RNA knockdown using gapmer Antisense Oligonucleotides (ASOs)

IDT products used in this protocol:
Antisense oligonucleotides, gBlocks™ Gene Fragments,
PrimeTime™ qPCR Probe Assays, PrimeTime™ Gene Expression Master Mix

Background

Antisense oligonucleotides (ASOs) are chemically modified oligonucleotides designed to degrade a target RNA through RNase H1-mediated cleavage or to sterically block other biomolecules, such as proteins or nucleic acids, from binding to a target RNA site. For both mechanisms of action, ASOs typically contain a phosphorothioate backbone to increase nuclease resistance and promote non-specific protein binding to facilitate intracellular uptake. Additionally, ASOs also typically contain 2′-ribose modifications, such as 2′-O-methoxyethyl (2′MOE) or locked nucleic acids (LNA® (Qiagen) or IDT's Affinity Plus™ oligos), to increase the binding affinity to the target RNA and further enhance nuclease resistance. RNase H1 is an endogenous enzyme that binds to an RNA:DNA heteroduplex and subsequently cleaves the RNA. For RNase H1 degradative ASOs, a stretch of DNA bases (optimally 8–10) must be present within the ASO to form the heteroduplex required for RNase H1 recognition. A common strategy for optimal RNase H1 ASO design is the "gapmer", whereby a stretch of 10 DNA bases is flanked on both sides by T_m-enhancing modifications such as 5 2′MOE bases (5-10-5 design) or 3 locked nucleic acid bases (3-10-3 design) with a phosphorothioate backbone throughout the oligonucleotide. For steric blocking ASO design, the DNA "gap" domain is eliminated by substituting with 2′-ribose chemical modifications to prevent the ASO from functioning as an RNase H1 degradative ASO. While both types of ASO strategies are supported by IDT, this protocol is focused on the delivery of RNase H degradative gapmer ASOs into cell culture. For further information on ASO design strategies, refer to the eBook **Designing RNase H1 "Gapmer" Antisense Oligonucleotides** located on the IDT website. This reference discusses multiple design parameters (e.g. ASO GC content and binding affinity, ASO secondary structure or self-dimerization potential, RNA target accessibility, off-target binding, etc.) that should be taken into consideration when designing gapmer ASOs to avoid sites predicted to be problematic and increase the probability that a potent site is identified. However, even when ASO design guidelines are followed it is often challenging to predict potent sites *in silico*. Therefore, we recommend screening 5-10 ASO sites that meet the design parameter criteria and empirically identifying the most active site from this subset. For assistance with ASO design, **contact us** and include species of interest, the target FASTA sequence or accession number, and the desired ASO application (RNA knockdown, steric blocking, splice-switching).

This protocol provides a demonstrated method to deliver gapmer ASOs in cell culture with cationic lipid transfection. To confirm that the gapmer ASO transfection protocols and reagents are working properly, a positive control example is provided using 2'MOE or Affinity Plus gapmers targeting the human long non-coding RNA (lncRNA) *MALAT1* (metastasis associated in lung adenocarcinoma transcript 1) which is highly expressed in multiple cell lines. A negative control sequence is included to ensure that target knockdown and associated phenotype is specific to the on-target gapmer ASO and to assess inherent toxicity that sometimes occurs when introducing chemically modified ASOs into cells.

Knockdown results are measured using reverse transcription quantitative PCR (RT-qPCR) and calculated by comparing RNA levels between the on-target gapmer ASO, the negative control gapmer ASO, and a mock control containing only the cationic lipid transfection reagent. It is recommended to compare the knockdown results from two distinct 5' nuclease qPCR assays that are distantly located on the target transcript and confirm data concordance between both assays. This comparison will help control against inaccurate results by ruling out mis-quantification from the following:

1. The absence or presence of unannotated isoforms overlapping the qPCR assay
2. Artifactually retained RNA fragments that did not degrade after RNA cleavage
3. Assay enzyme interference (e.g., blocking either the reverse transcriptase or the DNA polymerase with the ASO), giving false-negative or false-positive results.

