List of Contents:

Materials and Experimental Methods.

Materials. Goat anti-rabbit (cat# A10533) and anti-rat (cat# A10536) secondary antibodies and phalloidin-Alexa532 were purchased from Invitrogen. Goat anti-chicken secondary antibody, rabbit anti-histone complex H3 (cat# ab1791), rabbit anti-stathmin1 (cat# ab52630), and rat anti-α-tubulin (cat# ab6160) were purchased from Abcam. Rabbit anti-KLC4 (cat# HPA030169) and FITC-conjugated mouse anti-Vinculin (cat# F7053) were obtained from Sigma. Alexa647-conjugated mouse anti-WASP (cat# sc-13139 AF647) was purchased from Santa Cruz Biotechnology. PSmOrange-Tubulin was obtained from the Verkhusha Lab (addgene plasmid 31919).[1] Oligonucleotides were purchased from IDT (Coralville, IA), dye and quencher-bearing strands were purchased with HPLC purification. Images were collected with a 14-bit depth CCD camera (Luca, Andor) on a Nikon Ti Eclipse epi-fluorescence microscope with an automated stage and focus, using a 60x oil immersion plan-Apo objective.

DNA Probe Design: All sequences were selected using a custom MATLAB script that generates random domains of specified lengths having pre-determined GC% range, while excluding previously generated domains or other prohibitive sequences (*i.e.* G quadruplexes), and avoiding secondary structures (*e.g. hairpins*). The generated domains are ranked according to their normalized two-state hybridization energies with existing probe strands using mFold. The domains are then screened through the BLAST database to minimize probe sequence homology with the mRNA transcriptome. The final domain sequences are then selected manually from this list and concatenated with other domains to create full oligonucleotide sequences that will be incorporated into a probe complex. Other global criteria such as

temperature, strand concentration, and salt concentration are specified prior to domain design. All oligonucleotide sequences can be found in the supplement (Table S1).

Preparation of Antibody-Oligonucleotide Conjugates. Secondary antibodies (20 μg) were incubated with NHS-$(PEO)_4$-Azide (20 μmol in anhydrous DMSO, Thermo Scientific) in 100 mM $NaHCO_3$ at room temperature for 45 minutes. Following purification via gel filtration using a Tris-buffered Bio-Spin 30 column (Bio-Rad), hexynyl-modified oligonucleotide were added and a Cu(I) catalyze click reaction was performed using the Click-It Reaction Buffer Kit (Invitrogen) according to the provided protocol. The reaction was incubated at room temperature for two hours then moved to 4 C overnight. The resulting DNA-functionalized antibodies were purified once more using gel filtration. Conjugation was verified with non-reducing SDS-PAGE.

Cell Culture and Fixation: HeLa cells were cultured in Dulbecco's Modified Eagle Media (DMEM) with GlutaMAX (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL Pen/Strep at 37 C with 5% CO_2. Cells were seeded on cover glass (no. 1.5) and grown to 50-60% confluency prior to fixation. For mOrange labeled tubulin experiments, PSmOrange-Tubulin was transfected using Fugene (Promega) at a 2:5 ratio according to the manufacturer's protocol. Media was exchanged 24 hours post transfection and cells were fixed after an additional 24 hours of growth. Cells were fixed in freshly prepared 4% paraformaldehyde. Samples were then rinsed with PBS and quenched with $NaBH_4$ (1 mg/mL in PBS). The samples were washed with PBS, permeabilized with 0.2% TritonX-100, and washed once more. Cover slips were dried under airstream, then affixed to custom-fabricated 10 well chambers using precision cut double sided adhesive and stored in buffer at 4 C for up to one week.

