MATERIALS AND METHODS

Materials and reagents

DNA oligonucleotides were synthesized by Sangon Biotech (Shanghai, China). Ammonium persulfate (APS), N,N,N′,N′-tetramethyl ethylenediamine (TEMED) and GelGreen dye were obtained from Beyotime Biotechnology (Shanghai, China). 5-(N-Ethyl-N-isopropyl)-amiloride (EIPA) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). (−)-Epigallocatechin gallate (EGCG), chlorpromazine (CPZ), methyl-β-cyclodextrin (MβCD) and nocodazole were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS) and phosphate buffered saline (PBS) were bought from Gibco (Waltham, MA, USA). The Cell Counting Assay Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Tokyo, Japan). The FITC Annexin V Apoptosis Detection Kit was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

Synthesis of tetrahedral DNA nanostructures (TDNs)

TDNs were constructed as previously reported.[1] The specific sequences of S1, S2, S3, S4 and Cy5-labeled S1 (Cy5-S1) are shown in Table 1. The designed oligonucleotides were mixed to a final concentration of 5 μM per strand in Tris-HCl buffer supplemented with $MgCl_2$ (10 mM Tris-HCl, 50 mM $MgCl_2$, pH 8.0). The mixture was heated at 95 °C for 10 min, and then rapidly cooled to 4 °C for another 20 min. Finally, the TDNs solution was purified by ultrafiltration through a 30 kDa molecular weight cut off (MWCO) filter (Millipore, Burlington, MA, USA).

Synthesis of 6H × 73 nt, 4 × 4 × 64 nt and 6 × 6 × 64 nt DNA nanostructures

6H × 73 nt, 4 × 4 × 64 nt and 6 × 6 × 64 nt DNA nanostructures were designed using Cadnano software and synthesized according to our previous study.[2] The specific sequences of the core strand, Cy5 handle strand and Cy5 single strand are shown in Table 1. In brief, all DNA strands were mixed to a final concentration of 200 nM per strand in TE buffer supplemented with $MgCl_2$ (10 mM Tris-HCl, 1 mM EDTA, 10 mM

MgCl$_2$, pH 8.0 for 6H × 73 nt; 5 mM Tris-HCl, 0.5 mM EDTA, 40 mM MgCl$_2$, pH 8.0 for 4 × 4 × 64 nt and 6 × 6 × 64 nt). The solutions were subsequently subjected to the designed thermal annealing program and finally purified by ultrafiltration (MWCO 30 kDa filter for 6H × 73 nt, MWCO 50 kDa filter for 4 × 4 × 64 nt, MWCO 100 kDa filter for 6 × 6 × 64 nt) at least three times.

Preparation of EGCG@TDNs

After incubation of EGCG and TDNs for 6 h at 4 °C, the mixture was purified by ultrafiltration through a MWCO 30 KDa filter at 16200 g for 10 min to remove free EGCG. The absorption spectra of TDNs and EGCG@TDNs and the fluorescence emission spectrum of the mixture of GelGreen and EGCG@TDNs were analyzed using a multimode reader (Tecan, Switzerland). The entrapment efficiency was calculated by using a multimode reader to measure the absorption of EGCG at 272 nm.

Native polyacrylamide gel electrophoresis (PAGE) analysis

Native PAGE analysis was performed to confirm successful synthesis and determine the stability of TDNs. The 8% native PAGE gel was composed of 30% acrylamide/bis-acrylamide (29:1, 2 mL), distilled H$_2$O (4.5 mL), 10× TBE buffer (0.75 mL, 890 mM Tris-boric, 20 mM EDTA, pH 8.2), 10× ammonium persulfate (APS, 75 μL) and TEMED (7.5 μL). TDNs solution (10 μL) was mixed with 6× loading dye (2 μL) and run at a constant voltage of 100 V for 100 min at 4 °C. The gel was then immersed into 1× GelGreen solution for 30 min, visualized by ultraviolet illumination (254 nm) and photographed using a Tanon 3500 gel image system (Tanon, China).

Stability studies

TDNs were incubated in Tris-HCl buffer containing MgCl$_2$ (10 mM Tris-HCl, 50 mM MgCl$_2$) at pH 5.0, 6.1, 7.2 and 8.0 for 24 h at 37 °C to determine the stability of the DNA nanostructures. TDNs were incubated in artificial perilymph (137 mM NaCl, 5 mM KCl, 2 mM CaCl$_2$, 1 mM NaHCO$_3$, pH 7.4), mimicking a physiological environment, for 24 h at 37 °C. The stability of TDNs to nucleases was determined by

incubating in 50% FBS at 37 °C for 1, 3, 6, 12 and 24 h. All samples were finally analyzed by native PAGE after incubation.

Characterization of TDNs, 6H × 73 nt, 4 × 4 × 64 nt and 6 × 6 × 64 nt DNA nanostructures and EGCG@TDNs

The morphologies of TDNs,4 × 4 × 64 nt, 6 × 6 × 64 nt and EGCG@TDNs were evaluated by transmission electron microscopy (FEI Talos L120C, ThermoFisher Scientific, USA) at 120 kV. Purified TDNs,4 × 4 × 64 nt, 6 × 6 × 64 nt and EGCG@TDNs solutions (5 µL) were adsorbed onto glow discharged carbon-coated copper grids. The grids were then stained with 1% aqueous uranyl formate solution containing 25 mM NaOH for 30 s.

The morphologies of TDNs and 6H × 73 nt were determined by atomic force microscopy. Purified TDNs and 6H × 73 nt solution (5 µL) were adsorbed for 2 min onto the surfaces of freshly cleaved mica chips. The samples were then washed off using 40 µL 1× TE buffer containing 2 mM $MgCl_2$ and 1 mM $NiCl_2$. Images were captured by a MultiMode System under peak force tapping mode (Bruker, Germany). Zeta potentials of TDNs and EGCG@TDNs were characterized on a Zetasizer Nano ZS (Malvern Instruments Ltd, UK). The final concentration of TDNs used for detection was 250 nM.

Cell culture

The House Ear Institute-Organ of Corti 1 (HEI-OC1) cell line was kindly provided by Yiqing Zheng (Sun Yat-sen University, Guangzhou, China). The HEI-OC1 cell line was cultured in high-glucose DMEM containing 10% FBS without antibiotics under permissive conditions (33 °C, 10% CO_2), as we previously described.[3]

Cellular uptake of DNA nanostructures.

