ENDO-PORTER DELIVERY OF MORPHOLINO OLIGOS

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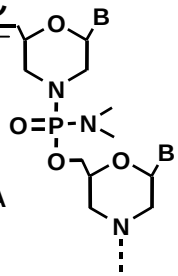
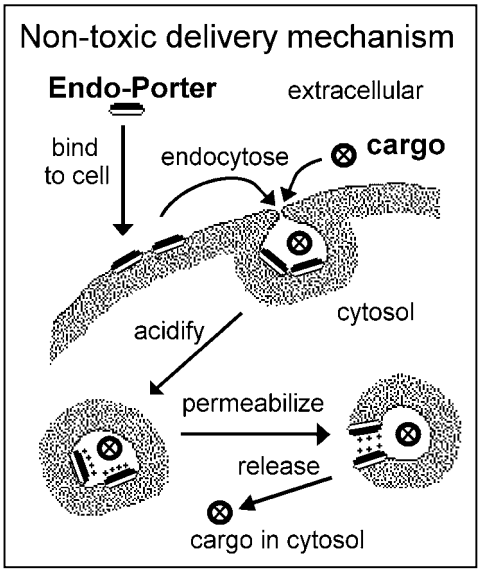


I. INTRODUCTION

A. Endo-Porter Delivery

Endo-Porter is a reagent for delivering Morpholino oligos, peptides or proteins into the cytosol of cultured cells. Morpholino oligos are the dominant tool for knocking down gene function and altering mRNA splicing in developmental biology research where cytosolic delivery is assured by microinjection into the early embryo. Early methods (Scrape Delivery and Osmotic Delivery) for delivery of Morpholinos to cells in culture were limited to certain cell types and were not overwhelmingly effective. Special Delivery oligos (Morcos, P.A., 2001) entered cells through the normal process of endoctyosis, thereby reducing cell toxicity, but was limited to serum-free delivery with a fixed concentration of Morpholino. **Endo-Porter** is a peptide-based reagent which retains the mechanism that relies on endocytosis, works in the presence of serum, and allows delivery of multiple oligos at a range of oligo concentrations.



Delivery is least toxic when the process does not disrupt the plasma membrane. The mechanism of **Endo-Porter** delivery (see figure) relies on Morpholinos and **Endo-Porter** being taken up from the media into the same endosome. **Endo-Porter** is an amphiphilic peptide with a sharp transition pH, with a hydrophobic face which associates with cell membranes once added to culture medium. Morpholinos in the medium are co-endocytosed with **Endo-Porter**. Natural acidification of the endosome protonates **Endo-Porter** that, in its ionic form, permeabilizes the endosome and releases the endosome contents into the cyotosol.

Gene Tools provides **Endo-Porter** in two formulations, a DMSO formulation and an aqueous formulation.

Endo-Porter in pure DMSO is for high efficacy delivery of Morpholinos. When used at the recommended concentration of 6 µM Endo-Porter peptide, the medium contains less than 1% DMSO. The DMSO is thought to inhibit peptide complex formation prior to delivery.

Endo-Porter-Aqueous is provided for those systems and cells in which DMSO is toxic or triggers differentiation. 1.0mM **Endo-Porter** peptide is provided in 0.3 M mannitol. Delivery of Morpholinos using **Endo-Porter-Aqueous** is reduced by approximately 30% compared to delivery using the DMSO formulation.

II. DELIVERING MORPHOLINO OLIGOS with Endo-Porter

A. Preparation of Reagents

Morpholino Stock Solutions

Custom-Sequence Oligo: To one vial containing 300 nanomoles of sterile freeze-dried custom-sequence Morpholino oligo add 0.6 ml of sterile water to give 0.6 ml of a 500 µM oligo stock solution.

Standard Control Oligo: To one vial containing 100 nanomoles of sterile freeze-dried Standard Control Morpholino oligo add 0.2 ml of sterile water to give 0.2 ml of a 500 µM oligo stock solution.