Analysis of Probe Erasing. Samples were blocked 2% BSA, 1 mg/mL Herring sperm DNA, and 0.5 μM polyT DNA in PBS, pH 7.4 (buffer BB) for 2 hrs. Rat anti-α-tubulin (1:250) was incubated on samples overnight at 4 C in PBS supplemented with 10% buffer BB (PBS-BB). Cells were washed 3 times with PBS, followed by a 2 hr incubation with an oligonucleotide-conjugated (TS1, TS2, or TS3) goat anti-rat secondary antibody at 10 μg/mL in PBS-BB. Cells were then washed 3 times with PBS and probe complex was added to the cells for 2 hours at 100 nM in TAE containing 12.5 mM Mg^{2+} and 10% buffer BB (TAE-BB), stained with DAPI (1.3 μM), and washed an additional 3 times with PBS. Erasing complexes were then applied to the cells at 1 μM in TAE-BB, and allowed to react overnight (~12 hrs) at room temperature. Cells were stained again with DAPI then washed 3 times with PBS. Images were collected between reactions steps. After alignment and background subtraction, initial ON images was used to generate a thresholded mask, eliminating pixels with intensity less than 2500. The ON/OFF ratios of these regions (> 400 regions were generated for each image) was then measured directly by comparing pixel intensities of the initial ON values to the OFF images. For mOrange-tubulin colocalization experiments, images were obtained as above using a probe complex incorporating an Alexa488 dye molecule. Pixel-by-pixel analyses and Pearson's coefficients were obtained using the ImageJ pulgin JACoP.[2]

Two Marker/One Dye Imaging: Samples were treated as in erasing experiments with minor modifications. Cells were blocked with buffer BB and incubated overnight with antibodies towards α-Tubulin and Histone H3 or α-Tubulin and KLC4 in PBS-BB. Samples were washed

with PBS, then TS1 conjugated goat anti-rat and TS4 conjugated goat anti-rabbit were added to the samples at 10 μg/mL in PBS-BB for 2 hours. α-Tubulin was activated with 100 nM probe complex and erased with 1 μM eraser complex in TAE-BB followed by the activation of the second marker (100 nM probe complex). Images were taken in between reaction steps. Off images were taken after a 10 second bleaching step. All images were aligned as before, false colored, and merged.

Six Marker Imaging: Samples were blocked as before and incubated overnight with rat anti-α-tubulin, rabbit anti-Stathmin 1 (1:200), and chicken anti-Vementin (1:200) at 4 C in PBS-BB. Following washing, cells are incubated with TS1-conjugated goat anti-rat, TS2-conjugated goat anti-chicken, and TS3-conjugated goat anti-rabbit at 10 μg/mL each in PBS-BB. All markers were activated simultaneously by adding 100 nM of each probe complex in TAE-BB. Erasing was performed overnight followed by incubation with 1 μM of each eraser complex in TAE-BB. Alexa647-conjugated mouse anti-WASP (1:500) and FITC-conjugated mouse anti-Vinculin (1:500) were then incubated on the cells for two hours in PBS-BB. Cells were stained with DAPI and Phalloidin-532 then washed. Images were taken between all reaction steps and aligned as before prior to being false colored and merged.

Literature Cited:

[1] O. M. Subach, G. H. Patterson, L. M. Ting, Y. Wang, J. S. Condeelis, V. V. Verkhusha, *Nat. Methods.* **2011**, *8*, 771-777.

[2] S. Bolte, F. P. Cordelieres, *J. Microsc.* **2006**, *224*, 213-232.

Supplemental Figures.

