Functional Analysis

miRCURY LNA™ microRNA Inhibitors and Target Site Blockers

Instruction manual v1.0

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EXIQON
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Product summary

Content

This manual addresses the use of two of Exiqon's microRNA products for functional analysis:

miRCURY LNA™ Inhibitor and miRCURY LNA™ Family Inhibitor products for *in vitro* studies:

Product No*	Product description	Label	Amount supplied
410000-XX to 424999-XX	miRCURY LNA™ Inhibitor	Ready-to-label, 5′ or 3′ FAM	5 nmol of nucleotide, dried down in vial
425000-XX to 449999-XX	miRCURY LNA™ microRNA Power Inhibitor	Ready-to-label, 5′ or 3′ FAM	5 nmol of nucleotide, dried down in vial
450001 – 4500xx	miRCURY LNA™ Inhibitor	Ready-to-label	5 nmol of nucleotide, dried down in vial
460001 – 4600xx	miRCURY LNA™ microRNA Power Family Inhibitor	Ready-to-label	5 nmol of nucleotide, dried down in vial

*Product No suffix digits varies depending on the type of label applied.

miRCURY LNA™ Target Site Blocker products for *in vitro* studies:

Product No*	Product description	Label	Amount supplied
480001-00	miRCURY LNA™ microRNA Target Site Blocker, Premium	Ready-to-label	5 nmol of nucleotide, dried down in vial
480003-00	miRCURY LNA™ microRNA Power Target Site Blocker, Premium	Ready-to-label	5 nmol of nucleotide, dried down in vial
480004-00	miRCURY LNA™ microRNA Power Target Site Blocker, *in vivo* ready	Ready-to-label	5 nmol of nucleotide, dried down in vial

Additional required materials

- Nuclease-free TE-buffer (10mM Tris, 0.1mM EDTA, pH 7.5 or 8.0)
- Microcentrifuge
- DNase-free microcentrifuge tubes or microtiter plate
- Cell culture plates
- Cell culture medium
- Transfection reagent

Product description

miRCURY LNA™ microRNA **Inhibitors** are antisense oligonucleotides with perfect sequence complementary to their target. When introduced into cells, they sequester their target microRNA in highly stable heteroduplexes thereby effectively preventing the microRNA from hybridizing with its normal cellular interaction partners.

miRCURY LNA™ microRNA **Target Site Blockers** are antisense oligonucleotides designed to compete with microRNA/RISC for a microRNA target site of a particular mRNA. When introduced into cells, a target site blocker will mask the microRNA target site by hybridizing strongly with it, thereby effectively preventing the microRNA from interacting with the specific target mRNA of interest while not affecting the activity of the endogenous microRNA *per se*.

The sequences of the oligonucleotides and their LNA™ spiking patterns have been carefully designed to achieve uniform high potency for all miRCURY LNA™ microRNA Inhibitors and miRCURY LNA™ Target Site Blockers regardless of the GC-content of their target. This is accomplished by ensuring T_m normalization around an optimal temperature while keeping the level of self-complementarity to a minimum.

Applications

MicroRNA Inhibitors are primarily used to study microRNA function by assessing the biological consequences of inhibiting microRNA activity. By a different mode of action, the microRNA target site blockers are also used to study microRNA function. In this case the biological consequences of blocking microRNA interaction with a specific mRNA target can be evaluated.

The effect of inhibiting a microRNA (using microRNA inhibitors) or preventing the microRNA from binding to a particular mRNA target (using microRNA target site blockers) can be studied in numerous ways, such as using cellular assays to monitor cell proliferation, cell differentiation, or apoptosis. The effect on gene expression can also be measured at the mRNA or protein level of putative microRNA targets.

Shipping and storage

This product is shipped dried down at room temperature. The unopened vial should be stored at -20°C or below. Fluorescence-labeled oligonucleotides should be protected from light to avoid bleaching. Shelf life is at least 6 months after shipping date when stored in this manner. Exposure to higher ambient temperatures during shipment does not pose any risk to the stability of the oligonucleotides.

Oligonucleotides are degraded by repeated freeze-thaw cycles, especially when in solution. After resuspension, it is recommended to aliquot the product before storage. For storage at -20°C, please use a constant temperature freezer.

Do not store in frost-free freezer with automatic thaw-freeze cycles.