Note: Data for the example provided in this protocol is normalized against two internal reference controls: *HPRT* (NM_000194) and *SFRS9* (NM_003769)

Consumables and equipment

Consumables—IDT

Item	Ordering information
Antisense oligonucleotides (2′ MOE and Affinity Plus gapmer ASOs) • Predesigned on-target and negative control (NC) ASOs*	www.idtdna.com
gBlocks™ Gene Fragments	www.idtdna.com
PrimeTime™ qPCR Probe Assays**	www.idtdna.com
PrimeTime Gene Expression Master Mix	1055770, 1055772, 1055771
IDTE pH 7.5 buffer [sterile Tris-EDTA (TE)]	11-05-01-05
RNaseAlert® Kit (optional)	11-02-01-02
Nuclease Decontamination Solution (optional)	11-05-01-01

* See Table 1 for an outlay of the ASO sequences in an easy-to-visualize format.

** Sequences can be manually entered as custom PrimeTime qPCR Probe Assays at IDTDNA.com; selecting 5′ dye/3′ quencher combination when ordering will automatically input the fluorophore and quencher modification codes. See the positioning of the fluorophores and quenchers in Table 2.

RNaseAlert is a registered trademark of Life Technologies, Inc.

Consumables—Other suppliers

Item	Supplier	Cat #
96-well PCR plates	General laboratory supplier	Varies
384-well qPCR plates		
MicroAmp™ Optical Adhesive Film	Thermo Fisher Scientific	4311971, 4360954
Cell line (HeLa or other cell line of choice)	General laboratory supplier	Varies
Dulbecco's Modified Eagle Medium (DMEM)		
Fetal Bovine Serum (FBS)		
OptiMEM™	Thermo Fisher Scientific	31985070, 31985062
Phosphate-buffered saline (1X PBS, pH 7.4)	General laboratory supplier	Varies
Trypsin–EDTA Solution		
Lipofectamine® 2000	Thermo Fisher Scientific	11668027
Polypropylene microcentrifuge tubes, 1.5 mL	General laboratory supplier	Varies
96-well culture plates		
Ethanol, 95%, RNase-free		
SuperScript™ IV First-Strand Synthesis System	Thermo Fisher Scientific	18091050, 18091200
SV 96 Total RNA Isolation System (if few samples)*	Promega	Z3500

* Use the 96-well plate format when processing multiple samples. When processing a few samples, use the tube-based SV Total RNA Isolation System.

Table 1. Sequences of targeting and negative control (NC) gapmer ASOs.

Sequence Name	Oligonucleotide sequence (5' -> 3')
MALAT1 site-5042 2'MOE PS ASO	**G*****G*****C*****A*****T***A*T*G*C*A*G*A*T*A*A***T*****G*****T*****T*****C**
NC 2'MOE PS ASO	**G*****C*****G*****A*****C***T*A*T*A*C*G*C*G*C*A***A*****T*****A*****T*****G**
MALAT1 site-1800 LNA® PS ASO	C*G*T*T*A*A*C*T*A*G*G*C*T*T*T*A
NC Affinity Plus PS ASO	G*A*C*T*A*T*A*C*G*C*G*C*A*A*T*A

Black = DNA; **Red** = 2'MOE; Black underscore = Affinity Plus; "*" = PS linkages

Table 2. Sequences of PrimeTime primers and probes with 5' dye/3' quencher combinations.

Assay	Sequence name	Oligonucleotide sequence (5' -> 3')
MALAT1 2638-2726	*MALAT1* For-2638	GTGCTACACAGAAGTGGATTC
	MALAT1 Rev-2726	CCTCAGTCCTAGCTTCATCA
	MALAT1 Probe-2668 HEX	/5HEX/AGGAAGACA/ZEN/GCAGCAGACAGGATT/ 3IABkFQ/
MALAT1 7443- 7535	*MALAT1* For-7443	CACCGAAGGCTTAAAGTAGGAC
	MALAT1 Rev-7535	GCTGACACTTCTCTTGACCTTAG
	MALAT1 Probe-7481 FAM	/56-FAM/CGCTTTGTT/ZEN/ GTCTCTCCTGCCACA/3IABkFQ/
HPRT 517-591	*HPRT* For-517	GACTTTGCTTTCCTTGGTCAG
	HPRT Rev-591	GGCTTATATCCAACACTTCGTGGG
	HPRT probe-554 FAM	/56-FAM/ATGGTCAAG/ZEN/ GTCGCAAGCTTGCTGGT/3IABkFQ/
SFRS9 594-690	*SFRS9* For-594	GTCGAGTATCTCAGAAAAGAAGACA
	SFRS9 Rev-690	CTCGGATGTAGGAAGTTTCACC
	SFRS9 Probe-625 HEX	/5HEX/ATGCCCTGC/ZEN/GTAAACTGGATGACA/ 31ABkFQ/

