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Chapter 12

Albumin-Binding Fatty Acid–Modified Gapmer Antisense Oligonucleotides for Modulation of Pharmacokinetics

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Abstract

Prolonged circulation and modulation of the pharmacokinetic profile are important to improve the clinical potential of antisense oligonucleotides (ASOs). Gapmer ASOs demonstrate excellent nuclease stability and robust gene silencing activity without the requirement of transfection agents. A major challenge for in vivo applications, however, is the short blood circulatory half-life. This work describes ~~utilisation~~utilization of the long circulation of serum

albumin to increase the blood residence time of gapmer ASO. The method introduces fatty acid modifications into the gapmer ASOs design to exploit the binding and transport property of serum albumin for endogenous ligands. The level of albumin–/gapmer ASOs interaction, blood circulatory half-life and biodistribution was dependent on number, position, and fatty acid type (palmitic or myristic acid) within the gapmer ASO sequence and either phosphorothioate or phosphodiester backbone modifications. This work offers a strategy to optimiseoptimize gapmer ASO pharmacokinetics by a proposed endogenous assembly process with serum albumin that can be tuned by gapmer ASO design modifications.

Key-words

Gapmer antisense oligonucleotides

Palmitic acid

Myristic acid

Serum albumin

Circulatory half-life

Pharmacokinetics

1. Introduction

Antisense oligonucleotides (ASOs) are synthetic single stranded nucleic acids that bind to complementary mRNA through Watson–Crick base pairing and activate RNase H-mediated cleavage to act as gene silencing therapeutics [1, 2]. In combination with flanked modified oligonucleotides such as locked nucleic acid (LNA) [3], the so-called gapmer ASOs are highly stable against nucleases and offer flexible gene silencing approaches due to unassisted

intracellular gymnotic delivery [4, 5]. However, the short circulatory half-life has remained one of the major challenges following systemic administration due to their small size (~6 ~~K~~kDa) that results in rapid renal clearance [6, 7]. Human serum albumin (HSA) has been exploited as a potent drug half-life extension strategy [8–10] due to its property as a natural transport protein facilitated by binding sites for endogenous and exogenous ligands and its long circulatory half-life by engagement with the cellular recycling neonatal Fc receptor (FcRn) [11, 12]. Multiple fatty acid binding sites on albumin [13, 14], therefore, can be ~~utilised~~utilized to extend the circulatory half-life of gapmer ASOs by including fatty acids into the nucleic acid design.

This chapter describes albumin interaction with ASOs with either palmitoyl or myristoyl modifications varying in position and number, with either a phosphorothioate or phosphodiester backbone and the effect on gapmer pharmacokinetics. The level of albumin-binding affinity had a significant impact on the circulatory half-life in mice, with a maximal 2.4-fold increase of circulatory half-life in mice (66 –min) that correlated with the highest albumin-binding affinity of two-palmitoyl phosphorothioate gapmer ASO in vitro [15]. Fatty acid gapmer ASOs also show maintained unassisted in vitro gene silencing activity in different cell lines [16]. Systemic administration of fatty acid gapmer ASO that exhibit prolonged circulation may be a promising gene silencing strategy for therapeutic applications.

2. Materials

2.1. Oligonucleotide S~~s~~ynthesis

All basic chemical reagents used were purchased from Sigma-Aldrich (St. Louis, MO, USA)

or Fluka (Honeywell lab, Morristown, NJ, USA) and used without further purification. Use ultrapure water (resistivity, 18.2 MΩ cm at 25 °C) and analytical grade reagents to prepare all solutions. Prepare and store all reagents at room temperature unless otherwise specified.

1. Standard DNA phosphoramidite monomers, solid supports and cyanine 5.5 (Cy5.5) phosphoramidite and additional reagents from Sigma-Aldrich, GE Healthcare (Chicago, IL, USA) or Glen Research (Sterling, VA, USA).
2. Locked nucleic acid (LNA) phosphoramidite monomers from Exiqon, now a QIAGEN company (Hilden, Germany).
3. Synthesize the 2′ -*N*-palmitoyl-amino-LNA-T and 2′ -*N*-myristoyl-amino-LNA-T phosphoramidite monomers according to literature procedure [17].
4. Anhydrous acetonitrile: activated molecular sieves (3 Å, 8–12 mesh) is added to acetonitrile (HPLC grade) and the mixture is kept for 24 h. The dryness of the solvent is confirmed on a Coulometric Karl-Fischer titrator. (*see* **Note 1**).
5. Solution of nucleoside phosphoramidite monomers: activated molecular sieves (3 Å, 8–12 mesh) are added into the 0.1 M solutions of standard DNA and LNA monomers in anhydrous acetonitrile (≤15 ppm) and the solutions are dried for 24 h (*see* **Note 2**).