The cellular uptake characteristics of TDNs, 6H × 73 nt, 4 × 4 × 64 nt and 6 × 6 × 64 nt DNA nanostructures were determined by confocal laser scanning microcopy (CLSM) imaging and flow cytometric analysis. HEI-OC1 cells were seeded and

cultured for 12 h. The cells were incubated with 150 nM Cy5-S1, 150 nM Cy5-labeled TDNs (Cy5-TDNs), 50 nM Cy5-labeled 6H × 73 nt (Cy5-6H × 73 nt), 30 nM Cy5-labeled 4 × 4 × 64 nt (Cy5-4 × 4 × 64 nt) or 30 nM Cy5-labeled 6 × 6 × 64nt (Cy5-6 × 6 × 64nt) for 6 h to observe cellular uptake. The cells were incubated with Cy5-TDNs at different concentrations (0, 10, 25, 50, 150 and 300 µM) for 6 h to investigate concentration-dependent characteristics. The cells were incubated with 150 nM Cy5-S1 or Cy5-TDNs in a time series (1, 3, 6, 12 and 24 h) to observe time-dependent properties. For CLSM imaging, cells were washed with PBS (×3) and fixed with 4% paraformaldehyde for 30 min. Subsequently, the cells were stained with Hoechst for 30 min and finally visualized by CLSM. For flow cytometric analysis, the cells were resuspended in 0.25% trypsin-EDTA and finally quantified by flow cytometry (LSRFortessa Cell Analyzer, BD, USA). The data were analyzed by Flow Jo software (BD, USA).

Endocytosis mechanistic studies

To investigate the endocytosis mechanism of TDNs, HEI-OC1 cells were incubated with 150 nM Cy5-TDNs for 6 h at 4 °C or pre-incubated with 20 µM inhibitors, including CPZ (clathrin-dependent endocytosis), MβCD (caveolin-dependent endocytosis), EIPA (micropinocytosis) or nocodazole (microtubules) for 1 h followed by co-incubation with 150 nM Cy5-TDNs and 20 µM inhibitors for another 6 h at 33 °C. The cells were then washed with PBS (×3). Intracellular fluorescence intensity was finally determined using a high content screening system (Operetta CLS, PerkinElmer, USA).

In vitro release study

The release of EGCG from EGCG@TDNs was measured using a dialysis method.[4] In brief, 500 µL sample solutions were transferred into dialysis bags (MWCO: 3.5 kDa) and then dialyzed against 100 mL PBS solution (pH = 6.0) at 37 °C with gentle shaking. Aliquots (100 µL) were taken at predetermined timepoints from dialysate and equal volumes of fresh PBS were added back into the outside dialysate. The increase of

EGCG concentration was evaluated using a multimode reader to measure the absorption of EGCG at 272 nm.

Cell viability assay

Cell viability was determined using a CCK-8 kit. HEI-OC1 cells were seeded in 96-well plates and cultured for 12 h. To establish the RSL3-induced lipid peroxidation cell model, cells were incubated with different concentrations of RSL3 (0, 0.5, 1, 2, 3 and 4 µM) for 24 h. Cells were incubated with TDNs (25–500 nM) or EGCG (1–50 µM) for 24 h to evaluate their cytotoxicity. Cells were co-incubated with TDNs/1 µM EGCG/1 µM EGCG@TDNs and 4 µM RSL3 for 24 h to evaluate their activity against RSL3-induced lipid peroxidation. Finally, 100 µL medium containing 10 µL CCK-8 working solution was added to each well and incubated for 2 h at 33 °C. The absorbance of each well was measured using a multimode reader (Tecan, Switzerland) at a wavelength of 450 nm.

Western blot analysis

HEI-OC1 cells were seeded in 6-well plates and cultured for 12 h. The cells were then co-incubated with TDNs/1 µM EGCG/1 µM EGCG@TDNs and 4 µM RSL3 for 24 h. The cells were harvested using RIPA lysis buffer containing protease inhibitors. Samples were boiled at 100 °C for 10 min to denature and separated by SDS-PAGE. The proteins were transferred to PVDF membranes, which were then incubated with blocking buffer (Beyotime, China) for 2 h at room temperature followed by incubation with primary antibodies against GPX4 (Proteintech, USA) and GADPH (ABclonal, China) overnight at 4 °C. Finally, the membranes were incubated with secondary antibodies for 1 h at room temperature and visualized using enhanced chemiluminescence (ThermoFisher Scientific, USA) on an Amersham Imager 600 imaging system (GE, USA).

Intracellular ROS detection

The fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was

used to detect intracellular ROS production. HEI-OC1 cells were seeded in 96-well confocal imaging plates and cultured for 12 h. The cells were co-incubated with TDNs/1 µM EGCG/1 µM EGCG@TDNs and 4 µM RSL3 for 24 h. Finally, the cells were stained with 10 µM DCFH-DA dye (Yeasen, China) for 30 min and visualized by CLSM. Intracellular fluorescence intensity was determined using a high content screening system (Operetta CLS, PerkinElmer, USA).

Mitochondrial membrane potential (MMP) assay

JC-1 dye (Invitrogen, USA) was used for monitoring mitochondrial membrane potential. HEI-OC1 cells were seeded in 6-well plates and cultured for 12 h. The cells were co-incubated with TDNs/1 µM EGCG/1 µM EGCG@TDNs and 4 µM RSL3 for 24 h. Subsequently, the cells were stained with 20 µM JC-1 dye for 30 min. After resuspension in 0.25% trypsin-EDTA, the cells were finally quantified by flow cytometry (LSRFortessa, BD, USA) and the data were analyzed using Flow Jo software (BD, USA).

Cell apoptosis assays

Cell apoptosis assays were performed using an Annexin V-FITC/PI double staining apoptosis detection kit. HEI-OC1 cells were seeded in 6-well plates and incubated for 12 h. The cells were co-incubated with TDNs/1 µM EGCG/1 µM EGCG@TDNs and 4 µM RSL3 for 24 h. The cells were then resuspended in trypsin free of EDTA and stained by FITC Annexin V and PI for 15 min according to the manufacturer's protocol. Finally, the cells were analyzed by flow cytometry (LSRFortessa, BD, USA) and the data were analyzed using Flow Jo software (BD, USA).

Animals

4-Week-old C57/BL6 male mice were obtained commercially from Shanghai Jihui Laboratory Animal Care Co., Ltd. (Shanghai, China). 6-Week-old male guinea pigs (weighing 220–250 g) were purchased from Shanghai Laboratory Animal Center (Shanghai, China). All animal studies were approved by the Ethics Committee of the

Shanghai Jiao Tong University School of Medicine.