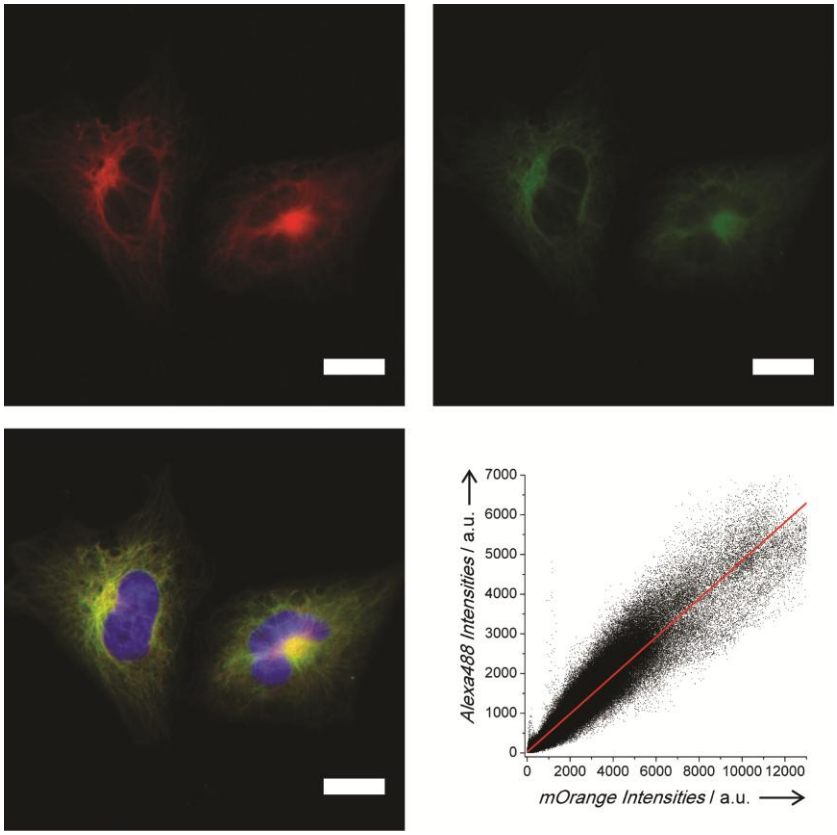

Figure S1. Analysis of the fidelity of DNA-probe localization and dispersion within HeLa cells. Cells were transfected with a mOrange-tubulin construct, fixed and then stained for α-tubulin using dynamic DNA probes incorporating a Alexa488 dye molecule. mOrange-labeled microtubules (red) colocalized with signals generated using dynamic DNA probes (green). (Scale bars: 20 μm). Selective microtubule recognition and the uniform dispersion of probes throughout the sample is further confirmed by a pixel-by-pixel correlation analysis of the mOrange and Alexa488 signals, pearson's coefficient, $p = 0.971$.

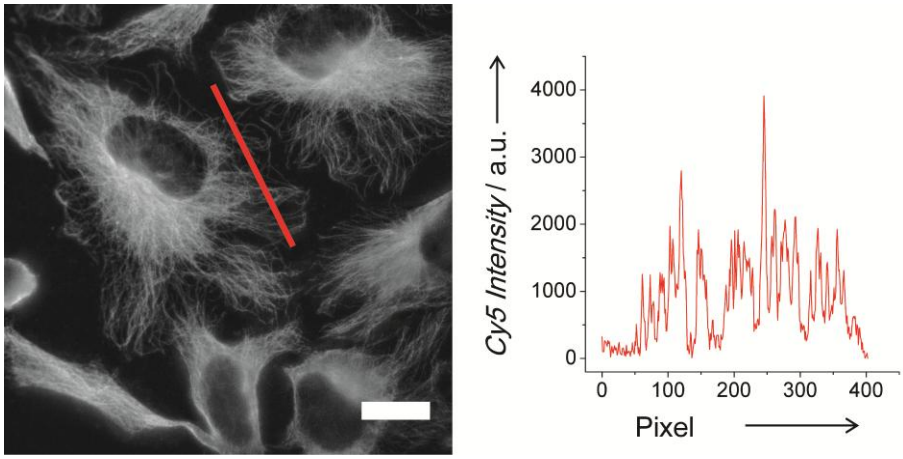

Figure S2. Analyses of signal-to-background ratios produce by standard antibody labeling strategies. Line profile analysis of α-tubulin detected with the same primary used in Figures 1 and 2 labeled with a dye-conjugated secondary antibody. Signal-to-noise ratios vary from 1:10 – 1:20 depending upon the location of the filament, similar to DNA-based image in Figure 1. (Scale bar: 20 μm).