2.2. RP-HPLC, IE-HPLC, and MALDI-MS

1. A-buffer (0.05 M triethylammonium acetate, pH 7.4): (1) Triethylammonium acetate stock solution (1 M, pH 7.4): 1 mol triethylamine (139.88 mL) is

emulsified in ultrapure water (600 mL) and the resulting mixture is cooled in a water/ice bath. To this 1 mol glacial acetic acid (57.25 mL) is added slowly and the solution is then warmed to room temperature. Triethylamine or acetic acid can be used to adjust pH to 7.4 if necessary. The solution is transferred into a 1 L volumetric flask, and ultrapure water is added to have the solution volume reach 1 L. (2) 1 M stock solution (250 mL, pH 7.4) is diluted to 5 L using ultrapure water (*see* **Note 3**). The resulting solution is filtered using a nylon membrane (Waters, 47 mm, 0.2 μm) before use.

2. B-buffer (A-buffer/acetonitrile = 1:3, v/v): Mix A-buffer (500 mL) and acetonitrile (1500 mL, HPLC grade) extensively (*see* **Note 3**). Filter the resulting solution using a nylon membrane (Waters, 47 mm, 0.2 μm) before use.
3. Solution of sodium acetate (3 M): Dissolve 0.75 mol sodium acetate (61.53 g) in ultrapure water (150 mL). Transfer the solution into a 250 mL volumetric flask and add ultrapure water to have the final volume at 250 mL.
4. Solution of sodium perchlorate (5 M): Dissolve 1.25 mol sodium perchlorate (153.05 g,) in 150 mL ultrapure water. Transfer the solution into a 250 mL volumetric flask and add ultrapure water to have the final volume at 250 mL.
5. 3-Hydroxypicolinic acid (HPA) matrix: Dissolve 100 mg diammonium hydrogen citrate in 1 mL ultrapure water, of which 10 uμL is taken and mixed with 10 mg HPA in 0.99 mL ultrapure water.
6. C-buffer (sodium perchlorate, 1.0 M): Dissolve 3 mol sodium perchlorate

(367.32 g) is dissolved in 2400 mL ultrapure water. Transfer the solution to a 3000 mL volumetric flask and add ultrapure water to have the final volume at 3000 mL (*see* **Note 3**). Filter the resulting solution using a nylon membrane (Waters, 47 mm, 0.2 μm) before use.

7. D-buffer (0.25 M Tris–HCl, pH 8.0): Dissolve 0.75 mol trisaminomethane (90.84 g) in 2400 mL ultrapure water, to which add concentrated hydrochloric acid to adjust the pH to 8. Transfer the solution into a 3 L volumetric flask and add ultrapure water to have the final volume of 3 L (*see* **Note 3**). Filter the resulting solution using a nylon membrane (Waters, 47 mm, 0.2 μm) before use.

8. Reversed-phase HPLC (RP-HPLC, Waters System 600 HPLC equipment) with a Waters XBridge BEH C18-column (5 μm, 100 mm × 19 mm).

2.3. Electrophoretic Mmobility Sshift Aassay

Prepare all solutions of oligonucleotides (RNA, DNA, gapmer ASOs) using nuclease-free water. Gapmer ASOs are stored at –20 °OC and thawed on ice before use. All the chemical reagents are analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA) unless noted.

1. Fatty acid gapmer ASOs (either palmitoyl or myristoyl modifications varying in one or two fatty acids, both at 3′ end or one either at the 3′ and 5′ end, with phosphodiester or phosphorothioate backbone).