Endo-Porter Delivery Reagent

Endo-Porter is provided as a 1.0 mM sterile solution prepared in either DMSO or 0.3 M mannitol solution. **Endo-Porter** can be stored at room temperature or refrigerated and requires no further preparation before use.

B. Preparing Cells for Endo-Porter Delivery

Cells can be cultured in plates or flasks and for best delivery should be 80-100% confluent.

C. Protocol for Delivery of Morpholinos with Endo-Porter

1. Replace spent culture medium with fresh complete medium (containing up to 10% serum).

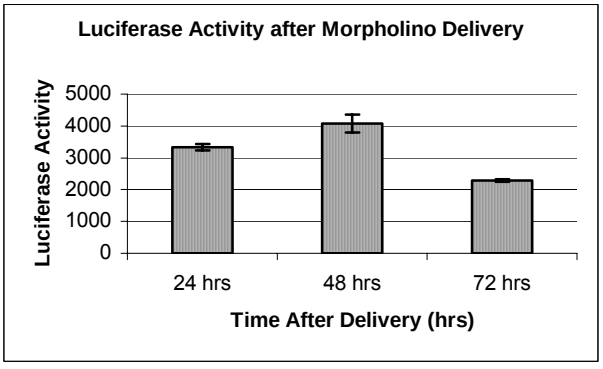
2. Add Morpholino stock solution to the desired concentration and swirl well to mix.

Morpholinos are typically effective at concentrations as low as 1 µM (2 µl of 500 µM stock per ml of media). However, for initial experiments and especially if you are evaluating Morpholino delivery via fluorescence we recommend starting with 10 µM Morpholino oligo concentration (20 µl of 500 µM stock per ml of media) and then reducing or increasing your Morpholino concentration based on your results.

3. Add 6 µl of **Endo-Porter** for every 1ml media and immediately swirl to mix. This provides a final **Endo-Porter** concentration of 6 µM.

4. Once all samples have been treated return plates/flasks to the incubator.

Cells can be assayed as soon as 16 hours after treatment and **Endo-Porter** can be left in the medium up to 72 hours without damage to your cells. We have found minimal toxicity and maximal delivery by assaying 48 hours after delivery (see figure below). In the HeLa positive antisense test system that generated these results, delivering the Standard Control Morpholino (3 µM) corrects a splicing error and puts luciferase in frame (Kang et al. 1998).



D. Storage of Reagents

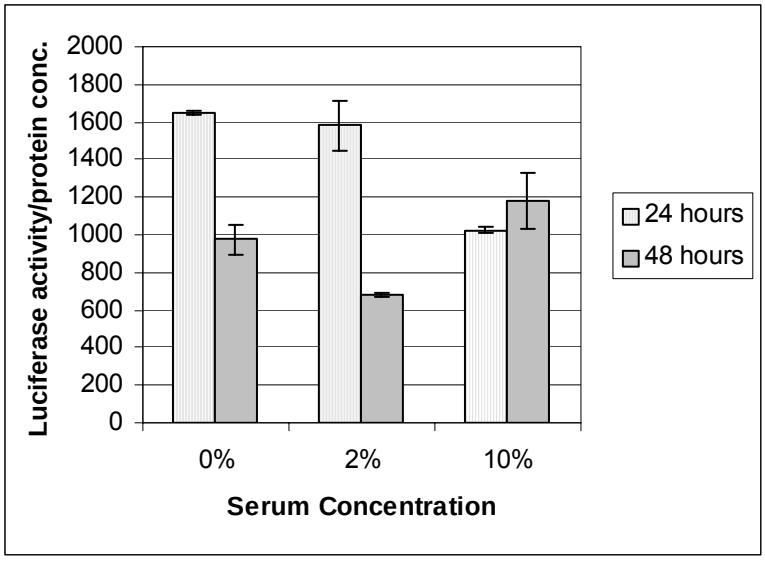
Morpholino stock solutions should be stored at room temperature in the original vial and preferrably at 1 mM. You can autoclave your stock in this original vial if you think you may have contaminated your stock. In addition, we recommend heating your stock solution at 65°C for 5 minutes to assure that the Morpholino is fully in solution before each use.

