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Block decoys: Transcription-factor decoys designed for in vitro gene regulation studies

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ABSTRACT

Transcription-factor decoys are short synthetic oligodeoxynucleotides that sequester cognate transcription factors and prevent their binding at target promoters. Current methods of decoy formation have primarily been optimized for potential therapeutic applications. However, they are not ideally suited to in vitro investigations into multi-transcription factor-mediated processes that may require multiple regulatory elements to be inhibited in varying combinations. In this study we describe a novel method for chimeric decoy formation in which blocks containing discrete transcription factor binding sites are combined into circular molecules. Unlike currently available methods, block decoys allow rapid construction of chimeric decoys targeting multiple regulatory elements. Further, they enable fine-tuning of binding-site copy ratios within chimeras, allowing sophisticated control of the cellular transcriptional landscape. We show that block decoys are exonuclease-resistant and specifically inhibit expression from target binding sites. The potential of block decoys to inhibit multiple elements simultaneously was demonstrated using a chimeric decoy containing molar optimized ratios of three regulatory elements, NF-κB-RE, CRE, and E-box. The chimeric decoy inhibited expression from all three elements simultaneously at equivalent levels. The primary intended use of block decoys is in vitro gene regulation studies in which bespoke chimeras can be rapidly constructed and utilized to determine a promoter's functional regulation.

Transcription factors (TFs) bind to specific DNA motifs (regulatory elements (REs)) within gene promoters and enhancers to regulate the levels of gene transcription [1]. Disruption of TF binding to target sites is accordingly a well-established method to investigate promoter regulation. An effective method to achieve this is the use of transcription-factor decoys [2–4]: short synthetic oligodeoxynucleotides (ODNs) that contain a specific TF-binding motif. When introduced into a cell the decoys compete for available TFs, preventing their association at target promoters [5]. This site-specific sequestration of TFs makes decoys an attractive method to determine the functional contribution of individual REs to a promoter's activity.

The key determinants of decoy effectiveness are stability, specificity, and uptake [6]. Multiple methods of decoy formation have been developed to improve these factors, primarily focusing on their stability against intracellular nucleases. These include chemical modifications such as the use of phosphorothioate groups [5] and circular dumbbell ODNs that have enzymatically ligated termini [7], conferring resistance to exonucleases (the primary cause of intracellular degradation [8]). Although such advancements have greatly improved decoy functionality, particularly in potential therapeutic applications [9], currently available methods are not ideally suited to in vitro gene regulation studies.

As most promoters contain binding sites for multiple TFs, gene regulation studies utilizing decoys are likely to require multiple decoys, targeting varying combinations of different binding motifs. Ideally, where multiple REs are targeted at once they would be included on a single decoy molecule to avoid the uneven distribution of various decoys across the transfected cell population. Phosphorothioate and dumbbell decoys targeting two [10,11] and three [12] REs have been described (and shown to be far superior to using individual decoys against each site) but these formation methods do not allow for the rapid creation of bespoke chimeric decoys. Further, they do not provide the capability to fine-tune the molar ratio of different sites within one molecule.

Here, we describe a method of decoy formation in which chimeric, ratio-optimized, molecules can be synthesized from a set of input RE blocks. We show that block decoys are resistant to exonuclease degradation and can specifically inhibit multiple REs simultaneously. This novel method offers significant advantages for multitarget decoy studies. Using existing methods each

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chimera must be designed and synthesized individually. Block decoys allow potentially hundreds of different chimeric combinations to be quickly constructed from a pool of input RE blocks, saving time and substantial costs. The ability to tailor RE ratios enables more efficient inhibition, reducing the decoy concentration required and potentially facilitating more REs to be targeted simultaneously. Furthermore, the relative level of inhibition of each target can be altered, allowing sophisticated control of the cellular transcriptional landscape. Block decoys are therefore a new tool for investigating multiple TF-mediated phenotypes and are particularly suited to in vitro gene regulation studies.

Materials and methods

Construction of block decoys

The method of block decoy construction is shown schematically in Fig. 1. RE block molecules were developed by annealing two complementary, single-stranded 5′-phosphorylated DNA ODNs (Sigma, Poole, UK) in STE buffer (100 mM NaCl, 50 mM Tris–HCl, 1 mM EDTA, pH 7.8; Sigma). ODNs were heated at 95 °C for 5 min and then ramp cooled to 25 °C over 2 h to create RE blocks that contain a transcription factor binding site and a 4-bp TCGA single-stranded overhang at each 5′ terminus. RE blocks (12 μg) were then ligated with 5 units of high-concentration T4 DNA ligase (Life Technologies, Paisley, UK) at room temperature for 3 h to form block decoys. The cohesive ends enable RE blocks to be ligated together into extending concatamers. At sizes greater than 100 bp DNA molecules are likely to bend [13,14], allowing ligation of cohesive termini [15,16]. Therefore, ligation of input blocks theoretically results in covalently closed circular block decoys. Chimeric decoys were constructed by ligating varying molar concentrations of different RE blocks. The sequences of RE blocks employed were as follows (consensus site in italic): nuclear factor κB response element (NF-κB-RE), 5′-TCGAT*GGGACTTTCC*A-3′ and 5′-TCGAT*GGAAAGTCC*-*CA*-3′; cyclic AMP-responsive element (CRE), 5′-TCGATT*TGAC GTCA*TT-3′ and 5′-TCGAAA*TGACGTCA*AA-3′; enhancer box (E-box), 5′-TCGAAA*CACGTG*AGA-3′ and 5′-TCGATCT*CACGTG*TT-3′. Scrambled decoys contained the following scrambled consensus sites: NF-κB-RE, AATCGCAAGT; CRE, GACTAGAG; E-box, GCTCAG. All block decoys were extracted and stored at 350 ng/μl.