2. Recombinant human serum albumin.

3. Novex Gel Cassettes (8 cm × 8 cm × 1 mm).
4. ProtoGel (30 %).
5. Ammonium persulfate: 10 % (w/v) solution in Milli-Q water.
6. *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine (TEMED).
7. Running buffer: 1 × TBE (tris–borate–ethylenediaminetetraacetic acid [EDTA]) buffer (from 10 × stock; GIBCO, Life Technologies, Grand Island, NY, USA).
8. Loading buffer: 20 mg Orange G and 1.5 mL glycerol in 10 mL Milli-Q water.
9. Gapmer ASOs staining solution: Diluted in Milli-Q water from 10,000 × SYBR Gold Nucleic Acid Gel Staining Concentrate in DMSO).
10. Coomassie blue staining solution: 45 % methanol, 10 % acetic acid, and 3 g/L Coomassie Brilliant Blue R in Milli-Q water.
11. Coomassie Blue destaining solution: 10 % methanol and 7.5 % acetic acid in Milli-Q water.
12. XCell SureLock® Mini-Cell, Gel Doc™ EZ Imager.

2.4. Nanoparticle Ttracking Aanalysis

1. Palmitoyl gapmer ASOs and myristoyl gapmer ASOs (varying of 1 or 2 fatty acids, both at 3′ end or one either at the 3′ and 5′ end, with phosphodiester or phosphorothioate backbone).
2. Recombinant human serum albumin (Sigma-Aldrich, St. Louis, MO, USA).

3. Two-component 1 –mL syringe (Frisenette ApS, Knebel, Denmark).
4. Nanoparticle tracking analysis (NanoSight LM10, 405 –nm laser, Malvern Instruments SA, Worcestershire, UK), thermometer (OMEGA HH804, Engineering, Inc./Stamford, CT, USA).

2.5. In Vitro Cellular Gene Silencing ~~in vitro~~

1. Complete cell culture medium dependent on cell lines.
2. RNA extraction: TRIzol, chloroform, isopropanol, absolute ethanol, glycogen (optional).
3. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK).
4. Real-Time Polymerase Chain Reaction (PCR): SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). 384-well standard PCR Plate –and Adhesive PCR Plate Seals.
5. Forward and reverse primers of target genes and house-keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). GAPDH forward primer (Integrated DNA Technologies, Coralville, IA, USA): 5′ -GTCAGCCGCATCTTCTTTTG-3′; reverse primer: 5′ -GCGCCCAATACGACCAAATC-3′.
6. LightCycler 480 System (Roche Diagnostics, Basel, Switzerland).

2.6. ~~Oligonucleotides pharmacokinetic evaluation in vivo~~In Vivo Pharmacokinetic Evaluation of Oligonucleotides

1. Cy5.5-~~-~~label~~l~~ed fatty acid gapmer ASOs (with one or two palmitoyl modifications both at 3′ ~~'~~ end).
2. Seven- to eight-week-~~-~~old female C57BL/6 mice (Janvier Labs, Berthevin, France).
3. Capillary tubes coated with heparin (Hirschmann® Laborgeräte GmbH & Co).
4. Low-fluorescent diet (AIN-76A, Research Diets Inc., New Brunswick, NJ).
5. In Vivo Imaging System (IVIS, PerkinElmer, USA).

3. Methods

3.1. Oligonucleotide S~~s~~ynthesis

All oligonucleotide preparation and analysis are carried out at room temperature unless otherwise specified.

1. Mount solutions (0.1 –M) of standard DNA and LNA monomers in anhydrous acetonitrile onto the designated positions on the DNA synthesizer respectively (*see* **Note 4**).
2. Perform oligonucleotide synthesis on an ÄKTA oligopilot plus 10 system in 1.0 –µmol scale (polystyrene support) or Uni80 support using the phosphoramidite approach. The coupling time for standard DNA and LNA monomers is 720 –s and monitor stepwise coupling efficiencies in a continuous manner determined by the absorbance of the dimethoxytrityl cation at 500 –nm (*see* **Note 5**).

3. Incorporate palmitoyl-amino-LNA-T phosphoramidite, myristoyl-amino-LNA-T phosphoramidite and Cy5.5 phosphoramidite (0.05 M, in anhydrous acetonitrile) into oligonucleotides via a manual coupling procedure [18] using 5-[3,5-bis(trifluoromethyl)phenyl]-*H*-tetrazole (0.25 M, in anhydrous acetonitrile) as activator and extended coupling time (20 min) (*see* **Note 6**).

4. Cleavage of oligonucleotides from solid support and removal of nucleobase protecting groups are performed using 28% aqueous ammonia (1 mL) for 16 h at 55 °C (*see* **Note 7**).