Equipment

Item	Supplier
Vac-Man® 96 Vacuum Manifold	Promega
Vacuum pump (capable of 15–20 in. of Hg and vacuum trap)	
NanoDrop® spectrophotometer or other nucleic acid quantification system	Thermo Fisher Scientific
Hemocytometer	General laboratory supplier

Introduction

This protocol details the procedure used to perform RNA knockdown using gapmer ASOs. Appropriate negative controls and recommendations for data analysis are provided. The selected RNA target for this protocol is the human lncRNA *MALAT1*, and knockdown is performed in HeLa cells which are known to highly express this lncRNA.

Note: Experimental set-up for this protocol is designed to perform biological triplicates for each transfection condition and qPCR triplicates of each transfection well, providing a total of 9 qPCR data points per transfection condition.

Protocol guidelines

Working dilutions

Working dilutions should be prepared so that the equivalent volume of gapmer ASO is added to the transfection mix for every dose. When testing multiple gapmer ASO doses, pipetting can be simplified by preparing serial dilutions in 96-well deep well plates to enable the use of a multichannel pipette.

Determining the appropriate dose range for each cell line may require optimization due to variabilities in the transfection efficiency of different cell lines. For example, cells that are difficult to lipofect may require a higher concentration of gapmer ASO to compensate for inefficient delivery. In cells that are easy to lipofect (e.g., HeLa, A549, HEK 293 or Huh-7 cells), a relatively high gapmer ASO dose may not improve potency but instead may result in toxicity.

We recommend starting with a dose range between 30 nM to 1 nM and then subsequently adjusting concentrations up or down depending on the knockdown results and whether any toxicity is observed. It is advised to use the lowest dose possible that achieves maximum knockdown levels to reduce the potential for toxicity and off-target effects (OTEs).

The *MALAT1* gapmer ASOs used in this protocol exhibit high potency and a nice dose response in the 3 nM to 0.1 nM dose range.

Transfection reagents

The most effective transfection reagents for delivering oligonucleotides are often cell-line dependent. While we find that Lipofectamine® 2000 (Thermo Fisher Scientific) efficiently delivers gapmer ASOs to a wide variety of commonly employed cell lines an alternative transfection reagent may be more suitable for other cell types. Using a positive control, such as the *MALAT1* gapmer ASOs used in this protocol, will assist with determining the optimal delivery reagent and conditions for each cell type employed.

Tip: Holding the lipid transfection reagent volume constant while adjusting the gapmer ASO concentration for the dose response generally gives more effective lipofection results. For example, in this protocol the *MALAT1* gapmer ASO doses are 3 nM, 1 nM, 0.3 nM, and 0.1 nM, and each dose is transfected with 0.5 µL of Lipofectamine 2000/well in a 96-well plate.

Prepare lipoplexes

To simplify pipetting, the lipoplexes can be prepared in a sterile 96-well plate instead of individual 1.5 mL microcentrifuge tubes. This plate format is compatible with the use of a multichannel pipette which will increase the efficiency of the liquid transfer. In this protocol, triplicate transfection wells are oriented in rows (e.g., the first biological triplicate would be in well positions A1, A2, and A3).

Cell confluency

Cell confluency at the time of transfection may influence the intracellular gapmer ASO concentration in the transfected cells. When using the same transfection conditions, we have sometimes observed that a lower cell density can have higher gapmer ASO activity than a higher cell density. To reduce inter-experimental variability, ensure that cell confluency at the start of transfection is similar between experiments.

Important: Transfection of chemically modified oligonucleotides can sometimes result in cytotoxicity. Having a higher confluency (e.g. 80–90%) at the time of transfection can reduce the toxicity potential from a wide variety of chemically modified ASOs.

Bacterial contamination and toxicity

High levels of bacterial contamination introduced during the transfection can often be macroscopically visualized as cloudy media that has changed color due to changes in pH caused by gross bacterial growth. Slight bacterial contamination can be visualized microscopically as tiny moving granules. Every well of the transfection should be microscopically examined to help ensure sterility throughout the experiment.

Note: If contamination occurs, review aseptic techniques and ensure that all tools, equipment, and consumables are sterile. Filter all suspect nucleic acids through a 0.2 µm syringe filter and quantify before use.