Intratympanic injection procedure and RWM administration

C57/BL6 mice and guinea pigs were anesthetized and placed on a heating pad (37 °C). For RWM administration, a posterior auricle incision was made to expose the RWM niche of guinea pigs. Gelfoam containing 2.5 µM Cy5-TDNs solution (10 µL) was gently placed on the round window niche. For intratympanic injection, 100 µM EGCG or EGCG@TDNs solution (5 µL for C57/BL6 mice and 100 µL for guinea pigs) were injected into the middle ear through the tympanic membrane under a microscope to fill the entire middle ear cavity. Finally, the incomplete auditory bulla was sealed with dental cement and the incision was closed with sutures.

Validating the feasibility of TDNs reaching the inner ear

After RWM administration of Cy5-TDNs, guinea pigs were sacrificed at designated time points (0.5, 2 and 6 h). The cochleae were immediately isolated and washed with PBS. The distribution of Cy5-TDNs in cochleae was determined using an in vivo imaging system (IVIS Lumina III, PerkinElmer, USA). For imaging of RWM tissues, the cochleae were fixed with 4% (w/v) paraformaldehyde for 2 h at room temperature. The RWM tissues were isolated from cochleae with a microspring scissor followed by Hoechst staining for 30 min. All samples were visualized by CLSM and the mean fluorescence intensity of images were analyzed by ImageJ software. To determine fluorescence intensity of Cy5-TDNs in perilymph, perilymph was extracted from a small hole drilled at the basal turn of the cochlea. Perilymph (~7 µL) was mixed with distilled water (100 µL) and fluorescence intensity was measured using a multimode reader (Tecan, Switzerland). Ex: 620 nm, Em: 670 nm.

In vivo release profiles

Perilymph was extracted at different time points (5 min, 0.5, 1, 2, 4 and 6 h) after intratympanic administration of EGCG or EGCG@TDNs. All samples were stored at −80 °C for LC–MS/MS analysis. The mobile phases for HPLC consisted of water

containing 1% formic acid (A) and acetonitrile containing 1% formic acid (B). Gradient elution was carried out at a constant flow-rate of 0.6 mL/min using a gradient program: 0–0.30 min 0%–5% B, 0.30–1.20 min 5%–95% B, 1.20–1.50 min 95%–95% B, 1.50–1.51 min 95%–5% B, 1.51–2.00 min 5% B. The retention times of the analyte (EGCG) and internal standard (diclofenac) were 1.16 and 1.44 min, respectively. For MS analysis, the precursor product ion pair was m/z 459.40→139.20 for EGCG and m/z 446.20→321.10 for diclofenac.

Noise-induced hearing loss mouse model

To establish the noise-induced hearing loss mouse model, C57/BL6 mice were placed, conscious and unrestrained, in a round cage separated into eight chambers where differences in noise intensity in each chamber varied less than 1 dB. Noise signal was generated using MATLAB software with a bandpass of 8–16 kHz at 106 dB and operated with an amplifier and loudspeaker for 2 h. An acoustimeter was used to calibrate to the designated sound pressure level before each noise exposure procedure.

Animal grouping and intratympanic administration

To evaluate the protective function of EGCG@TDNs against noise-induced hearing loss, 17 C57/BL6 mice were randomly divided into three groups: (1) TDNs + noise (n = 5); (2) EGCG + noise (n = 6); (3) EGCG@TDNs + noise (n = 6). The mice were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (10 mg/kg). TDNs, 100 µM EGCG or 100 µM EGCG@TDNs were administered by intratympanic injection. The animals were then transferred to a heating pad (37 °C). The noise exposure procedure was performed 8 h later when all mice were fully conscious. Auditory brainstem response (ABR) recordings were performed on d0, d1 and d14. To evaluate the safety of intratympanic injection procedure, TDNs, EGCG and EGCG@TDNs. 12 C57/BL6 mice were randomly divided into four groups: (1) sham (n = 3); (2) TDNs (n = 3); (2) EGCG (n = 3); (3) EGCG@TDNs (n = 3). TDNs, 100 µM EGCG or 100 µM EGCG@TDNs were administered by intratympanic injection

and ABR recordings were performed on d0, d1, d7, d14.

Auditory function evaluation—ABR recordings

The auditory function of animals was evaluated by ABR recordings. In brief, anesthetized animals were placed on a heating pad (37 °C) in a standard sound-proof room. Three electrodes were inserted subdermally at the pinna (reference electrode), vertex (recording electrode), and rump (ground electrode). Pure tone stimuli of 4, 5.6, 8, 11.3, 16, 22.6 and 32 kHz were delivered through a closed-field MF-1 speaker. The sound level of each measurement was started from 90 dB to 0 dB in 5 dB steps. The ABR threshold was defined as the lowest sound level at which a noticeable response was observed.

Histological analysis

The cochleae were immediately isolated after animals were sacrificed on d14 and then fixed with 4% paraformaldehyde at 4 °C overnight. After decalcification in 0.12 mM EDTA at room temperature for 6 h, specimens were embedded in paraffin and sliced for hematoxylin and eosin staining. The expression of 4-hydroxynonenal was detected by 3',3'-diaminobenzidine staining and images were analyzed by ImageJ software to collect integrated optical density (IOD) values and calculate the average density (IOD/area)

Statistical analysis

Data analyses were performed in GraphPad Prism 8.0 software. Experimental data were presented as mean ± standard deviation (SD). One-way ANOVA and two-way ANOVA with Bonferroni correction were used for statistical analysis and p values < 0.05 were considered statistically significant.