Analysis of block decoy structure

Block decoy population size distribution was analyzed by ethidium bromide agarose gel electrophoresis utilizing molecular weight markers (Hyperladder II; Bioline, London, UK). To confirm block decoy circularization, 1.5 μg of purified block decoy was added to 5 units of high-concentration T4 DNA ligase before gel analysis. To test the stability of block decoys against exonuclease, 4 μg of block decoy was incubated with 300 units of exonuclease III (Promega, Southampton, UK) and the mixture was incubated at 37 °C. A mixture of linear ODNs spanning the molecular weight range of the block decoys was used as a positive control.

Construction of RE-specific reporter vectors

A promoterless vector was subcloned from pSEAP2control (Clontech, Oxford, UK) by PCR amplification of appropriate vector regions with Phusion high-fidelity polymerase (New England Biolabs, Hitchin, UK). A minimal core promoter from the human cytomegalovirus (CMV) was synthesized (Sigma) and cloned into the XhoI and EcoRI sites directly upstream of the secreted alkaline phosphatase (SEAP) open reading frame (ORF). The core promoter sequence used was as follows: 5′-AGGTCTATATAAGCAGAGCTCGTTTAGTGAACCGTCAGATCGCCTAGATACGCCATCCACGCT GTTTTGACCTCCATAGAAGAC-3′. A second reporter plasmid was created by replacing the SEAP ORF with the Turbo green fluorescent protein (GFP) ORF. To create binding-site reporter plasmids, synthetic oligonucleotides containing 7× repeat copies of NF-κB-RE, CRE, and E-box were synthesized (Sigma), PCR amplified, and inserted into KpnI and XhoI sites upstream of the CMV core pro-

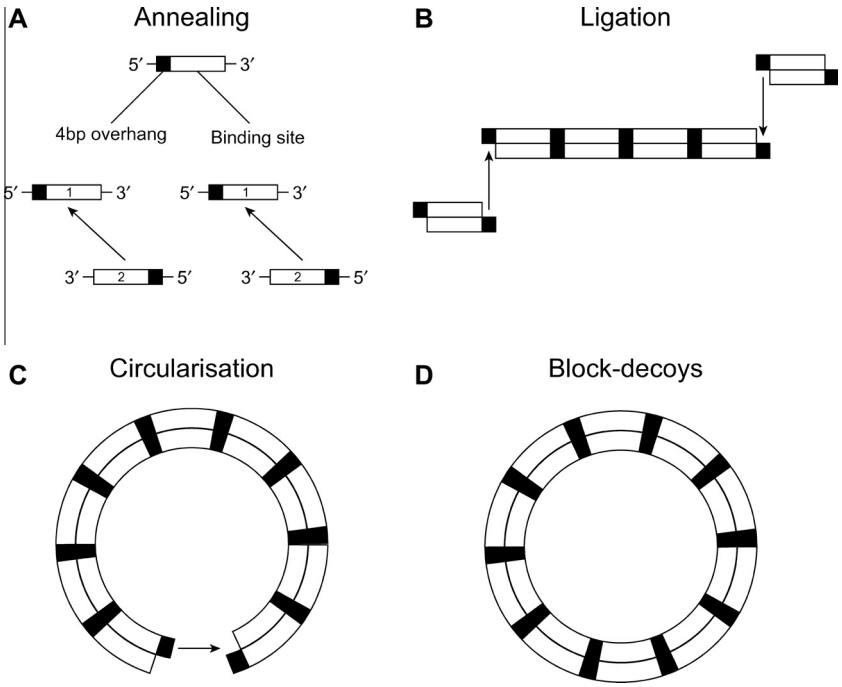

Fig.1. Schematic of block decoy formation. (A) Single-stranded oligonucleotides are annealed to form regulatory element blocks containing a transcription factor binding site and a 4-bp single-stranded overhang at the 5′ terminus. (B) Regulatory element blocks are ligated together into extending concatamers, (C) which circularize, allowing intramolecular ligation of cohesive termini, yielding (D) covalently closed circular block decoys containing multiple copies of the target binding site.