Residual chemicals during the gapmer ASO synthesis and/or purification process can sometimes result in cytotoxicity. This can be mitigated by ethanol precipitating the gapmer ASOs prior to use to remove trace toxic substances. If toxicity persists, try one of these possible solutions:

- decrease the gapmer ASO dose
- increase cell confluency (see **Cell confluency**)
- redesign the gapmer ASO sequence

  Tip: Toxicity may also be attributed to a reduction in target RNA levels, especially if the targeted RNA is involved in a cellular pathway important for viability.

Understand RNA degradation

RNA integrity is critical to generating high quality data for assessing RNA knockdown experiments. If RNA degradation has occurred, it is important to identify and remove the source of the RNase contamination. The presence of RNases in reagents and on surfaces can be rapidly detected using the RNaseAlert Kit (IDT). Surface RNases can be inactivated by treating the contaminated surface with Nuclease Decontamination Solution (IDT).

Reference dye concentration for qPCR

Different qPCR systems may have variable specifications for the optimal amount of reference dye to use in the qPCR reactions. For example, the QuantStudio™ Flex real-time PCR System (Thermo Fisher Scientific) requires a "low" concentration of the reference dye (4 µL of reference dye per 1 mL of PrimeTime Gene Expression Master Mix). Other qPCR platforms may require a "high" concentration of the reference dye (40 µL of reference dye per 1 mL of PrimeTime Gene Expression Master Mix). Please refer to the PrimeTime Gene Expression Master Mix guide to verify the appropriate amount of reference dye required for your specific qPCR platform.

Serial dilutions for qPCR

One method for calculating RNA knockdown by qPCR is with the absolute quantification method, which uses a standard curve that can be generated with gBlocks Gene Fragments. Serial dilutions for the gBlocks standard curve should be prepared fresh, as the DNA can adhere to the side of the tubes and cause inaccurate quantification. This can be particularly problematic for the dilutions where there are only a small number of molecules present.

Tip: Adding 100 ng/µL yeast tRNA (Thermo Fisher Scientific) to the serial dilutions can help stabilize the dilutions by coating the side of the microcentrifuge tubes and reducing the opportunity for the standard curve gBlocks Gene Fragments to bind.

Protocol

Perform reverse transfection

This protocol outlines the procedure for reverse transfection of 2'MOE and Affinity Plus gapmers targeting human *MALAT1* or a non-targeting control (NC) in HeLa cells in a 96-well plate format. If different sized plates are desired (e.g., larger wells may be required to increase the amount of isolated RNA in cases where the target RNA has low expression in the selected cell type), adjust the volumes accordingly by surface area of the well. For example, double all reagent volumes if using a 48-well plate versus a 96-well plate.

This protocol is specific for transfection into HeLa cells; if using a different cell line, plate the cells at the specified confluency. Include a "reagent only" control where only the transfection reagent is applied to the cells (no gapmer ASO) to help assess the source of any toxicity or contamination that might occur. For the *MALAT1* gapmer ASOs used in this protocol, it is recommended to start with a dose range of 3 nM, 1 nM, 0.3 nM, and 0.1 nM (see Working dilutions for more information). All transfections should be performed in a sterile tissue culture hood.

Nucleic acids

1. Centrifuge lyophilized gapmer ASOs before opening, then reconstitute in sterile IDTE pH 7.5 buffer at a stock concentration of 100 µM (e.g., add 50 µL of IDTE to 5 nmol of gapmer ASO).
2. Measure the concentration of the gapmer ASO using a nucleic acid quantification system such as the NanoDrop Spectrophotometer (Thermo Fisher Scientific). Tips for oligo quantification using the NanoDrop are included in this DECODED™ article.
3. Prepare a 10 µM working dilution in IDTE pH 7.5 buffer. For the 3 nM, 1 nM, 0.3 nM, and 0.1 nM dose response used in this example experiment, prepare serial gapmer ASO dilutions of 0.3 µM, 0.1 µM, 30 nM, and 10 nM in IDTE pH 7.5 buffer (see Working dilutions for more information).

   Note: Gapmer ASO stock solutions and dilutions should be stored at –20°C. If accidental bacterial contamination of the gapmer ASO occurs, filter the gapmer ASO stock solution through a 0.2 µm syringe filter (Pall) and quantify before use.

