# SPECIAL DELIVERY PROTOCOL

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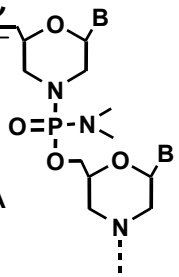


## I. INTRODUCTION

Special Delivery Morpholino oligos from Gene Tools have become an established method for delivering Morpholino oligos into cultured cells. The Special Delivery formulation provides substantially improved delivery into the cytosol/nuclear compartment of both adherent and suspension cells compared to previous scrape and osmotic delivery methods.

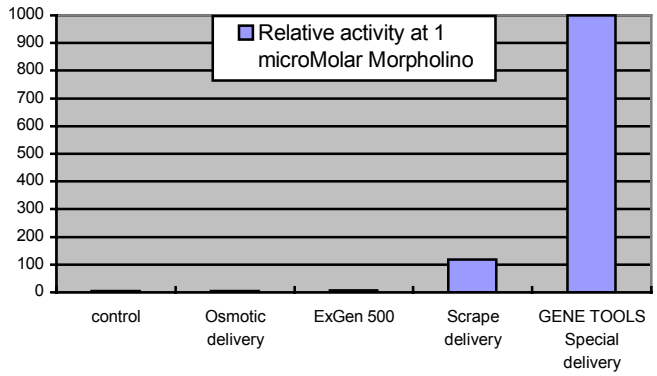
The Special Delivery Formulation consists of two components. One component comprises a pre-paired duplex of Morpholino oligo and partially complementary DNA oligo (A **green dot** should be attached to the bottom of the vial and **"-D**" should appear in the production number). The other component is a weakly basic delivery reagent, Ethoxylated Polyethylenimine (EPEI).

Delivery entails three simple steps:

1) Mix the Morpholino/DNA component with EPEI and incubate at room temp. 20 minutes.
2) Add the Morpholino/DNA/EPEI solution to your cells.
3) Three hours later remove the solution and replace with fresh culture medium.

The first step results in electrostatic binding of the anionic Morpholino/DNA duplex to cationic sites of the EPEI. Step 2 results in the electrostatic binding of the Morpholino/DNA/EPEI complex to anionic cell surfaces – leading to endocytosis of the complex. Subsequent acidification within the endosome ionizes additional weakly basic moieties of the EPEI, and this more extensively ionized EPEI permeabilizes the endosomal membrane causing release of the Morpholino oligo into the cytosol/nuclear compartment of the cell.

A related polyethylenimine reagent called ExGen 500 has been shown to be effective in delivering DNA into a variety of widely used tissue culture cell types (http://www.fermentas.com). The table below compares delivery by our special delivery formulation to that of scrape delivery, osmotic delivery, and delivery using ExGen 500.



## II. PREPARATION AND DELIVERY

### A. What GENE TOOLS provides

GENE TOOLS provides 300 nanoMoles of sterile pre-quantitated Special Delivery Morpholino oligo pre-paired with a partially complementary DNA oligo. The 300 nanoMoles of pre-paired oligo is enough for treating cells in 440 wells in 24-well plates. You also receive one 5-ml vial of green EPEI Special Delivery solution, which is sufficient for treating cells in 2,000 wells of 24-well plates.

### B. Preparing Stock Solutions

To one vial containing 300 nMoles of Special Delivery Morpholino/DNA add 600 μl sterile water to make a 0.5 mMolar stock solution. The green EPEI Special Delivery Solution is 200 μMolar in EPEI. If you have purchased a 100 nanoMole vial of Special Delivery Standard control Morpholino oligo then add 200 μl sterile water to make a 0.5 mMolar stock solution.

### C. Materials you supply for delivery:

1. Sterile 15.0 ml capped tubes
2. Sterile water
3. Non-adherent or adherent cells preferably at 80-100% confluency in the flask/dish of choice.
4. Prewarmed serum-free and serum-containing media for the cells you study.