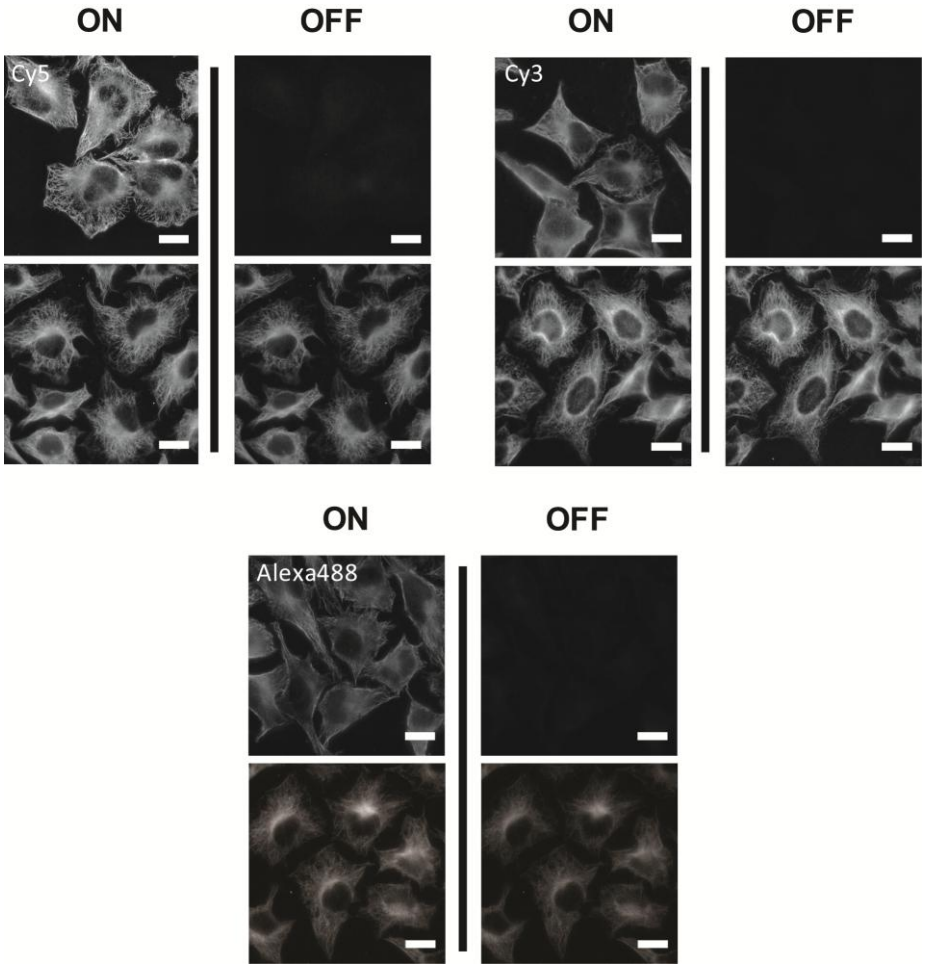

Figure S3. Erasing efficiencies of probe complexes possessing different sequences and incorporating different color dye molecules. The probe system (PC an E) and their dye molecular are indicated for each experiment. Representative label (ON) and erased (OFF) images are provided for each probe system, as well as for reactions where E was omitted from the erasing step in the procedure. (Scale bars: 20 μm)

