Achieving Efficient Delivery of Morpholino Oligos with Nucleofection®

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Morpholino oligos provide a stable, specific and effective antisense activity. Developmental biologists have long since used these properties by microinjecting or electroporating Morpholinos into embryos to allow for splice-blocking or gene knockdown. However, efficient delivery of Morpholinos in mammalian cell cultures can be a challenge. While complex formation with lipid-based reagents is ineffective due to the non-ionic Morpholino backbone, we show here that Nucleofection® can be used as an efficient delivery method. As such, Nucleofection® of Morpholinos offers a straightforward alternative to siRNA-mediated gene knockdown and miRNA maturation inhibition.

Introduction

Morpholino oligos (MOs), also known as phosphodiamidate Morpholino oligos, are designed to bind to complementary RNA sequence and sterically inhibit proteins from binding the RNA. Morpholinos are commonly used to knock down gene expression and alter mRNA splicing in a variety of organisms, tissues or cells (for a searchable database of references see http://www.gene- tools. com/Publications/). For gene knockdowns the MOs are designed to complement mRNA sequence in the 5′ untranslated region and/or the first 25 bases of mRNA coding sequence, sterically inhibiting the movement of the translation initiation complex toward the start codon. This prevents the large subunit of the ribosome from assembling and, as a consequence, causing reduced gene expression. Morpholinos are also used to modify pre-mRNA splicing by targeting the oligo to a splice junction or regulatory site, sterically blocking binding of snRNPs or other splice factors[1]. Splice-blocking Morpholinos have great promise for therapeutic use correcting and/or reducing the impact of the mutations that lead to β-Thalessemia[2], Duchenne muscular dystrophy[3] and other diseases caused by

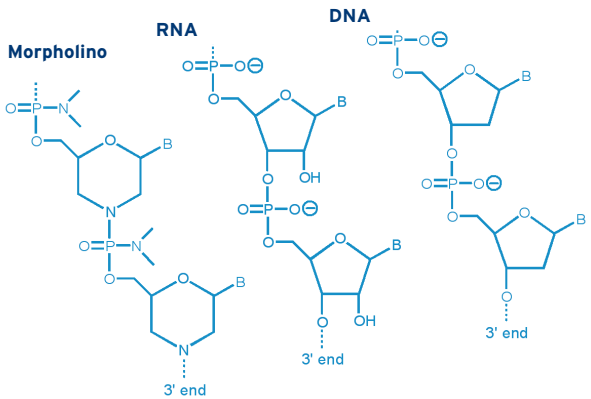


Figure 1: Comparison of the chemical structure of two-mers of Morpholino, RNA and DNA.
Two significant differences in the Morpholino backbone compared to RNA and DNA are the non-charged phosphodiamiate linkage between the subunits and replacement of the five-membered sugar ring with the six-membered Morpholine ring.

mis-splicing[4,5]. Morpholinos have also been used to block microRNA maturation[6] and microRNA targets[7], block ribozyme activity[8] and induce frameshifts[9].

However, even more so than RNA and DNA based oligos, Morpholinos are challenging to deliver into mammalian cells in culture. Standard lipid-based nucleic acid transfection reagents rely on an ionic interaction with a charged backbone and are not effective with the uncharged MO backbone (see Figure 1). While microinjection is suitable for delivery into organs (embryos or brains), most commonly used transfection methods for cultured cells are scrape loading[16], special delivery[17], conjugated cell-penetrating peptides[19], Endo-Porter delivery[18] or electroporation[14]. Here, we present, the results of our study using the Nucleofector® Technology for delivering Morpholinos.

Advantages of Morpholinos over DNA and RNA-based gene knockdown reagents:

- Very robust and stable molecules[10, 11] due to the non-biological, chemically stable backbone structure (**Figure 1**)
- Exquisite specificity: at low concentrations a Morpholino with just five mispairs along its length will have no antisense activity[12]
- Largely free of non-antisense effects as evidenced by rescue of the Morpholino phenotype via injection of the corresponding mRNA[13]
- Backbone is non-toxic
- Predictable targeting
- Steric block mechanism allows for additional applications such as altering mRNA splicing and blocking microRNA maturation

Material and Methods

The ON 705 HeLa cell line was grown to 75-80% confluency in DMEM/F-12 media (Invitrogen) with 10% FBS (Invitrogen). On the day of the experiment the cells were treated with 0.5% trypsin (Sigma), removed from the plate and spun down. The supernatant was removed and the cells resuspended in 100 µl Nucleofector® Solution R per manufacturer's, direction. The indicated amount of MO was added to each sample and mixed by vortexing. The sample was transferred to a Nucleofection® cuvette and transfected using Nucleofector® Program I-013. After Nucleofection®, 500 µL of serum free RPMI medium was added to each cuvette followed by transfer of contents to a 6-well plate. In the ON 705 HeLa cell line, the delivery of the Morpholino corrects a splicing mutation, splicing out a stop codon and putting luciferase in frame15. After 24 hours, the cells were lysed and luciferase (Promega) and protein (Bio-Rad) assays were carried out in duplicate for each sample. Delivery success is proportional to the light output and the data is normalized per protein.

