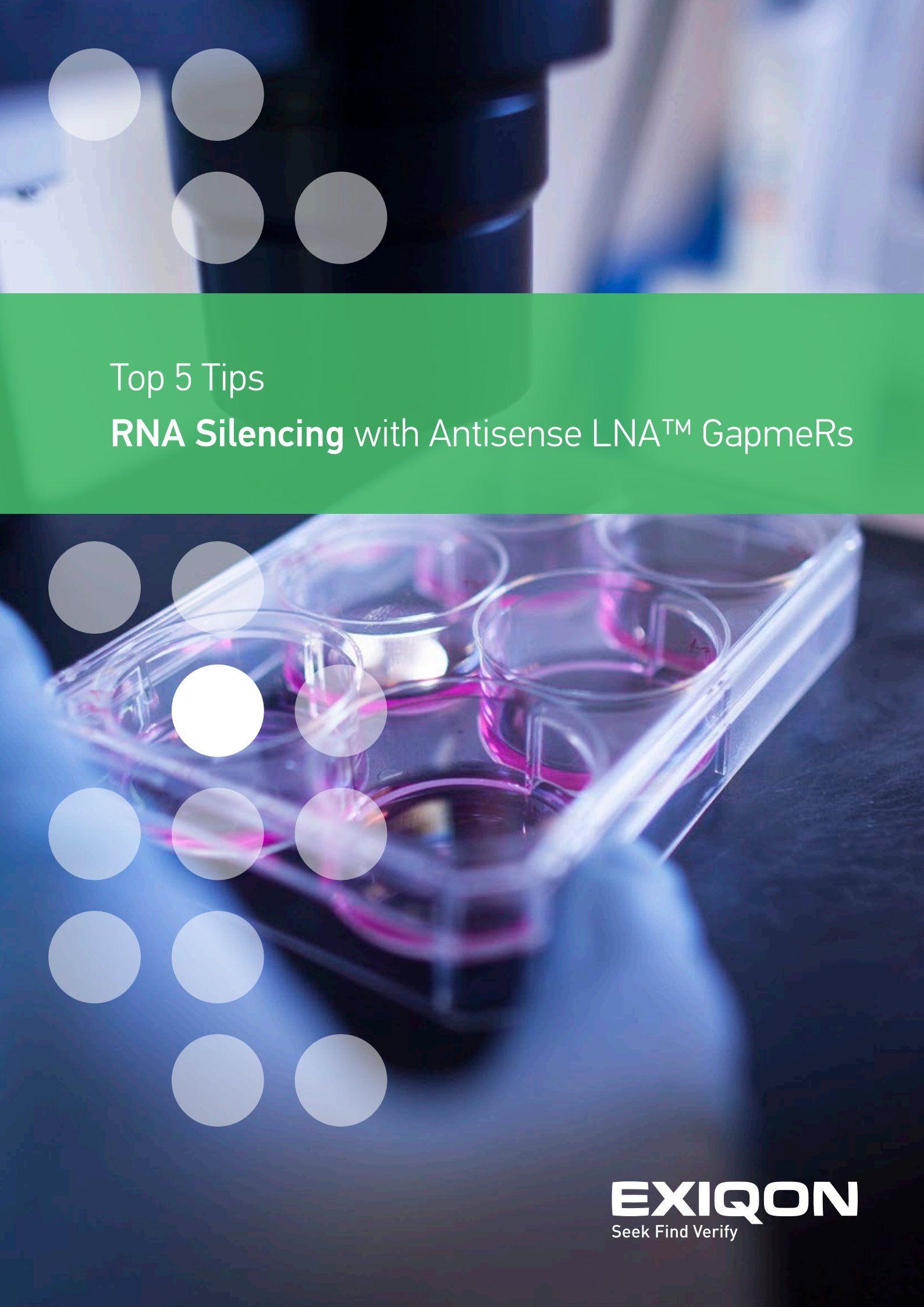

Top 5 Tips
RNA Silencing with Antisense LNA™ GapmeRs

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1

Design optimal Antisense LNA™ GapmeRs

- **Use Exiqon's LNA™ GapmeR design tool for best results.** Thousands of possible LNA™ GapmeR designs are evaluated against more than 30 design parameters to identify the LNA™ GapmeRs most likely to give potent and specific target knockdown.
- **Choose the highest ranking LNA™ GapmeRs** (rank 1 = best), and where possible with excellent design scores (indicated by green circles). On average there is a good correlation between rank and efficacy of the LNA™ GapmeR.