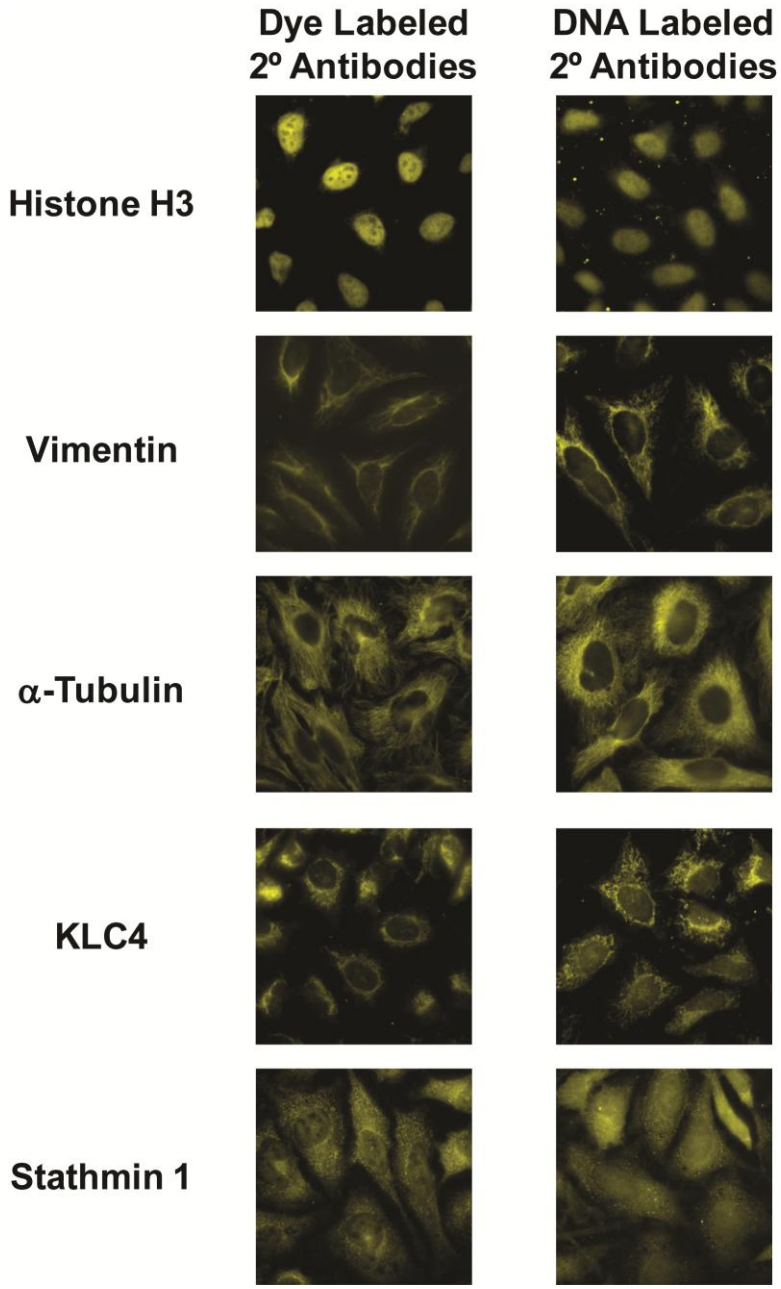

Figure S4. Comparisons of standard and dynamic DNA-mediated immunofluorescent labeling of different protein markers in cells. Characteristic images of histone H3, vimentin, α-tubulin, KLC4, and stathmin 1 detected using standard secondary antibody detection methods where fluorophores are conjugated directly to antibodies are provided in the left panel. The images on the right show the same markers detected with DNA probes using DNA-conjugated secondary antibodies.

