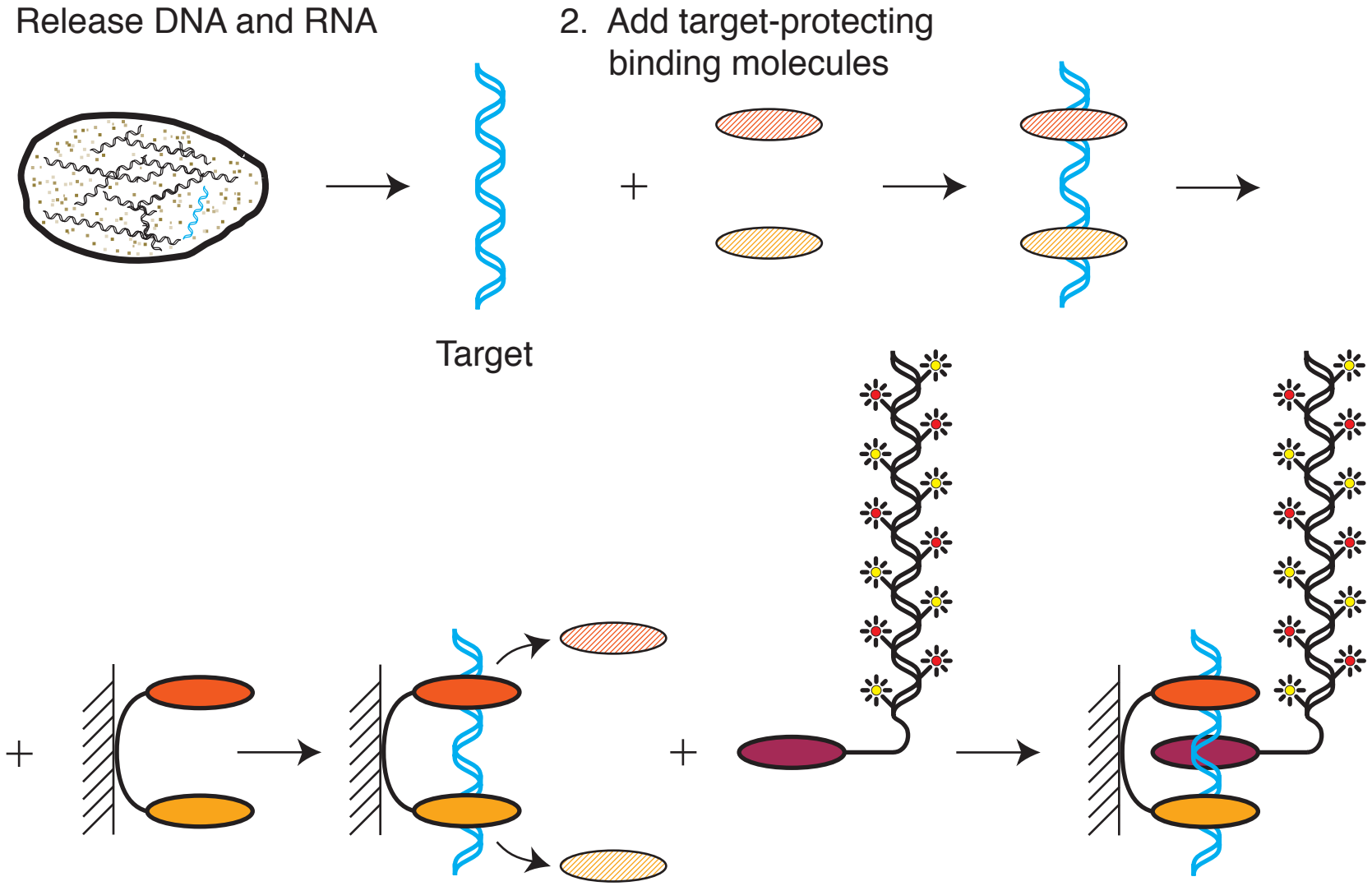

Figure 1

Pathogen Stabilization and Detection