Supplementary Figures and Table
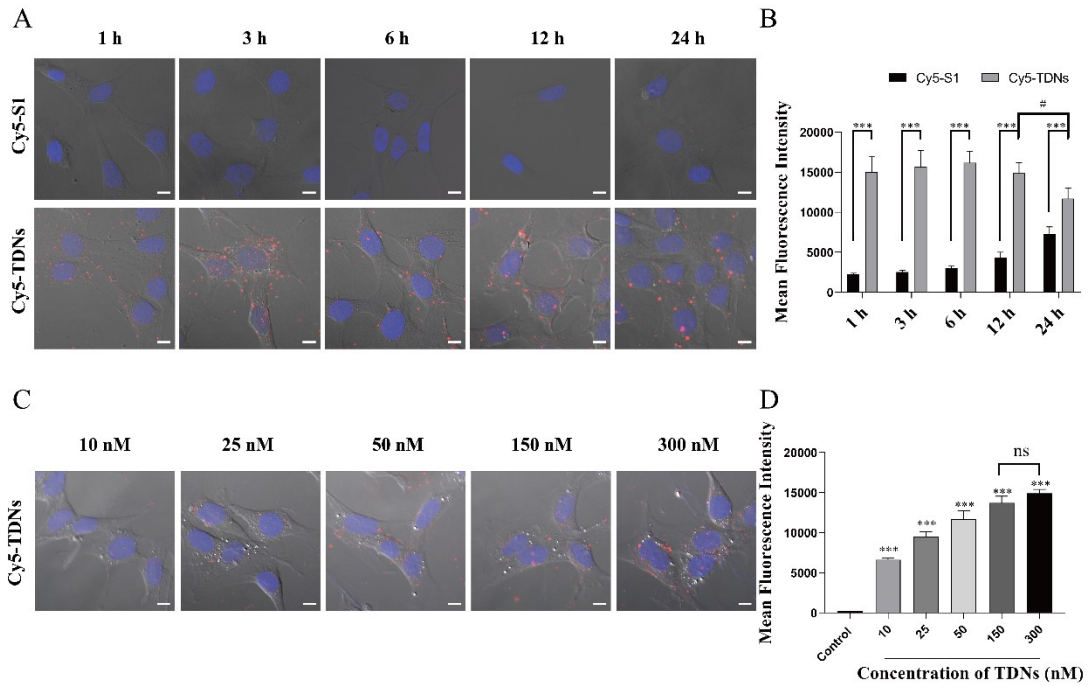
Figure S1. (A) Confocal images and (B) flow cytometry statistical analysis of HEI-OC1 cells incubated with 150 nM Cy5-TDNs or S1 at different time points (1, 3, 6, 12, 24 h). Scale bar = 10 μm. Statistical significance: $^{\#}P < 0.05$, $^{***}P < 0.001$. (C) Confocal images and (D) flow cytometry statistical analysis of HEI-OC1 cells incubated with a series of concentrations (10, 25, 50, 150, 300 nM) of Cy5-TDNs for 6 h. Scale bar = 10 μm. Statistical significance: $^{***}P < 0.001$ compared with control.
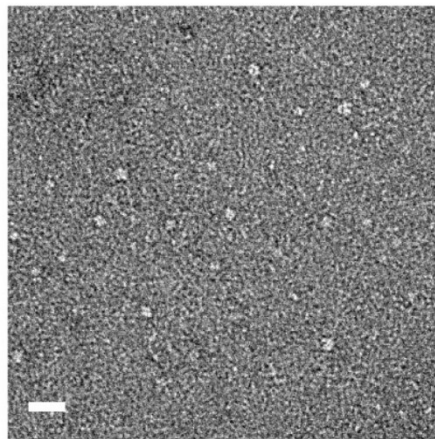
Figure S2. Transmission electron microscopy (TEM) image of TDNs. Scale bar = 20 nm.
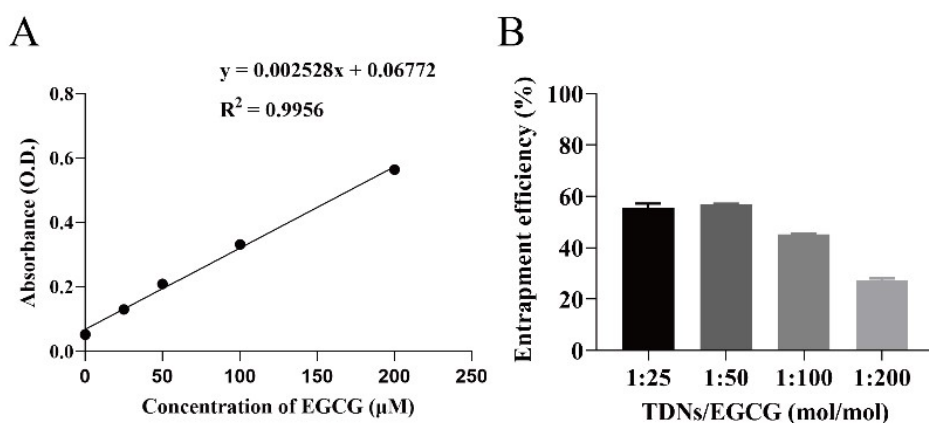
Figure S3. (A) Absorbance standard curve for quantification of EGCG loading into TDNs and (B) entrapment efficiencies of EGCG@TDNs under different TDN/EGCG molarity ratios (1:25, 1:50, 1:100, 1:200)
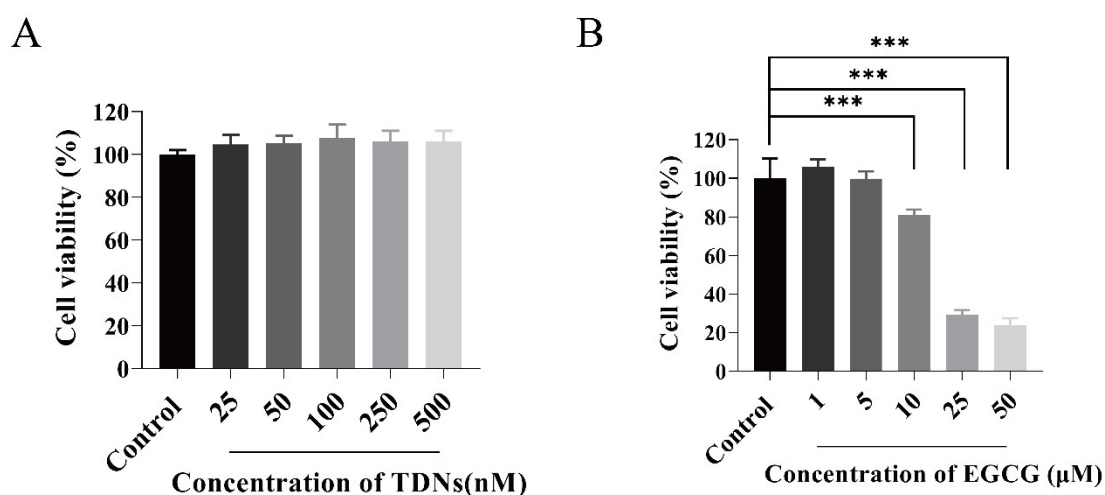
Figure S4. (A) Viability of HEI-OC1 cells incubated with a series of concentrations (0, 25, 50, 100, 250, 500 nM) of TDNs for 24 h. (B) Viability of HEI-OC1 cells incubated with a series of concentrations (0, 1, 5, 10, 25, 50 μM) of EGCG for 24 h. Statistical significance: ***$P < 0.001$.