Protocol

Important note

LNA™ oligonucleotides are susceptible to degradation by exogenous nucleases introduced during handling. Wear powder-free gloves when handling this product. Use DNase-free reagents and filter pipette tips. Whenever possible, work should be conducted under a tissue culture hood.

Resuspension

Step 1	Briefly centrifuge the screw cap vial at low speed (maximum 4,000 x g) to make sure that all material is collected at the bottom of the wells before removing the cap in step 2.
Step 2	Remove screw cap carefully.
Step 3	Add nuclease-free, sterile TE-buffer* using a pipette with a sterile filter tip to achieve the desired concentration. Stock solutions should not be lower than 10 µM (Adding 100 µL TE buffer* to 5 nmole microRNA inhibitor /target site blocker will yield a 50 µM solution).
Step 4	Let the vial stand for a few minutes at ambient temperature.
Step 5	Gently pipette up and down 5 times to resuspend.
Step 6	Repeat steps 4 and 5.
Step 7	We recommend aliquoting the inhibitor / target site blocker solution into sister tubes to limit the number of thaw-freeze cycles.

*TE-buffer: 10 mM Tris, pH 7.5 or 8.0, 0.1 mM EDTA

Step 8	Store at –20°C.
Step 9	Avoid thaw-freezing more than 5 times (working solutions can be stored at 4°C for a period of maximum two weeks).

Transfection guidelines

Transfection efficiency varies according to cell type and the transfection reagent used. The optimal combination of cell type, transfection reagent and transfection conditions must be determined empirically. Optimizing transfection efficiencies is crucial for maximizing intended antisense activity while minimizing secondary effects. Expect to spend some time finding the optimal transfection conditions.

One way of determining the optimal transfection conditions is to use a reporter plasmid in which expression of a reporter gene is regulated by the endogenous microRNA level in the chosen cell line through a microRNA target site in the 3'UTR. The effect of transfection can be assessed by measuring the relief of inhibition of reporter gene expression caused by microRNA inhibition or by masking of the microRNA target site in the case of a target site blocker. Typically, this type of experiment also involves a second reporter gene for normalization of variation in plasmid transfection efficiency. Reporter plasmids with microRNA target cloning sites in the 3'UTR of reporter genes are commercially available from several companies.

Alternatively, derepression of endogenous microRNA targets (validated or predicted) can be measured at either mRNA or protein level. Optimal transfection conditions are found by identifying efficient transfection reagents for each cell line and by adjusting:

- Amount of transfection reagent
- Amount of microRNA Inhibitor / Target Site Blocker
- Cell density at time of transfection
- Order of transfection (plating cells before transfection or plating cells at the moment of transfection)
- Length of exposure of cells to transfection reagent/oligonucleotide complex

Transfection conditions can also be optimized with a well characterized siRNA or gapmer that induces a quantifiable phenotype. Alternatively, siRNA or gapmer activity can be measured by qRT-PCR on the corresponding mRNA target.

Table 1.

Cell culture plate	96 well	24 well	12 well	6 well
Transfection reagent [A]	0.3 – 1.0 µL	1 – 3 µL	2 – 4 µL	3 – 36 µL
microRNA Inhibitor /Target Site Blocker [B]	5 pmole	25 pmole	50 pmole	150 pmole
Cell density (cells/well) [C]	6000	40000	80000	240000
Final volume per well	100 µL	500 µL	1000 µL	3000 µL

[A] Refer to the instructions provided by the transfection reagent supplier.
[B] The amount shown yields a microRNA Inhibitor /Target Site Blocker concentration of 50 nM.
[C] Optimal cell density varies with the cell type depending on cell size and growth characteristics. In general, 30 – 70% confluency is recommended.

Most protocols recommend maintaining mammalian cells in the medium used for transfection for 24 hours. The transfection medium should then be replaced with fresh medium to maximize viability of the cell culture. Normally, microRNA Inhibitors / Target Site Blockers display potent activity at final concentrations of 1-50 nM, but a more extensive range of 1-100 nM can be analyzed in optimization experiments. However, Power inhibitors are frequently toxic at concentrations >50 nM. Always remember to perform adequate controls to ensure that the resulting phenotype is due to antisense inhibition of the targeted RNA. The optimal time for analysis of the effect of transfection must be determined experimentally. However, normally antisense effects are assessed 24-72 hours after transfection. For some applications, such as cell differentiation assays the phenotypic readout may take place 7-10 days after transfection.