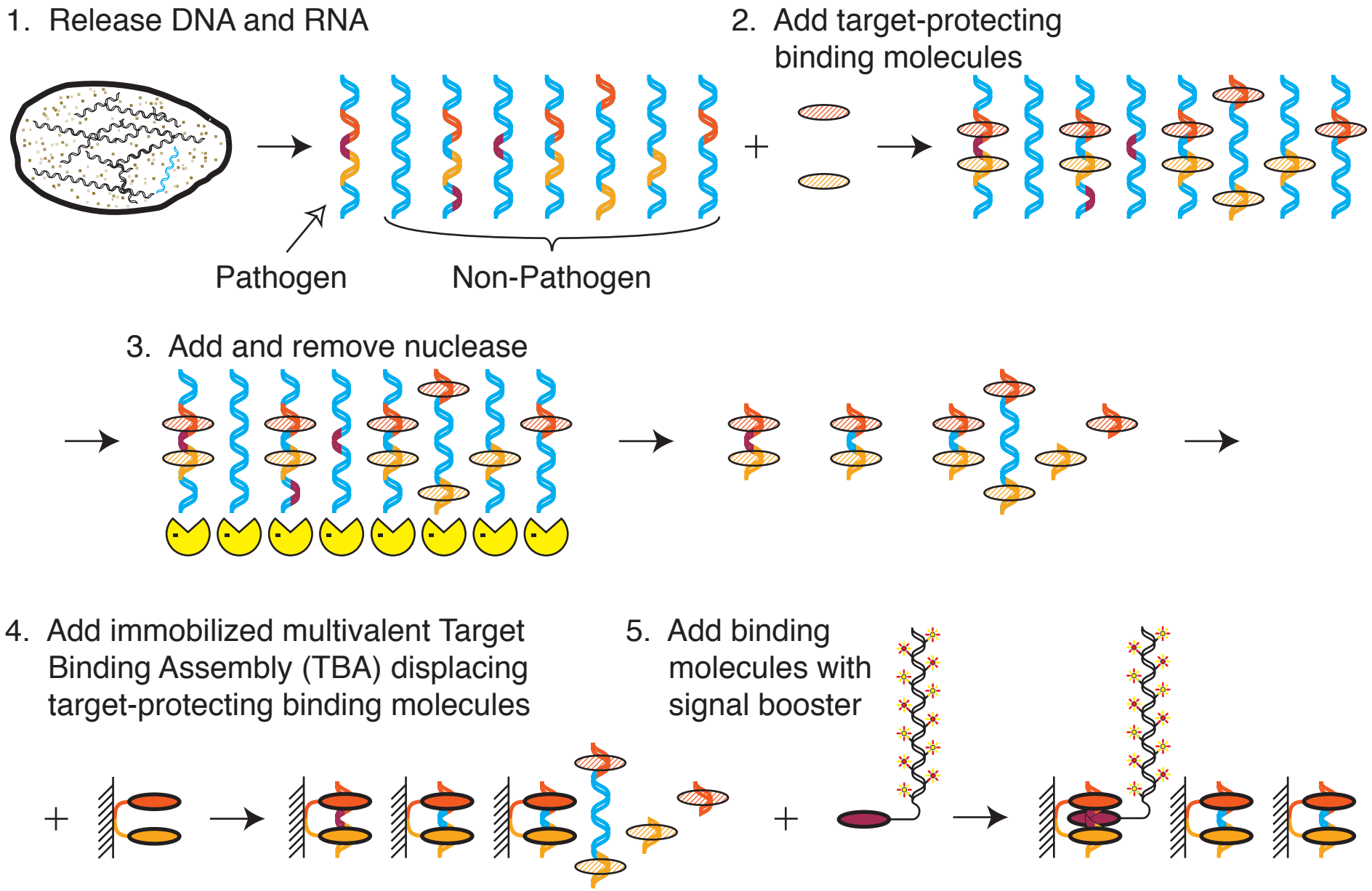

Figure 2

Multiple Binding