5. Purify the resulting oligonucleotides by reversed-phase HPLC (RP-HPLC). Start elution with an isocratic hold of A-buffer for 2 min followed by a linear gradient to 70% B-buffer over 38 min at a flow rate of 2.5 mL/min. Omit reversed-phase HPLC purification when the purities of the crude oligonucleotides was sufficiently high (>80% by ion-exchange HPLC analysis).

6. Combine fractions of isolated oligonucleotides and evaporate under a flow of nitrogen to remove the solvents (*see* **Note 8**).

7. The purified oligonucleotides are subjected to standard detritylation (80% aqueous acetic acid at room temperature) and then precipitate from abs. Eethanol by first adding aqueous solutions of 3 M sodium acetate (15 µL) and 5 M sodium perchlorate (15 µL) followed by addition of abs. Eethanol (1 mL, −20 °C). Store the resulting suspensions at −20 °C for 1 h, and remove the supernatants after centrifugation (13,200 rpm, 5 min, 4 °C), and wash the pellet with cold abs. Eethanol (2 × 1 mL; −20 °C) and dry for 20 min at

50 °C on a metal heat block under a flow of nitrogen.

8. Dissolve the pellets in ultrapure water (1 mL) and analyze by MALDI-TOF MS and analytical ion-exchange HPLC (IE-HPLC). Record mass spectra of oligonucleotides on a Bruker Daltonics Microflex LT MAIDI-TOF MS instrument in ES+ mode using HPA matrix. Record analytical IE-HPLC traces on a Merck-Hitachi Lachrom system equipped with a DNAPac PA100 analytical column (13 µm, 250 mm × 4 mm) heated to 60 °C. Perform elution with an isocratic hold of D-buffer (10 %), starting from 2 min hold using 2% C-Buffer in ultrapure water, followed by a linear gradient to 30 % C-buffer in 23 min at a flow rate of 1.1 mL/min.
9. Determine concentrations of purified oligonucleotides by UV absorption measurements at 260 nm.

3.2. Electrophoretic Mmobility Sshift Aassay

The binding affinity between fatty acid gapmer ASOs and rHSA can be estimated by visualizing the assembled rHSA/gapmer ASOs constructs at different molar ratios using an electrophoretic mobility shift assay.

1. For preparation of an 8 % native polyacrylamide gel: mix 2.7 mL of Protogel, 6.3 mL of Milli-Q water, and 1 mL of 10 × TBE running buffer. Add 100 µL of 10 % (w/v) APS solution and 10 µL of TEMED.
2. Cast gel solution into an empty gel cassette with a 10-well comb and wait for 40 min to allow for gelation.
3. Mix fatty acid-gapmer ASOs (either palmitoyl or myristoyl modifications

varying in 1 or 2 fatty acids, both at 3′ end or one either at the 3′ and 5′ end, with phosphodiester or phosphorothioate backbone) at 1 μM final concentration with rHSA at molar ratios of 40, 20, 10, 5, 2.5, 1.25, 0.63, and 0 (rHSA: gapmer ASOs) in a final volume of 20 μL for each sample. Incubate for 30 min at room temperature before loading onto gels.

4. Mix samples with loading buffer at volume ratio 1:1, and load 14 ~~u~~μL for each well in the gel. Run the gel at 125 V for 45 min in an XCell SureLock® Mini-Cell.

5. Stain the fatty acid-gapmer ASOs with 50 mL of 1× SYBR Ggold solution for 15 min and image the gel with a Gel Doc™ EZ Imager. To stain rHSA, incubate the gel with Coomassie blue staining solution for 15 min and destain in destaining solution overnight before imaging (*see* **Note 9**).

3.3. Nanoparticle T~~t~~racking A~~a~~nalysis

Fatty acid–modified gapmer ASOs may tend to aggregate in aqueous solutions. The addition of rHSA can ~~solubilise~~solubilize the aggregated fatty acid gapmer ASOs and demonstrate the formation of rHSA/fatty acid gapmer ASOs constructs by determining the nanoparticle number changes in solution using nanoparticle tracking analysis (NTA).

1. Incubate for 4 h at room temperature fatty acid gapmer ASOs (1 μM) either mixed with or without rHSA (40 uM). Dilute samples in 500 μL of Milli-Q water (*see* **Note 10**) are load into the sample chamber of NTA using a 1 mL syringe (*see* **Note 11**).