Endo-Porter is a stable peptide and can be stored in solution at room temperature for many months.

E. Frequently Asked Questions about Using Endo-Porter

1) What is the effect of serum on **Endo-Porter** delivery?

Endo-Porter is unique among delivery reagents as it works in complete (up to 10%) serum-containing media with only a small decrease in delivery efficacy. As shown below, the longer the **Endo-Porter** delivery period the smaller the difference between using serum-free and 10% serum-containing media. The data below was generated from delivering 3 μM Morpholino to 80% confluent HeLa positive antisense test system (Kang, 1998).



2) How do I scale the volume of **Endo-Porter** for different size plates and flasks?

Endo-Porter delivery of Morpholinos is a concentration-dependent process and you should expect no differences in delivery when using a lesser or greater volume of media.

3) Is **Endo-Porter** toxic?

Endo-Porter can **NOT** be washed off cells. Toxicity from **Endo-Porter** depends on the time and concentration that the cells are exposed to the **Endo-Porter** peptide. We have found minimal toxicity and maximum delivery using the recommended **Endo-Porter** concentration (6 μM) and assaying antisense activity at 48 hours.

4) May I optimize **Endo-Porter** delivery for my cell type?

Yes, in an initial study that follows your research protocol we recommend delivering your cargo (Morpholino, protein, peptide, etc.) using Endo-Porter in a range of concentrations from 2 to 10 μM (from 2-10 μl per ml of media) increasing in 2 μM increments and observing your cells for toxicity and delivery efficacy. We recommend you find the concentration that provides you with maximal delivery and minimal toxicity to use for further experiments.

5) How do I deliver proteins and peptides with **Endo-Porter**?

For proteins and peptides add the desired concentration to the medium, add 6 µl of **Endo-Porter** per ml of media and swirl to mix.

6) Which formulation of **Endo-Porter** should I purchase?

We recommend purchasing the **Endo-Porter** DMSO unless DMSO interferes with your system.

7) Does **Endo-Porter** deliver Morpholinos ***in vivo***?

Endo-Porter was specifically developed for *in vitro* applications. It may be effective in some *in vivo* systems where both Morpholino and **Endo-Porter** can be co-endocytosed into the same endosome. Gene Tools' is working on the development of methods for functional *in vivo* delivery of Morpholinos. New delivery products will be announced on www.gene-tools.com when they become available.

8) What procedures should I use to access delivery by fluorescence?

When using **Endo-Porter** delivery may be accessed via flow cytometry or visually using an inverted epifluorescent microscope. **Endo-Porter** can deliver enough Morpholino to the cell for antisense activity without depositing perceptible fluorescence in the cells, but during proof-of-delivery experiments we recommend using 10µM labeled Morpholino to see the fluorescence.

We recommend you look at live cells as the procedures involved in fixing cells may lead to leakage of Morpholinos into the cells and a false positive signal. Observe the cells periodically so you see changes in the pattern of the fluorescence over time. If you see diffuse fluorescence throughout the cytosol of the cells, you have delivered the Morpholino oligos. Using higher numerical aperture objective lenses (higher magnification objectives) can help increase the amount of light gathered from a cell and reveal dim fluorescence.

9) What cell types can **Endo-Porter** deliver?

We expect Endo-Porter to deliver cargo to any cell type that undergoes endocytosis followed by acidification of the endosome. Successful delivery has been reported for the following cell types:

Cell Lines
HeLa (ON705, CCL2)
Prostrate cancer
Glioma (U87, U250)
Kidney (MIMCD3)
Cortical (E13)
Hepatoma (H4 cells)
Neural crest stem (P19)
Neural
Xenopus
Primary mouse thymocytes

Primary Cells
Primary oligodendrocytes
Primary neurons
Sea urchin oocyte
Endometrial (*in vivo*)
Primary human fibroblasts

F. Reference

Morcos, P.A. (2001) Achieving Efficient Delivery of Morpholino Oligos in Cultured Cells. *genesis* 30(3), 94-102

Kang SH, Cho MJ, Kole R. (1998) Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry*. 37(18): 6235-39.