Site TBA Selection

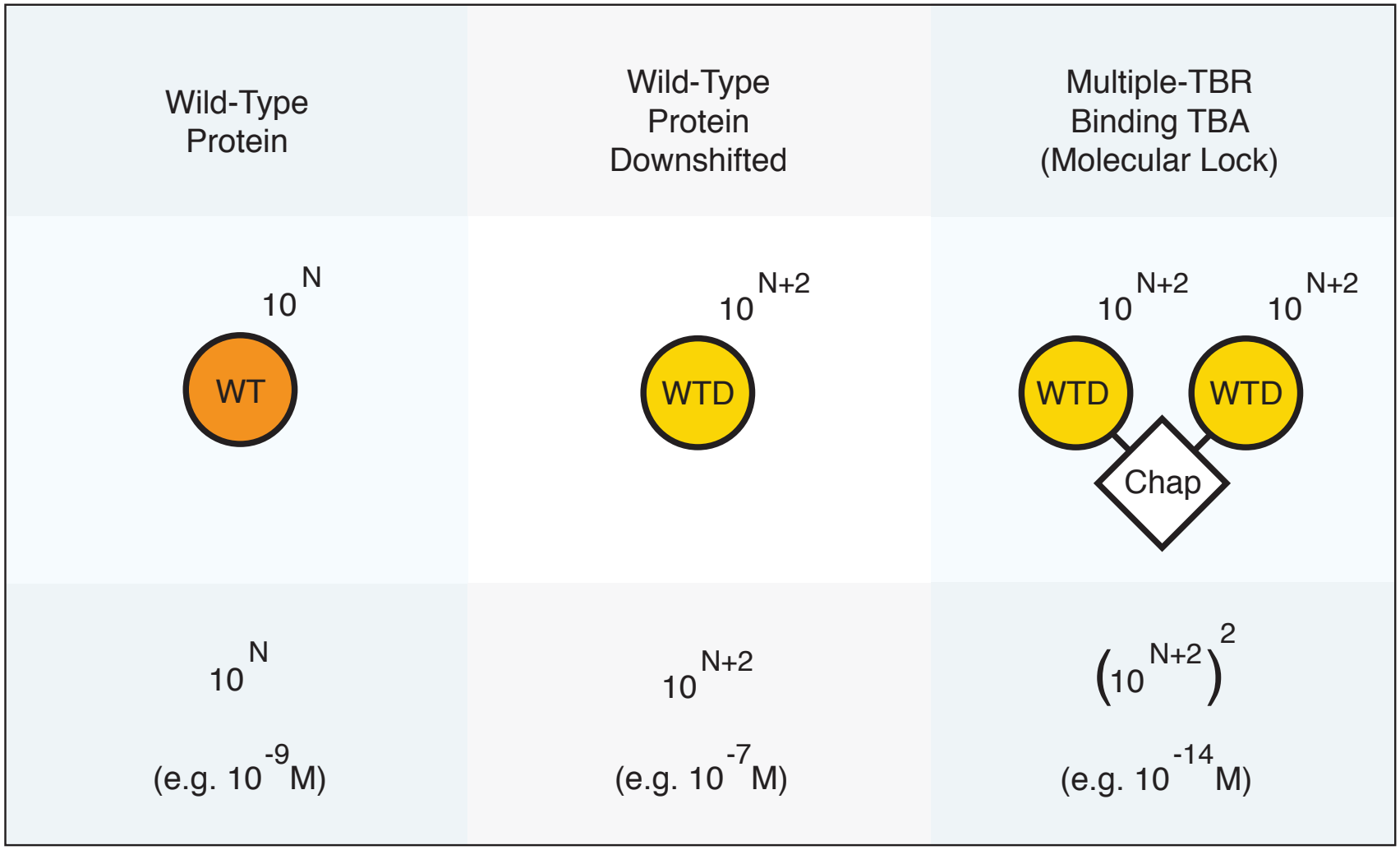

Figure 3