Reverse transfection

1. Guidelines for volumes used in this reverse transfection are calculated for biological triplicates, including a slight excess in volume to account for pipetting variability. For a triplicate transfection into a 96-well plate, combine 1.65 µL of Lipofectamine 2000 with 80.9 µL of Opti-MEM (for a single well, combine 0.5 uL of Lipofectamine 2000 with 24.5 µL of Opti-EM for a total volume of 25 µL), then multiply again by the total number of transfection conditions used (see Transfection reagents for more information). With the number of transfection conditions in this experiment—four gapmer ASOs with four doses each and a "reagent only" control—this Lipofectamine 2000/Opti-MEM dilution may be mixed in a 5 mL polypropylene tube to account for the volume.
2. Mix the combined Lipofectamine 2000/Opti-MEM by vortexing briefly or pipetting up and down several times.
3. For a triplicate transfection, combine 4.8 µL of the appropriate gapmer ASO dilution (e.g., use the 0.3 µM dilution for 3 nM dose) with 75.2 µL of Opti-MEM into separate 1.5 mL microcentrifuge tubes for a total volume of 80 µL (for a single well, dilute 1.5 µL of the gapmer ASO into 23.5 µL of Opti-MEM for a total volume of 25 µL; see Prepare lipoplexes).

4. For a triplicate transfection, add 80 µL of the diluted Lipofectamine 2000 to the diluted gapmer ASO for a total volume of 160 µL (for a single well, add 25 µL of the diluted Lipofectamine 2000 to the diluted gapmer ASO for a total volume of 50 µL) .
5. Gently mix the lipid complexes by pipetting up and down multiple times.
6. Add 50 µL of the gapmer ASO-lipid complex into triplicate wells in a 96-well transfection plate and incubate the lipid complexes at room temperature for a total of 20 minutes.
7. During the 20 minute incubation, trypsinize the HeLa cells and calculate the cells/mL using a hemocytometer.
8. Dilute the HeLa cells to 24,000 cells per 100 µL (240,000 cells per 1 mL) in DMEM containing 10% FBS (without antibiotics). Each well in the 96-well plate will be seeded with 100 µL of diluted HeLa cells in order to reach the desired ~90% confluency the next day (see Cell confluency). Scale up the total volume of diluted cells required (100 µL/well) for the number of wells being transfected.
9. After the 20 minute incubation, add 100 µL of gently mixed diluted HeLa cells on top of the lipid complexes for a final volume of 150 µL. Carefully mix by rocking the plate periodically for the first 15 min to allow cells to form an evenly confluent monolayer.
10. Incubate the cells at 37°C and 5% CO_2 for 24 hours.
11. After 24 hours, visualize the cells to ensure there is no toxicity or contamination.
    See Bacterial contamination and toxicity for more information.

Perform RNA isolation and RT-qPCR analysis

This section of the protocol demonstrates the procedure for isolating RNA, synthesizing cDNA in 96-well plate format, and quantifying RNA levels by qPCR in 384-well plate format. For more efficient throughput, use a multichannel pipette for liquid transfer. Always ensure that proper caution is taken when handling RNA (e.g., always wear clean gloves) and all reagents and plasticware are RNase-free (see Understand RNA degradation for more information).

RNA isolation

All buffer solutions for this procedure are provided in the SV 96 Total RNA Isolation System kit (Promega).

1. Prepare reagents for the SV 96 Total RNA Isolation System by adding 0.5 mL of β-mercaptoethanol (BME) to 50 mL of RNA Lysis Buffer, 275 µL of nuclease-free water to the lyophilized DNase I (do not vortex), 100 mL of 95% ethanol to the RNA Wash Solution, and 20 mL of 95% ethanol to the DNase Stop Solution.
2. Aspirate the transfection media off the cells and wash the cells with 100 µL of sterile 1X PBS.
3. Add 100 µL of RNA Lysis Buffer to each well. If necessary, the cell lysates can be stored in the transfection plate between –20°C to –80°C, taking care that the plate lid is firmly sealed (e.g., with tape or parafilm) to prevent evaporation.
4. Attach the vacuum manifold base to the vacuum via the vacuum port and place an RNA binding plate into the vacuum manifold base.
5. Mix the cell lysate by pipetting up and down, then carefully transfer the lysate from a well of the transfection plate to a well of the 96-well RNA binding plate. Apply the vacuum until the lysate is completely drawn.
6. Add 500 µL of RNA Wash Solution to each well of the RNA binding plate. Apply the vacuum until the RNA Wash Solution is drawn through the membranes of the RNA binding plate.