2. Set cameral level of ~~Nanosight~~NanoSight to 10~~ ~~13 (*see* **Note 12**) and record 5 videos of samples for 60 ~~ s~~econds~~ by advancing syringe of 100 ~~ µL each time. Temperature in sample chamber is recorded manually using a thermometer within the range of 22~~ ~~24 °C.

3. ~~Analyse~~Analyze videos of particle motions with threshold between 2 and ~~ 5 (*see* **Note 13**) to obtain nanoparticle sizes and concentrations. The nanoparticle concentrations can be interpreted as the aggregation of fatty acid gapmer ASOs with and without albumin.

3.4. In Vitro Cellular Ggene Ssilencing ~~in vitro~~

The unassisted gene silencing ability of gapmer ASOs offers great flexibility of formulation design and reduced cytotoxicity due to the absence of a transfection agent. Gapmer ASOs can be added directly into complete cell culture medium and silencing activity can be determined using real-time polymerase chain reaction (PCR).

1. Thaw cells and centrifuge to remove residual DMSO and grow in complete cell culture media for at least ~~two~~ 2 weeks before determination of gene expression levels.

2. Seed cells onto plates when cells are 80~~ ~~90% confluent in flasks. Optimize the number of cells seeded by determination of target gene expression levels to allow the amount of extracted RNA to be sufficient for Real-Time PCR measurement. For example, use 12-well plates for primary human chondrocytes with

1 × 10^5 cells/well in 1 mL complete medium and 24-well plates for Caco-2 cells with 5 × 10^4 cells/well in 0.5 mL complete medium.

3. Transfect cells with fatty acid gapmer ASOs when at 90% confluency in wells. On the day of transfection, refresh the complete cell culture media 1 h before transfection and add fatty acid gapmer ASO solutions directly to the media at final concentrations of 0.5 ~ 10 μM. (*see* **Note 14**). Mismatch control groups include gapmer ASOs with and without the same fatty acid modification (*see* **Note 15**).

4. After 36 72 h incubation with fatty acid gapmer ASOs (*see* **Note 16**), extract total RNA using TRIzol reagent according to the manufacturer's protocol. After resuspension of RNA in nuclease-free water, reversely transcript RNA into complementary DNA (cDNA) using High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol. (*see* **Note 17**) cDNA can be stored at 20 °C for at least 1 month or at 4 °C overnight.

5. Use the cDNA for real-time polymerase chain reaction (PCR) with SYBR Green master Mix to determine the gene expression levels normalized to house-keeping gene GAPDH. Dilute cDNA samples in nuclease-free water for 5 20 times and mix with corresponding forward and reverse primers in 384-well PCR plate (*see* **Note 18**). Add SYBR green master mix (from 2 × concentrated stock) to the samples (*see* **Note 19**) and seal plates with Adhesive PCR Plate Seals. Perform Real-time PCR using a LightCycler 480 System with thermal cycles following the manufacturer's protocol of SYBR

green. ~~Analyse~~Analyze real-time PCR data using LightCycler480 software (version 1.5) to obtain the cycle quantification (Cq) values and calculate gene expression levels using double delta analysis by normalization to the house-~~-~~keeping gene expression levels.

3.5. ~~Oligonucleotide~~ In Vivo P~~p~~harmacokinetic E~~e~~valuation of ~~in vivo~~Oligonucleotides

Circulatory half-life and biodistribution of fatty acid gapmer ASOs can be determined by detection of a far-red fluorophore Cy5.5 attached at the 5′ ~~-~~ end of oligonucleotides. Fatty acid gapmer ASOs can display an extended circulatory half-life that correlates to the albumin-binding affinity.

1. Maintain Female C57BL/6 mice of 7~~seven~~- to 8~~eight~~-week-old for 2 ~~-~~weeks prior to experiments and feed with low-fluorescent diet. Divide mice into experimental groups (fatty acid gapmer ASOs) and control groups (PBS injection and non~~-~~-fatty acid gapmer ASOs) with 4~~-~~-6 mice per group.
2. On the day of pharmacokinetic evaluation, administer 100~~-~~ ~~u~~µL of a 3.5 ~~-~~mg/kg Cy5.5-label~~l~~ed fatty acid gapmer ASOs intravenously by tail vein injection from a 5 ~~-~~mg/mL stock diluted in PBS. After injection, collect 10 µL blood samples either by sublingual puncture at 1 ~~-~~min or by tail-nicking at other time points. Isolate plasma samples by centrifuge and kept at 4 °C before fluorescence intensity scan in the In Vivo Imaging System with the emission filter settings

(720 –nm, 740 –nm, 760 –nm, and 780 –nm) for Cy5.5 fluorophore.