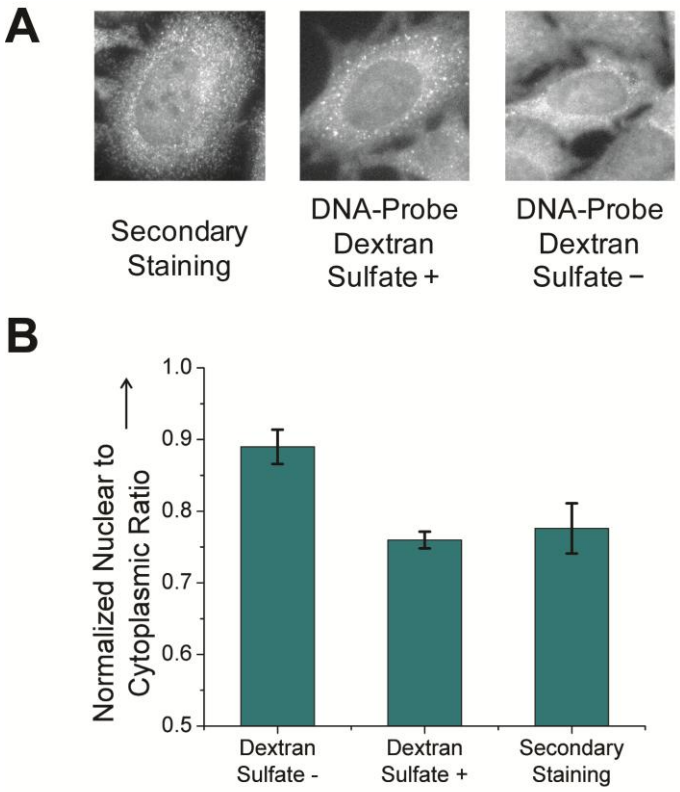

Figure S5. Effects of dextran sulfate on non-specific nuclear localization using dynamic DNA-based labeling. (A) Representative images of HeLa cells labeled with an anti-stathmin 1 antibody then detected using either conventional dye-labeled secondary antibodies or DNA-conjugated secondary antibodies and dynamic DNA probe complexes. DNA-mediated fluorescent images where samples were incubated with a blocking buffer containing 1% dextran sulphate (middle) better reproduce the nuclear to cytoplasmic localization patterns produced by conventional secondary detection (left) than those blocked without dextran sulphate (right). (B) Direct comparisons of the calculate nuclear to cytoplasmic ratios of the three samples confirm that blocking buffer containing dextran sulphate reduces the non-specific nuclear signals to levels similar to those produced by standard dye-labaeled secondary antibodies.