Table 1. DNA strand sequences

Primer Name	Sequence (5'-3')
Cy5 single strand	Cy5-ATTTTATTTCGGAGCGAAGC

DNA strands for TDNs

Primer Name	Sequence (5'-3')	Note
S1	ATTTATCACCCGCCATAGTAGACGTATCACCAGGCAGTTGAGACGAACATTCCTAAGTCTGAA	Core
S2	ACATGCGAGGGTCCAATACCGACGATTACAGCTTGCTACACGATTCAGACTTAGGAATGTTCG	Core
S3	ACTACTATGGCGGGTGATAAAACGTGTAGCAAGCTGTAATCGACGGGAAGAGCATGCCCATCC	Core
S4	ACGGTATTGGACCCTCGCATGACTCAACTGCCTGGTGATACGAGGATGGGCATGCTCTTCCCG	Core
Cy5-S1	Cy5-ATTTATCACCCGCCATAGTAGACGTATCACCAGGCAGTTGAGACGAACATTCCTAAGTCTGAA	Cy5 modified

DNA strands for 6H × 73 nt

Primer Name	Sequence (5'-3')	
[1,20]	CCAGGTTAAGTTTTTTTTTTTTTTTTTTTTTTTTGGCTCAATC	Core
[1,41]	ATACTCACGGTTAACTGTTGTGTCCTATGTTTACTTAGGGAT	Core
[1,62]	GGTACGAACTCAACGCACGCTCGGGTAGTCTCAAGAAGATAG	Core
[1,83]	AGAGCATAAATTCGCTAAACCGGAAAAGTATCCGCATCTCAC	Core
[3,20]	TCAGTGATCGTTTTTTTTTTTTTTTTTTTTTTAGTATTCATG	Core
[3,41]	TCGCCTTTTCGAACGGTGACACACTTGTGTTCATTCAGAGTA	Core
[3,62]	GCGGAAATATGGCCGCTTCGGTGCGCAGCGGGCCGCCTGTAA	Core
[3,83]	CGCTTAATCAGCCCGGGCTTTTGCTAATACGCTGAGCGATGC	Core
[5,20]	TGTTGTTGCGTTTTTTTTTTTTTTTTTTTTTTTGGGTTTCTGA	Core
[5,41]	ACCGGAACCCGGGCTAATGAAGCCGTGACCCCAGAGACTGTC	Core
[5,62]	TGATGCACCGGGAGCCAGCCACTTAGCTGTGTCAGTGTGATC	Core
[5,83]	GCGAGTTCCCTCTAGCTAGGGTGATGCCAAAAGGGTGGATAT	Core
[0,31]	AAACATAGGACGATTGAGCCACGCAACAACATTCATTAGCCCGCTTCGCTCCGAAATAAAAT	Cy5 handle
[0,52]	GAGACTACCCGATCCCTAAGTGGGTTCCGGTTGGCTGGCTCC	Core
[0,73]	GATACTTTTCCCTATCTTCTTCGGTGCATCACCCTAGCTAGA	Core
[0,94]	TTTTTTTTTTTGTGAGATGCGGGGAACTCGCTTTTTTTTTTT	Core
[2,31]	GAACACAAGTGCATGAATACTCTTAACCTGGACAACAGTTAA	Core
[2,52]	CCCGCTGCGCATACTCTGAATCCGTGAGTATAGCGTGCGTTGGCTTCGCTCCGAAATAAAAT	Cy5 handle
[2,73]	GCGTATTAGCATTACAGGCGGAGTTCGTACCGGTTTAGCGAA	Core
[2,94]	TTTTTTTTTTTGCATCGCTCATTTATGCTCTTTTTTTTTTTT	Core
[4,31]	GGGGTCACGGCTCAGAAACCCCGATCACTGATGTCACCGTTC	Core
[4,52]	ACACAGCTAAGGACAGTCTCTGAAAAGGCGACCGAAGCGGCC	Core
[4,73]	TTTTGGCATCAGATCACACTGATATTTCCGCAAAGCCCGGGCGCTTCGCTCCGAAATAAAAT	Cy5 handle
[4,94]	TTTTTTTTTTTTATATCCACCCTGATTAAGCGTTTTTTTTTTT	Core

DNA strands for 4 × 4 × 64 nt

Primer Name	Sequence (5'-3')	
[1,23]	CCAGGTTAAGTGGCTCAATCATACTCACGGTTAACTGTTGTGTCCTAT	Core
[1,39]	GTTTACTTAGGGATGGTACGAACTCAACGCACGCTTCGCTCCGAAATAAAAT	Cy5 handle
[1,55]	GCTCGGGTAGTCTCAAGAAGATAGAGAGCATAAATTCGCTAAACCGGA	Core
[1,71]	AAAGTATCCGCATCTCACTCAGTGATCGAGTA	Core
[3,39]	TTCATGTCGCCTTTTCGAACGGTGACACACTTGTGTTCATTCAGAGTA	Core
[3,71]	GCGGAAATATGGCCGCTTCGGTGCGCAGCGGGCCGCCTGTAACGCTTA	Core
[4,8]	ATCAGCCCGGGCTTTT	Core
[5,23]	GCTAATACGCTGAGCGATGCTGTTGTTGCGGG	Core
[5,39]	GTTTCTGAACCGGAACCCGGGCTAATGAAGCCGTGACCCCAGAGACTG	Core
[5,55]	TCTGATGCACCGGGAGCCAGCCACTTAGCTGT	Core
[5,71]	GTCAGTGTGATCGCGAGTTCCCTCTAGCTAGG	Core
[7,23]	GTGATGCCAAAAGGGT	Core
[7,55]	GGATATGGAGCTGGAACGGCTCTGATTTCTAGTGACCTGGTATAATGG	Core
[7,71]	CGGAGCTACCTGACCTCAATCATACGTCCCAC	Core