Multivalent TBAs and Binding Competition

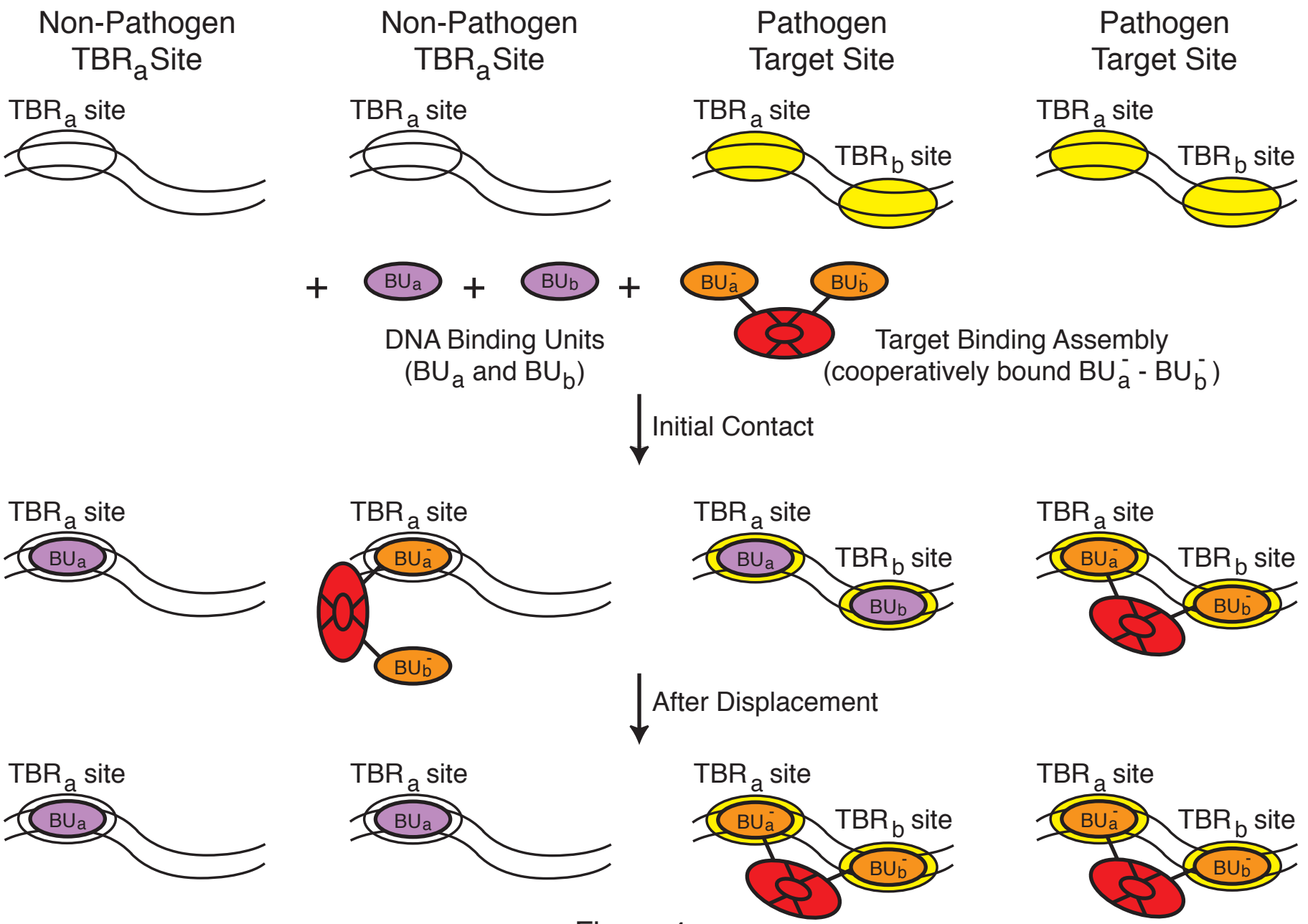

Figure 4