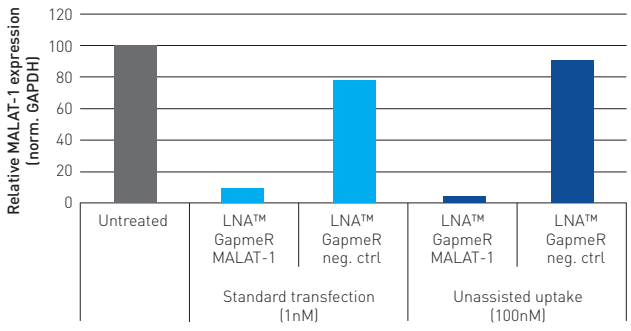
Design an LNA™ GapmeR for your favorite RNA at exiqon.com/gapmers

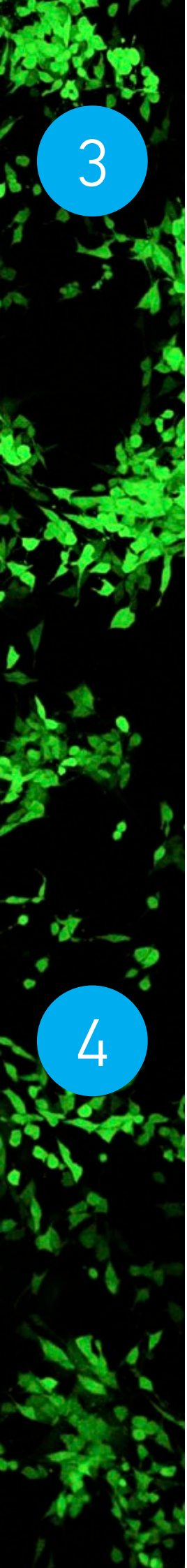
2

Optimize delivery conditions

- **Select an appropriate delivery method.** Determine the optimal transfection reagent and transfection protocol empirically for each cell type and target. In general, delivery methods used for siRNA can be used for delivery of LNA™ GapmeRs (lipid or amine based transfection reagents, or electroporation).
- **Consider Unassisted Uptake** (without any transfection reagent, gymnosis, Figure 1). Unassisted uptake may be beneficial for hard-to-transfect cells e.g. B-cells, primary cell lines, and suspension cells (Stein *et al.*, 2010), and may also avoid experimental artifacts introduced by transfection reagents. However unassisted uptake requires higher concentrations of LNA™ GapmeR (100 nM – 5 µM, compared with 0.1 – 50 nM for transfection), and the uptake kinetics are slower (effect usually apparent only after 48 hours).
- **Positive Control LNA™ GapmeRs.** Exiqon provides validated potent LNA™ GapmeRs against RNA targets expressed in a wide range of cell lines, ideal for optimization of delivery conditions. Choose one appropriate for your application or cell type.
- **Negative Control LNA™ GapmeR.** Use one of Exiqon's generic negative control LNA™ GapmeRs, or a custom designed negative control similar to your LNA™ GapmeR sequence.
- **Untreated Control.** Evaluate any effects of transfection that are not related to LNA™ GapmeR antisense activity.
- **Consider monitoring uptake using fluorescently labeled LNA™ GapmeRs.** However, it is still essential to confirm knockdown of the target RNA.

Figure 1: **Antisense LNA™ GapmeRs can be delivered by unassisted uptake (gymnosis) without transfection reagent. The results demonstrate almost complete elimination of Malat1 (an abundant nuclear lncRNA) both by classical transfection and by unassisted uptake.** Study details: A Malat1 LNA™ GapmeR and a negative control LNA™ GapmeR were delivered to HeLa cells by either standard transfection (blue bars) or added directly to the cell culture medium without transfection reagent (unassisted uptake, red bars). Cells were harvested after 48 hours, and analysed for Malat1 content by qPCR. Data was normalized to GAPDH and the relative Malat1 content was compared to untreated cells.