3. At 24 –h, terminate the mice and harvest organs such as kidneys, liver, spleen, lung and heart –for IVIS scanning as described above.

4. ~~Analyse~~Analyze fluorescence intensities by quantitation of regions of interest (ROI) and normalize to the PBS control group (*see* **Note 20**) using Living Image Software (version 3.2). Calculate the circulatory half-life of gapmer ASOs using a one phase decay model in GraphPad Prism software (version 7.0).

4. Notes

1. The amount of water in acetonitrile should be lower than 15 –ppm as measured on a coulometric Karl-Fischer titrator. If the water content is above this value, additional molecular sieves should be added for further water removal before re-measuring.

2. The molecular sieves are added after dissolving all the solid phosphoramidite monomers in anhydrous acetonitrile.

3. A homogeneous solution should be obtained after extensive mixing.

4. To prime all the positions on the DNA synthesizer, it is important to ensure that each tube is filled with the corresponding monomer solution.

5. Stepwise coupling efficiency in all cases should be >98.0%.

6. Each phosphoramidite monomer (0.1 –mmol) is dissolved in anhydrous acetonitrile (1.0 –mL), 0.5 –mL of which is taken and gently mixed with the activator solution (0.5 –mL, 5-[3,5-bis(trifluoromethyl)phenyl]-*H*-tetrazole in

acetonitrile, 0.25 –M) in a 1 –mL syringe. The syringe is placed on one end of the synthesis column and the solution is gradually injected into the column during 20 –min (starting with 0.2 –mL, followed by 0.05 –mL per minute).

7. The cleavage and deprotection should be carried out in a sealed tube, preventing the evaporation of ammonia.

8. RP-HPLC, IE-HPLC, and MALDI-TOF MS can be carried out on some fractions to guide the proper pooling of fractions.

9. Stain fatty acid-gapmer ASOs first and then stain rHSA for high quality gel pictures if both oligonucleotide and protein staining is required.

10. Optimal dilution factors for ~~Nanosight~~NanoSight analysis need to be determined by the number of particles with ideally 50 ~ –100 particles in the field of view.

11. Ensure no observable bubbles in ~~Nanosight~~NanoSight chamber when injecting samples with a syringe.

12. Higher camera levels of the ~~Nanosight~~NanoSight can maximize the ~~visualisation~~visualization of small particles but can overexpose particles (overexposed particles will show purple ~~colour~~color on screen).

13. When ~~analysing~~analyzing ~~Nanosight~~NanoSight videos of samples, keep threshold as low as possible to include as many particles as possible but number of blue crosses (background noise) should be below 5 per frame.

14. Optimal concentration of fatty acid-gapmer for cellular gene silencing is dependent on the cell type and gene expression level, corresponding siRNA formulated with transfection agents such as lipofectamine can be used as a

positive control but the media needs to be changed 4 –h after transfection to minimize cytotoxicity of transfection agents.

15. Blast the mismatch control sequence with the Nucleotides BLAST program (https://blast.ncbi.nlm.nih.gov/BlastAlign.cgi) to ensure no relevant off-target gene silencing effects.
16. Maximal gene silencing time point after addition of fatty acid-gapmers to media needs to be optimized based on cell types and gene targets.
17. Maximal 10 –µg RNA can be transcripted per sample using cDNA reverse transcription kit.
18. Optimal concentration of cDNA and primers for real-time PCR need to be optimized but can start with a relatively high cDNA concentration (5 ~ – 10 –× –dilution) and a final concentration of 250 –nM with each primer.
19. SYBR green master mix can be premixed with primers to be added at the same time for real-time PCR.
20. Spectral Unmixing Mode is used to generate an overlapping emission spectrum from different emission results obtained at 720 –nm, 740 –nm, 760 –nm, and 780 –nm for Cy5.5 fluorescence intensity analysis using Average Radiant Efficiency.

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