Bacteriophage λ Cro Protein

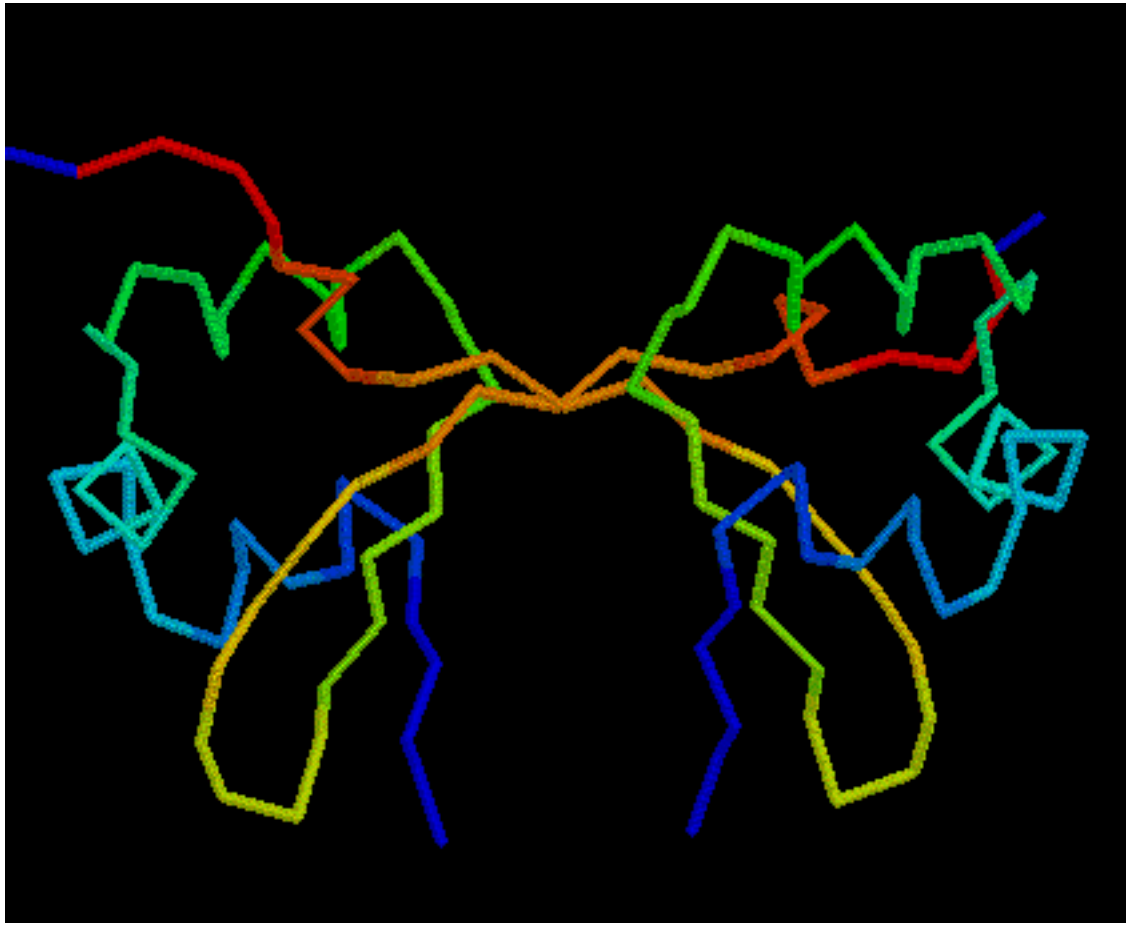

Figure 5