7. Prepare the DNase I solution by combining (in this order): 20 µL of Yellow Core Buffer, 2.5 µL of 0.09 M $MnCl_2$, and 2.5 µL of DNase I per well. Multiply these volumes by the number of wells required for the experiment. Mix by gently pipetting up and down.
8. Add 25 µL of DNase I solution to each membrane in the RNA binding plate and incubate at room temperature for 10 minutes. Unused DNase I can be stored at –20°C.
9. Add 200 µL of DNase Stop Solution to each well. Apply vacuum until the DNase Stop Solution is drawn through the membranes of the RNA binding plate.
10. Add 500 µL of RNA Wash Solution to each well. Apply vacuum until the RNA Wash Solution is drawn through the membranes of the RNA binding plate, then continue to apply the vacuum for 10 additional minutes to dry the membranes.
11. Remove the binding plate and gently blot the bottom tips onto a clean Kimwipes or paper towel.
12. Place a 96-well elution plate into the vacuum manifold bed. Then, place the manifold collar on top of the elution plate and put the binding plate on top of the manifold collar.
13. Add 100 µL of nuclease-free water to each well, ensuring that the water is in contact with the membrane. Incubate at room temperature for 1 minute.
14. Apply the vacuum for 1 minute to draw the eluant through the membranes of the RNA binding plate.
15. Cover the plate with a plate sealant. RNA may be stored at temperatures between –20°C and –80°C until ready to use.

Intact, high-quality RNA is required for accurate RT-qPCR quantification. Quantify the RNA samples and assess the RNA purity before cDNA synthesis. These analyses can be performed using a nucleic acid quantification system such as the NanoDrop Spectrophotometer (Thermo Fisher Scientific). RNA purity can be assessed by measuring the A260/A280 ratio; a value within the 1.8–2.1 range indicates high RNA purity. Residual chemical contaminants often absorb around 230 nm or can shift the 260 nm peak to a higher wavelength. Visualize the position of the RNA peak making sure there is no significant shift from 260 nm. Measure the A260/A230 ratio and ensure it is within the range of 2.0–2.2; a lower A260/A230 ratio can indicate the presence of a contaminant that may inhibit downstream enzymatic reactions.

Tip: Some inter-sample variability in RNA concentration is acceptable for RT-qPCR, as concentration variations will be accounted for with the two validated reference genes during qPCR normalization. However, best practice is to adjust the RNA samples to the same concentration. If any of the RNA samples significantly deviate from the average RNA input (e.g., a twofold reduction in concentration), this may indicate that toxicity occurred during transfection or that there was inefficient RNA extraction and/or RNA degradation, and the sample should be excluded from analysis.

cDNA synthesis

All components for cDNA synthesis are provided in the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). A "no reverse transcriptase (RT)" control will be included to assess genomic DNA contamination carryover from the RNA isolation step, as this can affect accurate quantification of the RNA.

1. Prepare a master mix of the following components and multiply each volume by the total number of RNA samples.

Components	Volume per sample (µL)
50 µM oligo dT	1
50 ng/µL random *HEX*amers	1
10 mM dNTP mix	1

Tip: This protocol is analyzing 51 RNA samples and a no-RT control (52 samples total). To account for pipetting variability, prepare enough master mix for 54 samples by combining 54 µL of each of the three components and vortexing to mix.

2. Pipette 3 µL of the master mix into 52 wells of a 96-well PCR plate.
3. Add 10 µL of each RNA sample, pipetting gently to mix. RNA from the "reagent only" transfection control can be used in the no-RT control reaction.
4. Cover the plate with a plate sealant such as MicroAmp Optical Adhesive Film.
5. Incubate the plate at 65°C for 5 minutes, and place on ice for at least 1 minute. If condensation has occurred, briefly centrifuge the plate before removing the plate sealant.
6. Prepare a master mix of the following components and multiply each volume by the total number of RNA samples.

Components	Volume per sample (µL)
5X SSIV Buffer	4
100 mM DTT	1
RT enzyme	1
Ribonuclease Inhibitor	1

Tip: This protocol is preparing 51 RT-containing cDNA reactions; to account for pipetting variability, prepare enough for 53 samples by combining 212 µL of 5X SSIV Buffer and 53 µL of each of the other four components, vortexing to mix.