### D. Protocol for treating cells in 3 wells of a 24-well culture plate

1. To a sterile 15 ml centrifuge tube:
   a) Add 188.8 μl $H_2O$
   b) Add 5.6 μl of the 0.5 mMolar Special Delivery Morpholino/DNA stock solution
   c) mix
   d) add 5.6 μl of the green 200 μMolar EPEI Special Delivery solution
   e) **Vortex immediately** and let stand at room temperature for exactly 20 minutes

2. After the 20 minute incubation above at room temp, add 1.8 ml of serum-free medium and **vortex immediately** to generate the complete delivery solution.

3. Remove media from cells (requires centrifugation in the case of non-adherent cells) and then add 500 μl of the complete delivery solution to each of the 3 wells, briefly mix, and return to the incubator.

4. After incubation for 3 hours, remove complete delivery solution from cells and replace with fresh serum-containing medium (centrifugation is required for non-adherent cells).

Cells can be assayed as soon as 16 hours after media replacement. Morpholino oligos are stable and totally nuclease resistant so there is no need for re-delivery. In some cases turnover of the targeted protein may be slow so incubation for several days may be required to significantly reduce the level of previously synthesized protein. However, when cells are allowed

to undergo more than 5-10 divisions it may be necessary to treat again to compensate for the dilution of oligo due to cell divisions.

**E. Table for scaling the above protocol for <u>triplicate</u> samples in selected plates and flasks.**

| Solutions | 48 well plate | 24 well plate | 12 well plate | 6 well plate | 25 $cm^2$ flask | 75 $cm^2$ flask |
|---|---|---|---|---|---|---|
| **$H_20$** | 94.9 μl | 188.8 μl | 283.2 μl | 566.4 μl | 1.0 ml | 3.4 ml |
| **Morph/DNA stock** | 2.8 μl | 5.6 μl | 8.4 μl | 16.8 μl | 33.6 μl | 100.8 μl |
| **EPEI Special Delivery solution** | 2.8 μl | 5.6 μl | 8.4 μl | 16.8 μl | 33.6 μl | 100.8 μl |
| **Serum-free medium** | 0.9 ml | 1.8 ml | 2.7 ml | 5.4 ml | 10.8 ml | 32.4 ml |
| **Aliquot per well or flask (3 samples)** | 0.25 ml | 0.5 ml | 0.75 ml | 1.5 ml | 3.5 ml | 11 ml |

**F. Storage of reagents**

The EPEI Special Delivery Solution and the Morpholino/DNA stock solution should be stored tightly capped at 4 °C. Freezing and thawing of the Morpholino/DNA stock solution should not adversely affect results.

**G. Troubleshooting and Optimization**

1. The EPEI Special Delivery Solution is killing more than 40% of the cells.

First make sure that you used the right amount of EPEI Special Delivery Solution for your scaled experiment (see table above). Second, make sure you are not using cells that were confluent more than a day. Such cells tend to be very sensitive to changes in conditions. Finally, you can reduce the Morpholino/DNA stock and EPEI stock together by as much as 1/3 and still achieve respectable delivery. For example with 24 well plates (see table above) you could reduce the volumes of the Morpholino/DNA stock and EPEI Special Delivery solution to 3.7 μl each. We have found for particularly sensitive cells (i.e. neuroblastoma and MDAMB231 cells) that reducing the EPEI volume to 4.0 μl and increasing the Morpholino/DNA volume to 10.0 μl has not only eliminated toxicity but resulted in significant delivery. Since the product has not been tested in all cell types, it is possible that your cells are not amenable to delivery by EPEI Special Delivery Solution. In such an event, if the cells are adherent you can utilize scrape delivery to achieve respectable results. The DNA partially paired to Morpholino oligo does not interfere with scrape delivery of the Morpholino oligo.

2. Can I optimize delivery for the cells I study?

You may be able to achieve better delivery by varying the concentration of EPEI in your preparations, but excessive EPEI may result in increased toxicity. We suggest that you use the Morpholino/DNA amounts listed in the table above, but vary the EPEI volumes in 10% increments (change $H_20$ volumes accordingly). Alternatively you can vary the Morpholino/DNA and EPEI Special Delivery solution by equivalent 10% increments. Watch for toxicity. You may wish to accept some cell toxicity in order to achieve greater delivery.

## H. Reference

Description of Special Delivery system:
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