[9,23]	GGAAGGATTCATCCCCTTTAAGAATGATCGCACAGTCTTTGAGATGTT	Core
[9,39]	GAACATCTTTTGGTTGGCGTAAAGATCTCGCA	Core
[9,55]	TTCTGGTGCCAAAACTGCCGACTCGTTCAAGCACTATTATCTGGCTAT	Core
[9,71]	AAAAGATGTCTGTCAGCCCAAGCTTAGCATAA	Core
[11,39]	GCGGCTGGACGACCCCGCCGTGCGACGACTGACCGTCGAGCCCTGCGC	Core
[11,71]	GACAAACGCATATCCTCGGCCCGCCAAATTAATAGTCGATTACGCTTC	Core
[12,8]	GCCTAAAGGTCCTTCC	Core
[13,23]	ACGGTAATCGGGGGTGACTCCGGCCTCCTTGG	Core
[13,39]	TATATTAGCTTACCCACTCGCGTGGCACGTCTACGACTCGTCGAACGA	Core
[13,55]	GGACTGTTCTACATAGTGACTTGGTTTTTTTTT	Core
[13,71]	ACGAGGTTTCACTGGGCGCTATACAGGAAACT	Core
[15,23]	CGAGGGGCTCCCTGTC	Core
[15,55]	TGTCGAGCCCAGCGACCACTTCTGGAGAAAGATGCGGTGGCAGTAGTT	Core
[15,71]	GTGATTAGCTTGGGGGTTTTTTTTTGATATAC	Core
[0,15]	GTATGATTTTTTTTTT	Core
[0,47]	CTATCTTCATAGGACACAACAGTTAACCGTGAGGCATCACCTAGAAAT	Core
[0,79]	TTTTTTTTTCCGGTTTAGCGAATTTATGCTCTCCATATCCAGGTCAGG	Core
[2,15]	ATGAACACTTTTTTTT	Core
[2,47]	ACAGGCGGGTGCGTTGAGTTCGTATACTCTGAGTATTAGCGTTCCGGT	Core
[2,79]	TTTTTTTTTACTCGATCACTGAGTTAAGCGTTGCATCAGATCGCGATC	Core
[4,15]	GGGCTGATTTTTTTTTTTTTTTTTAAGTGTGTCACCGTTCGAAAAGGC	Core
[4,31]	TAGCCCGGAAAAGCCCCGCACGGCGGGGTCGTGCTTCGCTCCGAAATAAAAT	Cy5 handle
[4,47]	GGGGTCACGGCTTCATGACATGAACCCGCTGCGCACCGAAGCGGCCAT	Core
[4,63]	GAGGGAACCAGTCTCTGCGGGCCGAGGATATG	Core
[4,79]	TTTTTTTTTCCTAGCTAATTTCCGCTTTTTTTT	Core
[6,15]	AACAGCATTTTTTTTTTTTTTTTTTGAGCCCACTTAACCTGGCCATCCCT	Core
[6,31]	CCAGGTCACCCGCAACATCCTTCCCAACCAAA	Core
[6,47]	GTGGCTGGCCATTATAAAGTAAACTTGAGACTACCCGAGCGAGATGCG	Core
[6,63]	TATGATTGACAGCTAACACCAGAACTGACAGA	Core
[6,79]	TTTTTTTTTGTGGGACGGATACTTTTTTTTTTT	Core
[8,15]	TTCTTAAATTTTTTTTTTTTTTTTTTACCCTTTTGCTTCGCTCCGAAATAAAAT	Cy5 handle
[8,31]	AAAGACTGTGCGATCAGCCCCTCGTCTTTCTCCAGAAGTGGTCGCTGG	Core
[8,47]	GAGTCGGCAACATCTCCAGAGCCGTTCCAGCT	Core
[8,63]	ATAATAGTGCTTGAACGCTCGACACCCCCAAGCTAATCACTTTTTTTT	Core
[8,79]	TTTTTTTTTATAGCCAGTAGCTCCGGCTTCGCTCCGAAATAAAAT	Cy5 handle
[10,15]	CTCGACGGTTTTTTTTTTTTTTTTTCGCTCAGC	Core
[10,31]	CTTTACGCGCGCAGGGATTACCGTTGGGTAAGCTAATATACTATGTAG	Core
[10,47]	ATCGACTATGCGAGATTCAGAAACCTCCCGGT	Core
[10,63]	AGCTTGGGGAAGCGTAAACAGTCCCCCAGTGAAACCTCGTTTTTTTTT	Core
[10,79]	TTTTTTTTTTATGCTAACACTGACTTTTTTTT	Core
[12,31]	CACGCGAGGGAAGGACCTTTAGGCTTTTTTTTTTTTTTTTTTCAGTCGT	Core
[12,63]	GTATAGCGTCGTTCGACGAGTCGTAGACGTGCCCAGCCGCTTAATTTG	Core
[12,79]	TTTTTTTTAGTTTCCTCGTTTGTCGCTTCGCTCCGAAATAAAAT	Cy5 handle
[13,0]	TTTTTTTTCACCCCCG	Core
[14,31]	CCACCGCACCAAGGAGGCCGGAGTTTTTTTTTTTTTTTTTTGGGGATGA	Core
[14,47]	CCAAGTCAAACTACTGAGATGTTCAGTTTTGG	Core
[14,79]	TTTTTTTTTGTATATCACATCTTTTTTTTTTTT	Core
[15,0]	TTTTTTTTTGACAGGGA	Core