7. Carefully remove the plate sealant from the 96-well PCR plate. Pipette 7 µL of the master mix (containing the enzymes) into each of the 51 sample wells, pipetting to mix. For the no-RT control, add the components for one reaction without the RT enzyme (add 4 µL of 5X SSIV Buffer, 1 µL of 100 mM DTT and 1 µL of Ribonuclease Inhibitor), pipetting to mix. Cover the plate with a plate sealant, then briefly centrifuge the plate.
8. Incubate the plate at 23°C for 10 min, 50°C for 10 min, 80°C for 10 min, and then store at 4°C until ready to use.
9. Dilute each cDNA reaction twofold by adding 20 µL of nuclease-free water before use.

qPCR methods

Important: Use PCR template-free reagents, consumables, and equipment throughout this procedure. Always keep gBlocks Gene Fragments qPCR amplicons for standard curve analysis separate from all PrimeTime qPCR Probe Assays and components and designate a set of clean pipettes for the qPCR assay setup. For accurate and comprehensive data collection and reporting of results, refer to the Minimum Information necessary for Quantitative real-time PCR Experiments (MIQE) guidelines.

This protocol is designed to analyze 51 transfection wells, a no-RT control cDNA reaction, 6 dilutions of a standard curve and a no template control (NTC) to test for template contamination that can affect quantification accuracy. Two multiplexed master mixes will be prepared in 1.5 mL microcentrifuge tubes: (1) lncRNA assays *MALAT1* 2638–2726 and *MALAT1* 7443–7535 together in one tube, and (2) normalizer assays *HPRT* 517-591 and *SFRS9* 594-690 together in a second tube. All qPCR reactions are performed in triplicate, giving a total of 177 reactions per multiplexed assay mix.

1. Briefly centrifuge lyophilized PrimeTime qPCR Probe Assay tubes and reconstitute to a 20X stock concentration in IDTE pH 7.5 buffer using template-free pipettes in a clean PCR hood to mitigate contamination.

   Tip: For this example protocol, if using the PrimeTime Std qPCR Assay (500 reaction size), add 500 µL of IDTE pH 7.5 buffer and vortex to mix. Store PrimeTime qPCR Probe Assays at –20°C.

2. Prepare qPCR master mixes for the target RNA and the reference (normalizer) genes. Thaw the PrimeTime Gene Expression Master Mix and reference dye on ice, then vortex to mix.

   Note: If using the QuantStudio Flex real-time PCR System, add 4 µL of reference dye to every 1 mL of PrimeTime Gene Expression Master Mix (see Reference dye concentration).

3. Calculate the amount of master mix required for the number of qPCR reactions for the experiment.

   Using a 10 µL per reaction volume setup, prepare enough master mix for 200 reactions to account for pipetting variability. For each of the two multiplexed master mixes, combine 1 mL of PrimeTime Gene Expression Master Mix (with reference dye added) and 100 µL of each of the 20×PrimeTime qPCR Assays (i.e., both *MALAT1* assays in one tube and both normalizer assays in a separate tube), giving a total of 1.2 mL of each multiplexed master mix. Vortex to mix.

4. In an area designated for template handling, prepare the qPCR standard curves with pipettes separate from those used for qPCR setup. Briefly centrifuge the lyophilized gBlocks Gene Fragments to ensure product is at the bottom of the tube. Reconstitute the gBlocks Gene Fragments in IDTE pH 7.5 buffer to 10 ng/µL, then briefly vortex.

5. Incubate the gBlocks Gene Fragments at 50°C for 15–20 min to assist with resuspension. Vortex the samples, then briefly centrifuge to collect the contents at the bottom of the tube.

6. Quantify the gBlocks Gene Fragments using a nucleic acid quantification platform such as the NanoDrop Spectrophotometer to verify concentration. Calculate the molecules/µL by using the following formula: (C)(M)(1×10^{-15} mol/fmol)(Avogadro's number) = molecules/µL, where C is the gBlocks Gene Fragments concentration in ng/µL, M is the molecular weight of the gBlocks Gene Fragments in fmol/ng (found on the provided spec sheet), and Avogadro's number = 6.022×10^{23}.

   Tip: A 10 ng/µL stock of the *MALAT1* gBlocks Gene Fragments is converted to molecules/µL as follows: (10 ng/µL) (8.66 fmol/ng)(1×10–15 mol/fmol) (6.022×1023) = 5.22×1010 molecules/µL.