At sufficiently high concentrations all oligonucleotides are cytotoxic. The level of toxicity is sequence dependent and the sensitivity of cell lines varies considerably. MicroRNA functional analysis should therefore only be performed under optimized transfection conditions with the minimal required inhibitor concentration. Dose response experiments are often useful for determining the threshold concentration where the advantage of increasing the dose is

cancelled out by beginning symptoms of toxicity that negatively affect the phenotypic readout (bell shaped dose response curves). Typically the first signs of toxicity can be observed at 50-100 nM concentrations. Always perform adequate controls to ensure that the resulting phenotype is due to antisense inhibition of the targeted microRNA.

Electroporation

miRCURY LNA™ microRNA Inhibitors / Target Site Blockers can also be introduced into cells by electroporation. This is especially useful with cells that are notoriously difficult to transfect (i.e. non adherent cells such a lymphocytes, bone marrow stem cells and primary cancer cells). Please follow the instructions provided with the electroporation system.

Important note

microRNA qPCR is not a reliable method for measuring the level of microRNA knock down. Even if this method is often cited in literature, it is not recommended. microRNA inhibitors do not degrade their targets instead they form stable complexes with their target causing an accumulation of the microRNA due to reduced turnover. Moreover, failed transfections are often the result of accumulation of oligonucleotides inside vesicles so that the inhibitor and microRNA target are present in different subcellular compartments. Upon cell lysis, liberated vesicular inhibitors will form strong heteroduplexes with their microRNA target and therefore efficient and inefficient transfection cannot be distinguished by microRNA qPCR. In addition, LNA™ oligonucleotides can interfere with PCR primers and give rise to aberrant results.

Related products

Exiqon offers a broad variety of tools enabling new discoveries concerning the expression, function and spatial distribution of microRNAs:

miRCURY LNA™ microRNA Inhibitors, Power Inhibitors and Family Inhibitors
Unravel the function of microRNAs by microRNA inhibition. Sophisticated LNA™ design ensures potent inhibition of all microRNAs regardless of their GC content. Chemically modified, highly stable Power Inhibitors for unrivalled potency. Available for *in vitro* and *in vivo* studies.

miRCURY LNA™ microRNA Inhibitor Negative Controls
These oligonucleotides are designed to have no known microRNA targets in miRBase. The oligonucleotides are provided unlabeled or fluorescence labeled.

miRCURY LNA™ microRNA Inhibitor Library
For genome-wide high throughput screening of microRNA function.

miRCURY LNA™ Target Site Blockers
For inhibition of microRNA binding to a specific mRNA target.

LNA™ longRNA GapmeR
LNA™ gapmers are potent antisense oligonucleotides used for highly efficient inhibition of mRNA and lncRNA function. Designed using advanced algorithms, the RNase H-activating LNA™ gapmers offer excellent performance and high success rate.

miRCURY LNA™ Universal RT microRNA PCR
Fast and accurate determination of microRNA expression with real-time PCR.

miRCURY LNA™ microRNA Array, microarray kit and Power labeling kit
Pre-printed miRCURY LNA™ microRNA Array microarray slides, available for hsa, mmu & rno and other species. Kit includes hybridization and wash buffers as well as synthetic spike-in microRNAs. Power Labeling kit for fluorescent labeling of microRNAs from total RNA samples.

miRCURY LNA™ microRNA Detection Probes
For *in situ* hybridization and northern blotting of all annotated microRNAs.

References

- Griffiths-Jones, S. The microRNA Registry. Nucleic Acids Research 2004, 32, Database Issue, D109-11
- Torres AG, Fabani MM, Vigorito E, Gait MJ. MicroRNA fate upon targeting with anti-microRNA oligonucleotides as revealed by an improved Northern-blot-based method for microRNA detection. RNA 2011, 17, 933-43
- miRBase: www.mirbase.org
- www.exiqon.com

Literature citations:
Please refer to the relevant product: miRCURY LNA™ microRNA Inhibitor, miRCURY LNA™microRNA Power Inhibitor, miRCURY LNA™ microRNA Family Inhibitor, miRCURY LNA™ microRNA Power Family Inhibitor, miRCURY LNA™ microRNA Target Site Blocker or miRCURY LNA™ microRNA Power Target Site Blocker when describing a procedure for publication using one or more of these products when describing a procedure for publication using this product.

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