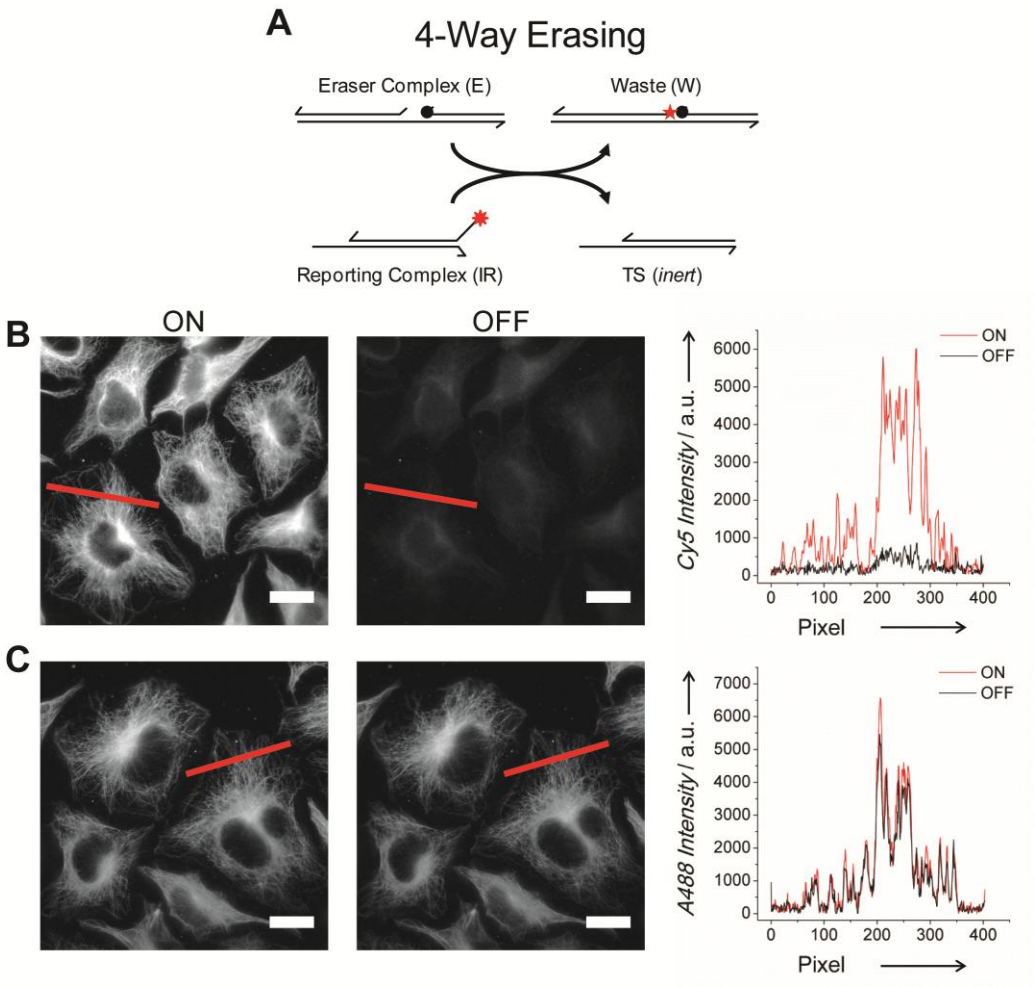

Figure S6. Analysis of four-way erasing probes incorporating a conserved quencher domain (A) Scheme of the four-way erasing mechanism for dynamic DNA probes that employ a conserved quencher domain (qd). The use of four-way erasing reactions is capable of reducing signals imparted by probe complexes incorporating a Cy5 dye molecule (B), but is unable to erase signals from probe complexes incorporating Alexa488 dye molecules (C). (Scale bars: 20 μm)

Supplemental Table.

Table S1. List of oligonucleotide sequences used in design of DNA probe systems. /5Hexynyl/, /5Cy5/, /5Cy3/, /5Alexa488/, /3IAbRQSp/, /3IABkFQ/ indicate a 5' hexynyl, Cy5, Cy3, Alexa488, 3' Iowa Black re quencher, or Iowa Black green quencher modification, respectively.

Strand	**Sequence (5'-3')**
RQ	GAT GCG AAG TCA GCG TTC/3IAbRQSp/
FQ	CGT AAT AGC GCT AGT CTC/3IABkFQ/
TS1	/5Hexynyl/TTT TTT TTT TGG CCA CCG AGA CAA TAC GCA GGA CCC
TS2	/5Hexynyl/TTT TTT TTT TGT GTA CCG GAA ACA TCG GCG AAT TAG
TS3	/5Hexynyl/TTT TTT TTT TGC CAT CGA CCC GTG CAT TAA GTG TCC
TS4	/5Hexynyl/TTT TTT TTT TAC CTA CGG TCT CCG GAA CTT ACG ATC
D1	/5Cy5/CCT TAA GTC CTG CGT ATT GTC TCG GTG GCC
L1	GTC GAG ACA ATA CGC AGG ACT TTT TTG AAC GCT GAC TTC GCA TC
K1	GGC CAC CGA GAC AAT ACG CAG GAC TTA AGG GAA CGC TGA CTT CGC ATC
D2	/5Cy3/CTT GTC AAT TCG CCG ATG TTT CCG GTA CAC
L2	GTC GGA AAC ATC GGC GAA TTT TTT TTG AGA CTA GCG CTA TTA CG
K2	GTG TAC CGG AAA CAT CGG CGA ATT GAC AAG GAG ACT AGC GCT ATT ACG
D3	/5Alex488N/TT TCT CAT AAC ACT TAA TGC ACG GGT CGA TGG C
L3	CTG ACC CGT GCA TTA AGT GTT TTT TTT TTG AGA CTA GCG CTA TTA CG
K3	GCC ATC GAC CCG TGC ATT AAG TGT TAT GAG TTT TTT GAG ACT AGC GCT ATT ACG
D4	/5Cy5/TT TGC AAA GTC GTA AGT TCC GGA GAC CGT AGG T
L4	GTG GTC TCC GGA ACT TAC GAT TTT TTT TTT TTG AAC GCT GAC TTC GCA TC
K4	ACC TAC GGT CTC CGG AAC TTA CGA CTT TGC TTT TTT GAA CGC TGA CTT CGC ATC