Cyclic Polymer Formation

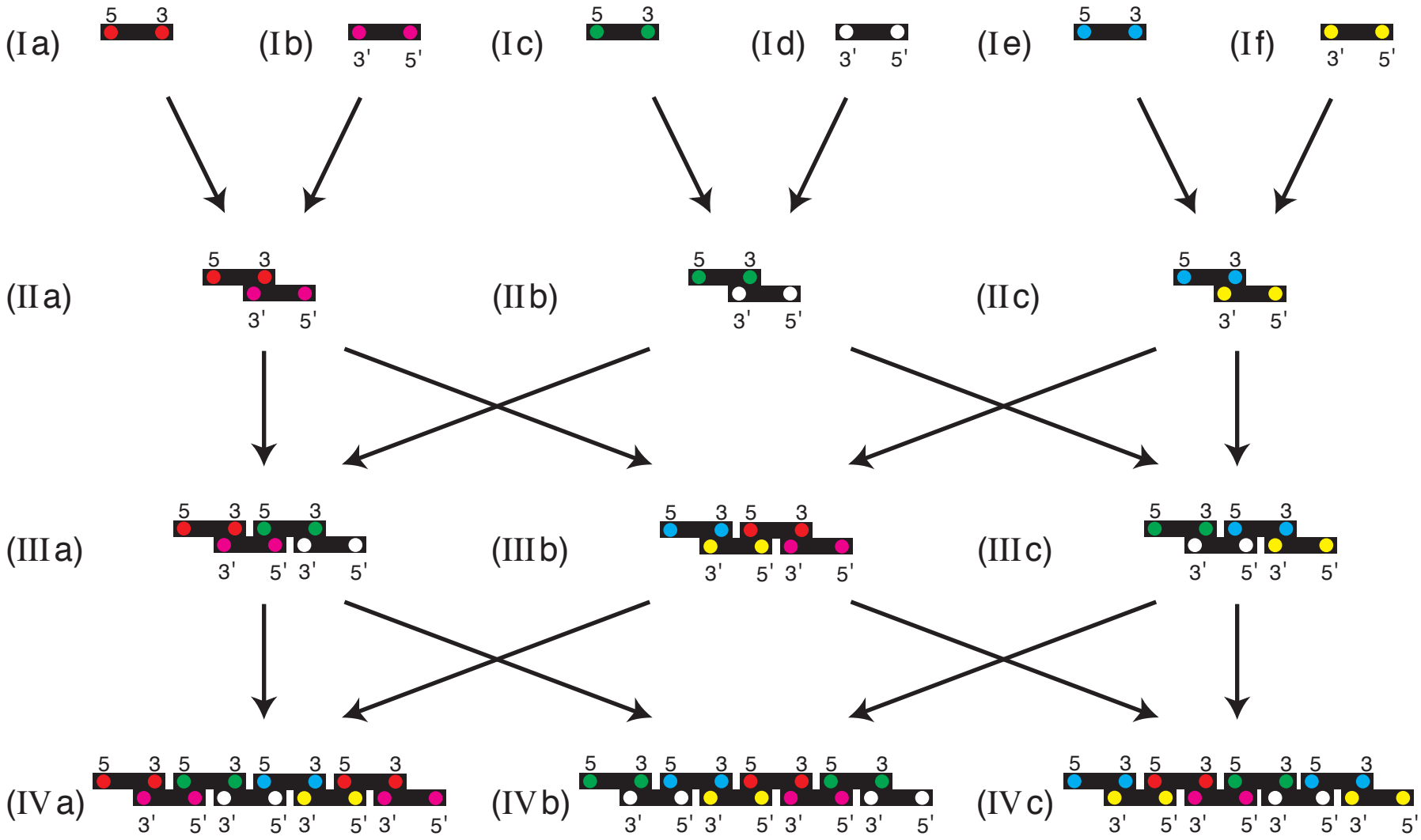

Figure 6