3

Establish a reliable assay to monitor RNA silencing

- **Choose an assay that reflects the state of the target RNA.** The assay should also have low intrinsic variance and ideally not be influenced by any factors other than the addition of the inhibiting oligonucleotide.
- **Know your target.** In most cases, one gene gives rise to multiple different transcripts. Study what is known about transcript variants deriving from your gene of interest using databases such as Ensembl, GenBank or the UCSC genome browser. Consider transcript variants when choosing qPCR primers for evaluation of target RNA knockdown.
- **Use multiple qPCR assays for long RNA targets.** We recommend using multiple qPCR assays located at different positions along the length of the target RNA to verify that the entire transcript has been degraded following RNase H cleavage.
- **Use a qPCR assay that does not overlap with the LNA™ GapmeR target site.** Exiqon offers custom LNA™ qPCR assays for any RNA target, and we can ensure the assay does not overlap with the LNA™ GapmeR target site. This minimizes the rare possibility that LNA™ GapmeR in the RNA extract may interfere with the qPCR assay, leading to a false-positive qPCR result.
- **Control for false-positive qPCR results.** Rule this out by performing a simple control: lyse untransfected cells, add LNA™ GapmeR (the same amount as added to transfected cells), then proceed with the RNA isolation and qPCR assay. This control is most relevant when using a high concentration of LNA™ GapmeR, e.g. for unassisted uptake (gymnosis).

Assay	Advantages	Disadvantages
RT-qPCR to monitor RNA levels.	Quantitative method to measure how LNA™ GapmeRs affect target RNA levels. Suitable for coding and non-coding RNA targets.	Assay should not overlap with the LNA™ GapmeR target site(s). Need to control for false-positive results.
Western blot to monitor protein levels.	Biologically relevant read-out for mRNA targets (transcript levels are not always directly proportional to protein levels).	Suitable only for coding transcripts. Requires specific antibody. Semi-quantitative. Stable proteins may show delayed knockdown.
Biological assays on the cellular level e.g. morphology, differentiation, cell growth, cell death, cell adhesion, migration.	Ultimate proof that the target is involved in a particular biological process.	Can be complex to interpret. Should always be used together with assays that monitor RNA levels.

4

Identify the best Antisense LNA™ GapmeRs and test the phenotype

- **Screen multiple LNA™ GapmeRs.** We recommend that you test 5 LNA™ GapmeRs to identify 2-3 LNA™ GapmeRs that give potent knockdown of the target RNA.
- **Use the lowest effective concentration of LNA™ GapmeR.** Perform a dose response study to find the lowest concentration needed for effective knockdown, identify the most potent LNA™ GapmeRs, and observe the phenotype at the lowest concentration (minimizing side effects). The recommended range of LNA™ GapmeR final concentrations is 0.1 – 50 nM for transfection, or 100 nM – 5 µM for unassisted uptake (gymnosis).
- **Monitor the timecourse of knockdown.** The time required to achieve knockdown will depend on the delivery method and the stability and turnover of the transcript and protein. LNA™ GapmeRs target primarily newly synthesized RNA transcripts in the nucleus, where RNase H is located. It may take longer to observe the effect with highly stable cytoplasmic transcripts or proteins.
- **Validate the phenotype using a second LNA™ GapmeR.** Ideally you should observe the same phenotype with at least two different LNA™ GapmeRs targeting different positions within the target RNA, in order to verify that the effect is due to knockdown of the target RNA. Also include a negative control LNA™ GapmeR.

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Test *in vitro* before using Antisense LNA™ GapmeRs *in vivo*

- **Screen multiple LNA™ GapmeRs *in vitro* to** increase the likelihood of identifying a super-potent LNA™ GapmeR. This is important if your goal is to achieve maximal silencing of the target RNA *in vivo*.
- **Test unassisted uptake *in vitro*** (in the absence of transfection reagent – gymnosis), using a cell line representative of the tissue you wish to target. Gymnosis may simulate the *in vivo* situation better than transfection, thus providing a better prediction of results *in vivo*.
- **Start with an *in vivo* pilot experiment to identify the optimal dose regime.** Using a limited number of animals, test a range of doses (5 – 25 mg/kg in mice) by a single subcutaneous administration. Measure the knockdown efficacy and duration of effect in relevant tissues, and monitor signs of toxicity by checking liver and kidney function (e.g. by testing blood for AST/ALT transaminases and creatinine/urea).

Exiqon's online LNA™ GapmeR design tool presents a ranked list of LNA™ GapmeRs making it easy to choose multiple LNA™ GapmeRs for testing. A range of purity options are available, including cost-effective options for screening of multiple LNA™ GapmeRs *in vitro*.

LNA™ GapmeRs enable RNA silencing in animal models (Xing et al., 2014 and Michalik et al., 2014), and are particularly effective against nuclear retained lncRNA targets *in vivo* (Figure 2). Exiqon offers *in vivo* Ready LNA™ GapmeRs in small quantities so you can test *in vitro*, before scaling up to large quantities for an *in vivo* experiment.