DNA strands for 6 × 6 × 64 nt

Primer Name	Sequence (5'-3')	
[1,23]	CCAGGTTAAGTGGCTCAATCATACTCACGGTTAACTGTTGTGTCCTAT	Core
[1,39]	GTTTACTTAGGGATGGTACGAACTCAACGCAC	Core
[1,55]	GCTCGGGTAGTCTCAAGAAGATAGAGAGCATAAATTCGCTAAACCGGA	Core
[1,71]	AAAGTATCCGCATCTCACTCAGTGATCGAGTA	Core
[3,23]	TTCATGTCGCCTTTTCGAACGGTGACACACTTGCTTCGCTCCGAAATAAAAT	Cy5 handle
[3,39]	GTGTTCATTCAGAGTAGCGGAAATATGGCCGC	Core
[3,55]	TTCGGTGCGCAGCGGGCCGCCTGTAACGCTTA	Core
[3,71]	ATCAGCCCGGGCTTTTGCTAATACGCTGAGCG	Core
[5,39]	ATGCTGTTGTTGCGGGGTTTCTGAACCGGAACCCGGGCTAATGAAGCC	Core
[5,71]	GTGACCCCAGAGACTGTCTGATGCACCGGGAGCCAGCCACTTAGCTGT	Core
[6,8]	GTCAGTGTGATCGCGA	Core
[7,23]	GTTCCCTCTAGCTAGGGTGATGCCAAAAGGGT	Core
[7,39]	GGATATGGAGCTGGAACGGCTCTGATTTCTAGTGACCTGGTATAATGG	Core
[7,55]	CGGAGCTACCTGACCTCAATCATACGTCCCAC	Core
[7,71]	GGAAGGATTCATCCCCTTTAAGAATGATCGCA	Core
[9,23]	CAGTCTTTGAGATGTTGAACATCTTTTGGTTG	Core
[9,39]	GCGTAAAGATCTCGCATTCTGGTGCCAAAACT	Core
[9,55]	GCCGACTCGTTCAAGCACTATTATCTGGCTAT	Core
[9,71]	AAAAGATGTCTGTCAGCCCAAGCTTAGCATAA	Core
[11,23]	GCGGCTGGACGACCCC	Core
[11,55]	GCCGTGCGACGACTGACCGTCGAGCCCTGCGCGACAAACGCATATCCT	Core
[11,71]	CGGCCCGCCAAATTAATAGTCGATTACGCTTC	Core
[13,23]	GCCTAAAGGTCCTTCCACGGTAATCGGGGGTGACTCCGGCCTCCTTGG	Core
[13,39]	TATATTAGCTTACCCACTCGCGTGGCACGTCT	Core
[13,55]	ACGACTCGTCGAACGAGGACTGTTCTACATAGTGACTTGGACGAGGTT	Core
[13,71]	TCACTGGGCGCTATACAGGAAACTCGAGGGGC	Core
[15,23]	TCCCTGTCTGTCGAGCCCAGCGACCACTTCTG	Core
[15,39]	GAGAAAGATGCGGTGGCAGTAGTTGTGATTAG	Core
[15,55]	CTTGGGGGTGATATACCTCACTAAACTCCTTC	Core
[15,71]	TAATGTGACTAAGCGTTAACTCCAAAGGAAAC	Core
[17,39]	CAGCTATCCCATATTCGATGTAAGCGGACATCTATGCGCCCGATATCC	Core
[17,71]	GTTCAAACTAGGGGGCTTCTGACCAAAGCTCATGAGAATTGTAAACAG	Core
[18,8]	GAACAGCTTAGAGCAG	Core
[19,23]	TCAAGTTCGGATACGCTACCTATGGACGGGGA	Core
[19,39]	TATGCGACACCATGATCCGGATGAGTTATGAAGATCGTCATTGGGATA	Core
[19,55]	TACAATCTCGAAGTAAAGCATAGCACCCTGCG	Core
[19,71]	AAGATAGGGTCACAGCAGTAATACCTCTACCT	Core
[21,23]	TGTTGGACGTAAGCGTGAAATAACGACCAGAA	Core
[21,39]	AGCACGAGGATTATCACGTGTACCTCGAAATA	Core
[21,55]	ATCGTTAGTTTCTCGACTGTTCCGAAACAGGC	Core
[21,71]	AGCGGTAGCCTCCCACTTAACCTATGCCTTGG	Core
[23,23]	TAACTGATTTTCTCCT	Core
[23,55]	GGTTCCGCTAGCTGATCTATGGTCCTTAATATAACCCTCGGAGGCAAG	Core
[23,71]	GGGAGAGGGTGAAACAGGCCACCTGGCTCGGG	Core
[25,23]	GAGTGCCCCCTCGGCAGCCGCCTCGGTTATACTCGTCAAGCCTGCTTT	Core
[25,39]	CCATAGCTAACCCGATTGTGCATTCCACGTGA	Core
[25,55]	GTCGTACTTAAAGCAAACGTGGGAGTGCCCGAGCGTCTGAGGTATGGT	Core
[25,71]	TTACCCACTATGTTGTCTCAAGTGCTAACAGT	Core
[27,23]	ACTAATTATCCATAACTGTGCACGTCAGGGAT	Core

[27,39]	TCTCACCGGTGGCCGTTGCTACATCAGTTTCT	Core
[27,55]	TTGCACTCCTGGGGAAACTATACTTACGCACT	Core
[27,71]	GGTTTGGAGGGATTAGGCAATTGCTAGGTTGC	Core
[29,39]	ATTGTAGGCACAATTTTTGATTCGATCACATACATGGGCAATAAAGCT	Core
[29,71]	GTAGCCTGAGGCGCGCAGCTGCACCGTGTCACCTTGATATTGAGGGAC	Core
[30,8]	ACCTCGTCATTCGGAT	Core
[31,23]	AAACCATACTGAGGGCAGTGGGCGAGATCGGC	Core
[31,39]	TAATCATTAGTGCTCCCTTAGTTAGTTCATAGTTCATTAGGGGCGGAA	Core
[31,55]	ATGGGCTGGTGCCCCACGTACAGTAACCAACC	Core
[31,71]	GGTGTACCTGTGGGTAGGGCCGAGCGCTGGGC	Core
[33,23]	TTACATGTGCAAGTGAAATATATGACTTGGTC	Core
[33,39]	GACCAGATCTGCTAGAAACCACCAACATCCTC	Core
[33,55]	CTTGACACCTTTCAATTTTTAGTAAACACCCA	Core
[33,71]	ACAGAGCTGAACTTCCCATGGTCGTTCTACTG	Core
[35,23]	TCGTATGATCATAGGA	Core
[35,55]	TCAAACTAAATACAGCGTACCGCCAGTTCTTGTCCTTATTGGCGATCG	Core
[35,71]	TGCATTCCAATGTTTGATGGCCGCGTGGCTTA	Core
[0,15]	GTATGATTTTTTTTTTT	Core
[0,47]	CTATCTTCATAGGACACAACAGTTAACCGTGACCAGCCGCGCGCAGGG	Core
[0,79]	TTTTTTTTTCCGGTTTAGCGAATTTATGCTCTCGCACGGCTTAATTTG	Core
[2,15]	CACCGTTCTTTTTTTT	Core
[2,47]	ACAGGCGGGTGCGTTGAGTTCGTAAAGTGTGTAAAGACTGTGCGAGAT	Core
[2,79]	TTTTTTTTTACTCGATCACTGAGTTAAGCGTTGAGTCGGCCTGACAGA	Core
[4,15]	TAGCCCGGTTTTTTTTT	Core
[4,47]	GTGGCTGGGCGGCCATATTTCCGCGGCTTCATGAGGGAACTTCCAGCT	Core
[4,79]	TTTTTTTTTCGCTCAGCGTATTAGCACAGCTAATAGCTCCGGGGGATGA	Core
[6,15]	ACACTGACTTTTTTTTTTTTTTTTTTGTTCCGGTTCAGAAACCCCGCAAC	Core
[6,31]	CAGAGCCGTCGCGATCCTTACATCGAATATGG	Core
[6,47]	CCAGGTCACTAGAAATAACAGCATCTCCCGGTGCATCAGACAGTCTCT	Core
[6,63]	TTCTTAAACCATTATAGGTCAGAAGCCCCCTA	Core
[6,79]	TTTTTTTTTGCGATCAGGGGTCACGCTTCGCTCCGAAATAAAT	Cy5 handle
[8,15]	GGCATCACTTTTTTTTTTTTTTTTTTGAAAAGGCGACATGAATACTCTGA	Core
[8,31]	CACCAGAAACCCTTTTGACAGGGACCACCGCA	Core
[8,47]	TATGATTGAGTTTTGGATGAACACCCCGCTGCGCACCGAAAAAAGCCC	Core
[8,63]	AGCTTGGGGTGGGACGCCCCCAAGACGCTTAG	Core
[8,79]	TTTTTTTTTTATGCTAGGGCTGATTTTTTTTT	Core
[10,15]	AGATGTTCTTTTTTTTTTTTTTTTTGAGCCCACTTAACCTGGCCATCCCT	Core
[10,31]	CGTTTGTCCAACCAAACTTTAGGCTGGGTAAG	Core
[10,47]	ATAATAGTAGGATATGAAGTAAACTTGAGACTACCCGAGCGAGATGCG	Core
[10,63]	ATCGACTAATAGCCAGCGAGTCGTGTATAGCG	Core
[10,79]	TTTTTTTTGAAGCGTAGATACTTTTTTTTTTT	Core
[12,15]	ATTACCGTTTTTTTTTTTTTTTTTTTGGGGTCGT	Core
[12,31]	GCCGGAGTCACCCCCGATCAGTTAATATTAAG	Core
[12,47]	AACAGTCCCCAAGGAGCTCGACGGTCAGTCGT	Core
[12,63]	CCAAGTCACTATGTAGGCGGAACCTGTTTCAC	Core
[12,79]	TTTTTTTTAACCTCGTGCGGGCCGTTTTTTTT	Core
[14,15]	GTCGCTGGTTTTTTTTTTTTTTTTTTAACATCTC	Core
[14,31]	CACGCGAGCAGAAGTGGTCCAACATGATAATC	Core
[14,47]	TTAGTGAGAGACGTGCCTTTACGCGCTTGAAC	Core
[14,63]	AGTTTCCTGAAGGAGTCTAACGATGTGGGAGG	Core

