Detection of Multiple Hairpins in a DNA Construct

Tiffany Wanyoike, Gilberto Garcia, Brian Cannon

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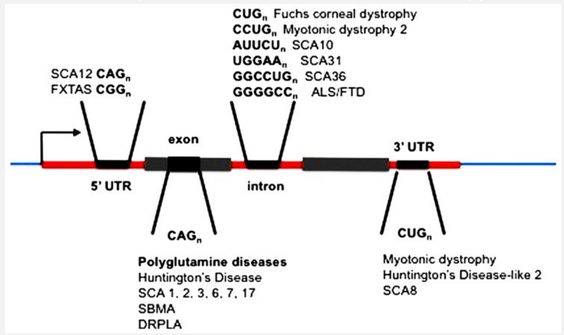
Abstract

Numerous genetic disorders such as fragile X syndrome, spinocerebellar ataxia, ALS, and Huntington's disease arise from repetitive DNA. Repeats in a DNA sequence can cause altered base pairing in single-stranded domains, which can lead to the formation of larger structures, such as hairpins. These structures can disrupt genome integrity and cell functioning. Detection of hairpin formation can be useful in understanding the structural dynamics behind them and possibly the development of treatments with preventative or reductive effects. This project sought to develop a triplex-forming strategy to identify the presence and number of repeat hairpins in double-stranded DNA. The triplex-forming oligonucleotide probes include alternating locked nucleic acids (LNAs) to improve the hybridization of the probe to the target hairpins. Using a single-molecule fluorescence microscope, the binding of the probes to individual target DNAs was measured versus the number of hairpin domains within the target DNA. The effectiveness of different probe sequences, concentration, and experimental conditions on target hybridization were examined.

Background

Many genetic diseases are caused by the expansion of genomic trinucleotide repeats beyond a specific threshold. Various neurological symptoms can result. (Note: modes of inheritance vary)



Disease	DNA repeat sequence	Size
Huntington's Disease	CAG	>40
Spinocerebellar ataxia type 1 (SCA1)	CAG	>39
Myotonic Dystrophy Type 1 (DM1)	CTG	50-1000
Amyotrophic Lateral Sclerosis (ALS)	G_4C_2	>30
Fragile X	CGG	>200

Experimental Design

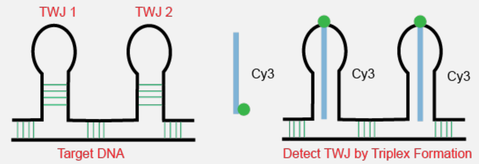


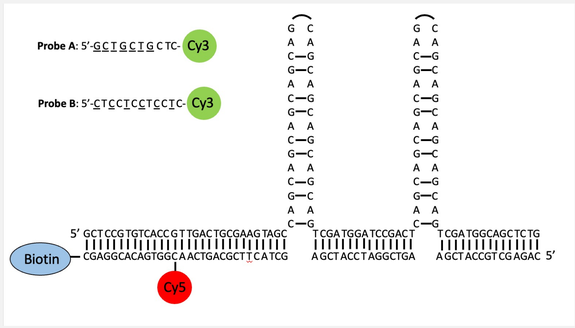
Figure 1: Two site binding target

Figure 2: Single site binding target

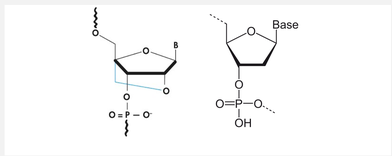
Figure 3: Control

DNA Target and LNA Probe Design

The target DNA, synthesized by IDT DNA (Coralville, IA) is double-stranded and contains one or two hairpin domains with the repeat sequence CAG. Each hairpin domain consists of ten repeats. The target DNA has two modifications for the single-molecule detection assay: 1) a 3' end biotin for surface immobilization and 2) a 5' end Cy5 dye for detection.



To target the hairpins, triplex-forming oligonucleotide (TFO) probes were developed. The TFO probe consists of a mixture of locked nucleic acids (LNA) and DNA. LNA nucleotides are an engineered version of DNA nucleotides.



LNA vs. DNA. The LNAs form stronger bonds with DNA nucleotides than DNA Watson-Crick base pairs. The probe has a 5' Cy3 dye for detection. Two different probe sequences were tested.

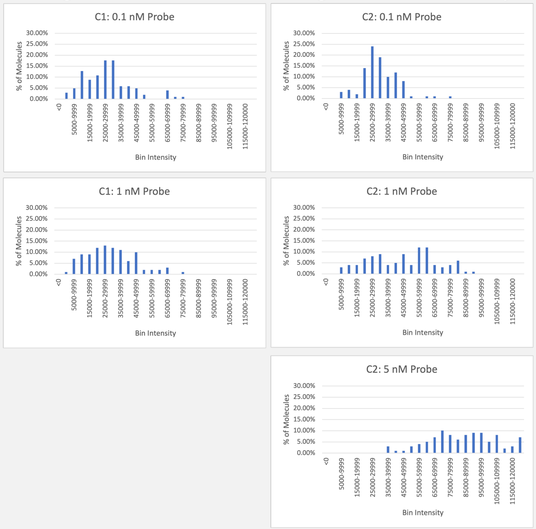
Methodology

The target DNA is immobilized on a quartz slide as part of a flow chamber. Using a single-molecule fluorescence microscope, the location of individual target DNA molecules are readily identified through the Cy5 emission upon excitation with 634-nm laser (red); left. The Cy3-labeled TFO probes were then added to the flow chamber for binding to the immobilized target DNA. The location of the bound probes are identified by excitation with the 532-nm laser (green); right.

By simultaneously imaging the Cy3 and Cy5 dyes for a single field of view, the data are analyzed for colocalization of the dyes. Colocalization of the dyes represents probe binding to the target DNA. Targets with one hairpin domain (cluster 1, c1) should be differentiated from targets with two hairpin domains (cluster 2, c2) by the number of Cy3 dyes detected.

Result

To analyze the data, time traces for the location of each Cy5 dye were generated. These time traces show the Cy3 intensity as a function of the observation time. The distributions of total intensities were determined for each target and probe combination. The time traces were also analyzed for photobleaching as a metric for counting the number of dyes bound to the target.



Conclusion

- At low probe concentrations, both cluster 1 and cluster 2 showed similar signals collected for hundreds of molecules.
- As the concentration of probe was increased, a pronounced high-intensity population appeared for cluster 2.
- At the highest probe concentration tested, 5 nM, the data suggest that complete binding of the two sites in cluster 2 occurred.
- We showed that the triplex forming oligonucleotide assay can clearly differentiate whether a sample has one or two extrusion domains.