7. Prepare tenfold serial dilutions of the gBlocks Gene Fragments in IDTE pH 7.5 buffer ranging from 2.5×10^{6} to 2.5×10^{1} molecules/µL (see Serial dilutions). As 4 µL of template will be loaded per qPCR well, this will provide a standard curve ranging from 1×10^{7} to 1×10^{2} copies.

8. Using a multichannel pipette, load 6 µL of the *MALAT1* multiplexed master mix into 177 wells of a 384-well qPCR plate.
9. In triplicate wells, dispense 4 µL of each cDNA reaction (including the no-RT control) and each dilution of the *MALAT1* standard curve. Dispense 4 µL of nuclease-free water for the NTC.
10. Seal the plate with MicroAmp Optical Adhesive Film and briefly centrifuge to ensure the contents are collected at the bottom of each well.
11. Repeat this plate setup (above steps 9–11) for the normalizer assays in a separate 384-well plate.
12. Run the qPCR plates on a real-time PCR instrument using the following cycling parameters: enzyme activation at 95°C for 3 min, followed by 40 cycles of two-step PCR (95°C for 15 sec, 60°C for 1 min). Input sample names and standard curve values.

Data analysis

1. Analyze the standard curves to assess the efficiency of the assays, the quality of the amplification reactions and the accuracy of pipetting. Acceptable standard curve slopes range between –3.1 and –3.6, equating to 90–110% efficiency (–3.32 is 100% efficient). The R2 value, which measures pipetting accuracy and assay performance, should be greater than 0.99. All test samples must lie within the range of the standard curve for accurate quantification.
2. Verify that there is no amplification in the no-RT control or the NTC negative control reactions from contaminating carryover genomic DNA or template, respectively. Examine the amplification curves generated for the reference genes for all samples to ensure minimal inter-sample deviation. An overall spread between all samples combined that is greater than 2 Cqs may be indicative of cell toxicity, poor sample integrity, or a suboptimal amplification process.
3. For each sample, calculate the average copy number values for the reference genes and for each assay of the target gene (*MALAT1*), then divide the target gene averaged value to its respective reference gene averaged value. Divide the normalized copy number value of each ASO treatment sample by the normalized copy number value of the appropriate negative control (e.g., Affinity Plus ASO treatment should be compared to the Affinity Plus negative control ASO at the same dose). All treatments should also be compared to the "reagent only" control to verify that the negative control ASO treatment did not largely affect *MALAT1* expression levels. Perform the calculations separately for both *MALAT1* qPCR assays and compare the results to assess assay concordance. An example of *MALAT1* knockdown results for this protocol is shown in Figure 1.

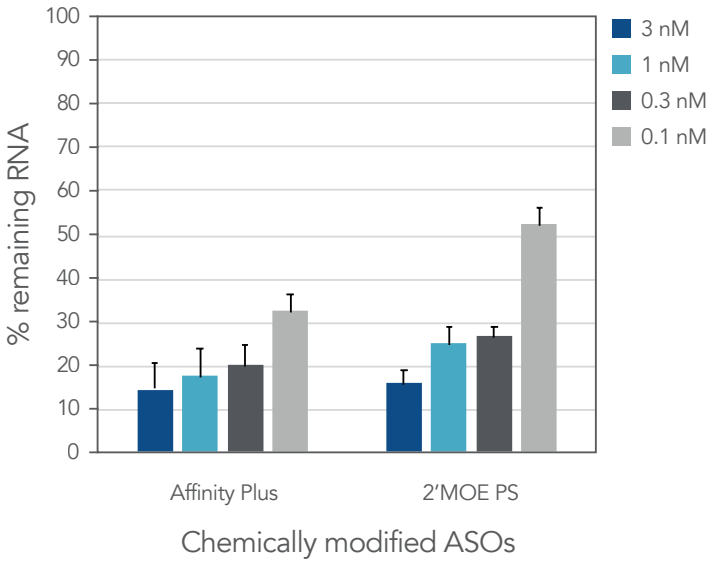

Figure 1. Knockdown of the nuclear human *MALAT1* lncRNA with 2′ MOE PS or Affinity Plus gapmer ASOs. Gapmer ASOs were transfected in triplicate into HeLa cells with Lipofectamine 2000. RNA levels were measured by RT-qPCR 24 h after transfection. LncRNA levels were calculated by normalizing to the internal reference genes *HPRT* and *SFRS9* and comparing to negative control gapmer ASOs with similar chemical composition at a similar dose.

RNA knockdown using gapmer Antisense Oligonucleotides (ASOs)

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