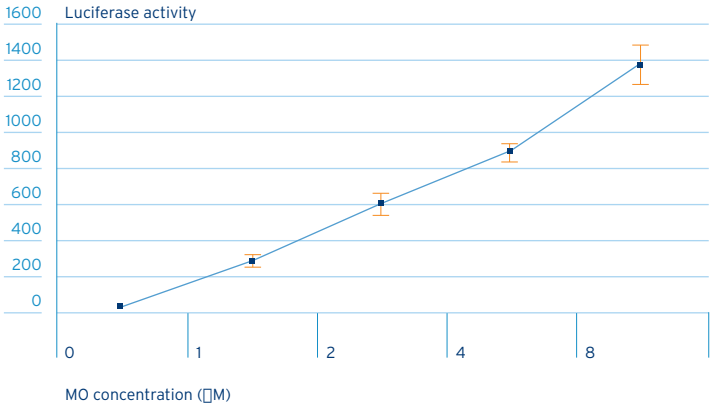
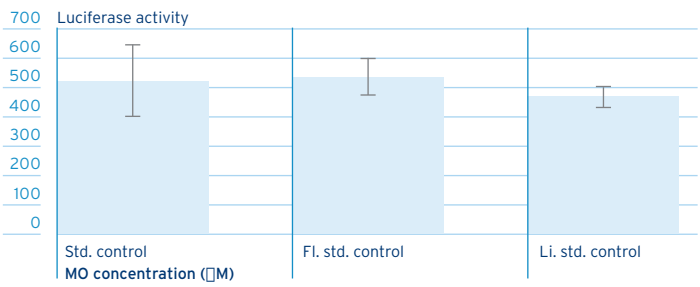


Figure 2: Dose-dependent delivery of Morpholinos into ON 705 HeLa cells. Cells were transfected with indicated concentrations of splice-correcting MO. In the ON 705 HeLa cell line, the Morpholino corrects a splicing mutation splicing out a stop codon and putting luciferase in frame. After 24 hours, the cells were lysed and luciferase activity and protein amount were determined. Delivery success is proportional to the light output (normalized to total protein amount).

Results/Discussion

Morpholinos were delivered uniformly in nearly 100% of the cells when imaged by fluorescence microscopy (data not shown). To further analyze delivery efficiency we used our established splice correction assay with a luciferase reporter stably expressed in ON 705 HeLa cells. The luciferase activity produced at the 1-8 µM range of MO reveals a consistent increase in MO activity (Figure 2). Generally, as shown by the efficacy of cytosolic microinjection of splice-modifying Morpholinos into embryos, delivery to the cytosol is sufficient to give nuclear splice-correcting activity of Morpholinos. However, average luciferase activities (Figure 2) seem to be slightly higher with Nucleofection® compared to activities commonly achieved with Endo-Porter. Effective MO amounts needed for Nucleofection® seem to be lower than those published for classical electroporation[14]. This suggests that Nucleofection® could be advantageous in terms of effective amounts due to its direct nuclear delivery or to smaller reaction volumes (higher concentrated cell suspension). In an additional experiment (Figure 3), the MO concentration was fixed at 2 µM and we compared delivery of Morpholinos with different 3' end modifications. The carboxyfluorescein (Fl) has two negative charges at physiological pH while the lissamine (Li) (sulforhodamine B) is a zwitterion with no net charge.

Figure 3: Modifications of 3' end do not alter delivery efficiency. Cells were transfected with 0.2 nmol (2 µM) of a standard MO, a carboxyfluorescein (Fl) or a lissamine (Li) labeled MO. 24 hours post Nucleofection®, delivery efficiency was analyzed by activity of splice-corrected luciferase (normalized to total protein amount).



We found that there were no significant differences in delivery of the MOs with 3' end groups compared to MOs with unmodified ends. In our lab, Nucleofection® has also been used effectively in C2C12 muscle cells to produce a quantitative shift in the splicing of dystrophin. In conclusion, Nucleofection® has been shown to allow efficient delivery of Morpholino oligos in cultured mammalian cells. Thus, by using Nucleofection® for delivery, Morpholinos can now be explored in difficult-to-transfect cell types and primary cells as potent alternative for other DNA- or RNA-based gene knockdown substrates.

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