> ***"[LNA™] GapmeRs showed similar effects to our KO mice, or even better in the adults (since there is likely some compensation by other mechanisms [in KO mice])."*** *Dr. Reiner Boon*
>
> Co- author of Michalik et al., Circ. Res. 2014. Read more here: http://www.exiqon.com/ls/Pages/michalik.aspx

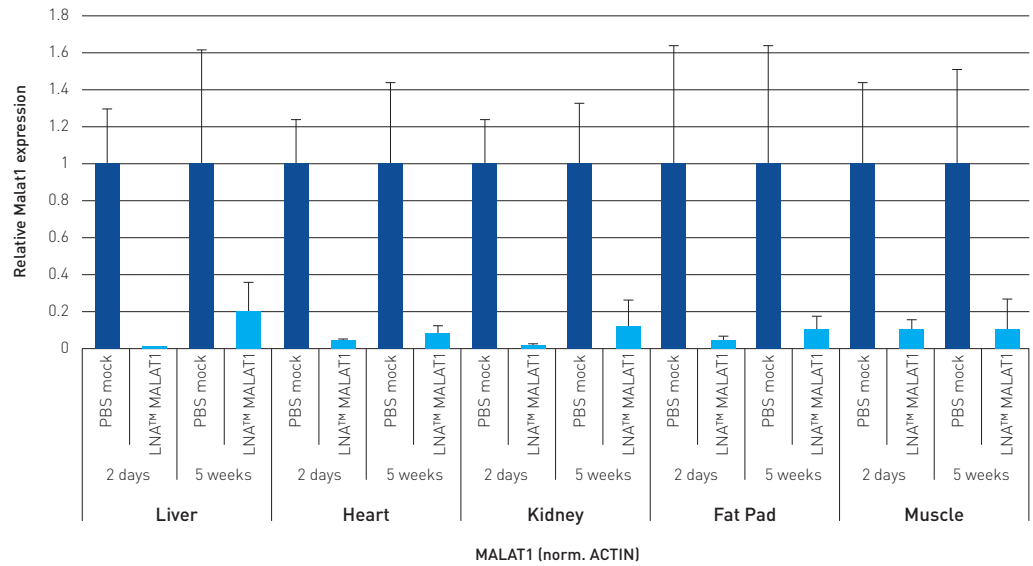

Figure 2: Efficient and long lasting knockdown *in vivo* with Antisense LNA™ GapmeRs in a broad range of tissues. LNA™ GapmeR for knockdown of Malat 1 in mice was injected subcutaneously over a period of 4 weeks. Samples from a broad range of tissues from the mice were collected up to 5 weeks after last LNA™ GapmeR administration, and analyzed for Malat1 RNA content by qPCR. The knockdown effect was still highly efficient in all tissues, even 5 weeks after the last dose of LNA™ GapmeR.

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Antisense LNA™ GapmeRs are an excellent alternative to siRNA for silencing mRNA and long non-coding RNA (lncRNA).

- **Silence any mRNA or lncRNA target** – even nuclear lncRNAs
- **No microRNA-like off-target effects**
- **Unassisted uptake** *in vitro* without transfection reagents
- **Active** *in vivo* (with no formulation)

Li *et al.*, Functional importance of eRNAs for estrogen-dependent transcriptional activation events. Nature 2013 June 27; 498 (7455):516-520.
Michalik *et al.*, Long noncoding RNA MALAT1 regulates endothelial cell function and vessel growth. Circ Res. 2014 Apr 25;114(9):1389-97.
Soifer *et al.*, Silencing of gene expression by gymnotic delivery of antisense oligonucleotides. Methods Mol Biol. 2012;815:333-46.
Stein *et al.*, Efficient gene silencing by delivery of locked nucleic acid antisense oligonucleotides, unassisted by transfection reagents. Nucleic Acids Res. 2010 Jan;38(1):e3.
Stratigi *et al.*, Spatial proximity of homologous alleles and long noncoding RNAs regulate a switch in allelic gene expression. Proc Natl Acad Sci 2015 Mar 31;112(13):E1577-86.
Xing *et al.*, lncRNA directs cooperative epigenetic regulation downstream of chemokine signals. Cell. 2014 Nov 20;159(5):1110-25.
Zhang *et al.*, Down-modulation of cancer targets using locked nucleic acid (LNA)-based antisense oligonucleotides without transfection. Gene Ther. 2011 Apr;18(4):326-33.

For more information go to: exiqon.com/gapmers

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