[14,79]	TTTTTTTTGCCCCTCGCATCTTTTTTTTTTTT	Core
[16,15]	GGCGCATATTTTTTTTTTTTTTTTCCTAGCTA	Core
[16,31]	AACTACTGGGATATCGGAACTTGAATCATGGT	Core
[16,47]	AATTCTCACTAATCACCCATATCCAGGTCAGG	Core
[16,63]	TGGAGTTACTGTTTACAGATTGTAGCTGTGAC	Core
[16,79]	TTTTTTTTGTTTCCTTATCCTTCCTTTTTTTT	Core
[18,15]	AGCTGTTCTTTTTTTTTTTTTTTTGATGTCCG	Core
[18,31]	TCATCCGGCTGCTCTACGAATCAAAAATTGTG	Core
[18,47]	TGACGATCTTCATAACGATAGCTGTGAGCTTTGCTTCGCTCCGAAATAAAAT	Cy5 handle
[18,63]	GTATTACTTATCCCAAGTGCAGCTGCGCGCCT	Core
[18,79]	TTTTTTTTAGGTAGAGGTTTGAACTTTTTTTT	Core
[20,15]	CATAGGTATTTTTTTTTTTTTTTTGCTCGACA	Core
[20,31]	GGTACACGTCCCCGTCTAATTAGTACGGCCAC	Core
[20,47]	GCTATGCTTATTTCGATCTTTCTCGTATATCA	Core
[20,63]	TAGGTTAACGCAGGGTGAGTGCAACTAATCCC	Core
[20,79]	TTTTTTTTCCAAGGCATCACATTATTTTTTTT	Core
[22,15]	GTTATTTCTTTTTTTTTTTTTTTTGGAAGGAC	Core
[22,31]	CGAGGGTTTTCTGGTCGGGCACTCATCGGGTT	Core
[22,47]	CGGAACAGCTTGCCTCCTAATATATCGTTCGA	Core
[22,63]	AGGTGGCCGCCTGTTTAGTACGACACAACATA	Core
[22,79]	TTTTTTTTCCCGAGCCCCCAGTGATTTTTTTT	Core
[24,15]	GAGGCGGCTTTTTTTTTTTTTTTTTAGGAGAAA	Core
[24,31]	CTTGACGAGTATAACCTCATACGACAAGAACTGGCGGTACGCTGTATT	Core
[24,47]	TCCCACGTAAAGCAGGGACCATAGATCAGCTAGCTTCGCTCCGAAATAAAAT	Cy5 handle
[24,63]	TCAGACGCTCGGGCACTAGTTTGACAAACATTGGAATGCATTTTTTTT	Core
[24,79]	TTTTTTTTACCATACCCCTCTCCCGCTTCGCTCCGAAATAAAAT	Cy5 handle
[26,15]	CGTGCACATTTTTTTTTTTTTTTTTACGCTTAC	Core
[26,31]	AATGCACAATCCCTGAACATGTAATCTAGCAGATCTGGTCATTGAAAG	Core
[26,47]	AGTATAGTTCACGTGGCTCGTGCTTCGAGAAA	Core
[26,63]	CACTTGAGAGTGCGTAGTGTCAAGGGAAGTTCAGCTCTGTTTTTTTTTT	Core
[26,79]	TTTTTTTTTACTGTTAGCTACCGCTTTTTTTT	Core
[28,15]	TGCCCATGTTTTTTTTTTTTTTTTGCGTATCC	Core
[28,31]	ATGTAGCAAGCTTTATTATGGTTGGAGCACTAATGATTATGGGGCAC	Core
[28,47]	ATATCAAGAGAAACTGGTCGCATATTACTTCG	Core
[28,63]	GCAATTGCGTCCCTCACAGCCCATTACCCACAGGTACACCTTTTTTTT	Core
[28,79]	TTTTTTTTGCAACCTACCTATCTTTTTTTTTT	Core
[30,31]	TAACTAAGATCCGAATGACGAGGTTTTTTTTTTTTTTTTTTATGTGAT	Core
[30,63]	CTCGGCCCTTCCGCCCCTAATGAACTATGAACCCTACAATGTGACACG	Core
[30,79]	TTTTTTTTGCCCAGCGCAGGCTACTTTTTTTT	Core
[31,0]	TTTTTTTTGCCCTCAG	Core
[32,31]	TGGTGGTTGCCGATCTCGCCCACTTTTTTTTTTTTTTTTTTGTTATGGA	Core
[32,63]	CGACCATGGGTTGGTTACTGTACGGAGGATGTCGGTGAGATTCCCCAG	Core
[32,79]	TTTTTTTTCAGTAGAATCCAAACCTTTTTTTT	Core
[33,0]	TTTTTTTTTCACTTGC	Core
[34,31]	AATAAGGAGACCAAGTCATATATTTTTTTTTTTTTTTTTTTTGCCGAGG	Core
[34,63]	GCGGCCATTGGGTGTTTACTAAAACGATCGCCAGCTATGGTTGCTTTA	Core
[34,79]	TTTTTTTTTAAGCCACGTGGGTAATTTTTTTT	Core
[35,0]	TTTTTTTTTCCTATGA	Core

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