Using Morpholinos to Control Gene Expression

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Morpholino oligonucleotides are stable, uncharged, water-soluble molecules used to block complementary sequences of RNA, preventing processing, read-through, or protein binding at those sites. Morpholinos are typically used to block translation of mRNA and to block splicing of pre-mRNA, though they can block other interactions between biological macromolecules and RNA. Morpholinos are effective, specific, and lack non-antisense effects. They work in any cell that transcribes and translates RNA, but must be delivered into the nuclear/cytosolic compartment to be effective. Morpholinos form stable base pairs with complementary nucleic acid sequences but apparently do not bind to proteins to a significant extent. They are not recognized by any proteins and do not undergo protein-mediated catalysis—nor do they mediate RNA cleavage by RNase H or the RISC complex. This work focuses on techniques and background for using Morpholinos. © 2017 by John Wiley & Sons, Inc.

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INTRODUCTION

Morpholino oligos (Morpholinos) are synthetic, uncharged *P*-chiral analogs of nucleic acids. They are typically constructed by linking together 25 subunits, each bearing a nucleic acid base. Figure 4.30.1 illustrates the structure of three Morpholino subunits joined by inter-subunit linkages. The Morpholino phosphorodiamidate backbone of Morpholinos consists of morpholine rings that bear methylene groups that are bound to modified phosphates in which the anionic oxygen is replaced by a nonionic dimethylamino group. The substituted phosphate is bound through a phosphorus-nitrogen bond to the nitrogen atom of another morpholine ring. One standard DNA nucleobase (adenine, guanine, cytosine, or thymine) is bound to each morpholine ring. The ends of Morpholinos are conventionally named 3′ and 5′ by analogy with the nomenclature for nucleic acids (though if one were to number the atoms of a Morpholino oligonucleotide backbone by IUPAC rules, the numbers assigned to the ends would be different). The secondary amine of the morpholine ring at the end of an unmodified Morpholino oligonucleotide is called the 3′ end of the oligo, whereas the 5′ end terminates with a carboxamidated phosphorodiamidate group (Fig. 4.30.1).

Antisense Morpholinos block the interactions of macromolecules with mRNA by base pairing with the targeted mRNA in a complementary fashion, thus preventing initiation complex read-through or modification of splicing in cells ranging from bacterial (Geller et al., 2005) to human (Suwanmanee et al., 2002). In particular, antisense Morpholinos have become a standard tool for developmental biologists to manipulate gene expression in embryos such as zebrafish and *Xenopus* sp. (Ekker and Larson, 2001). These modified

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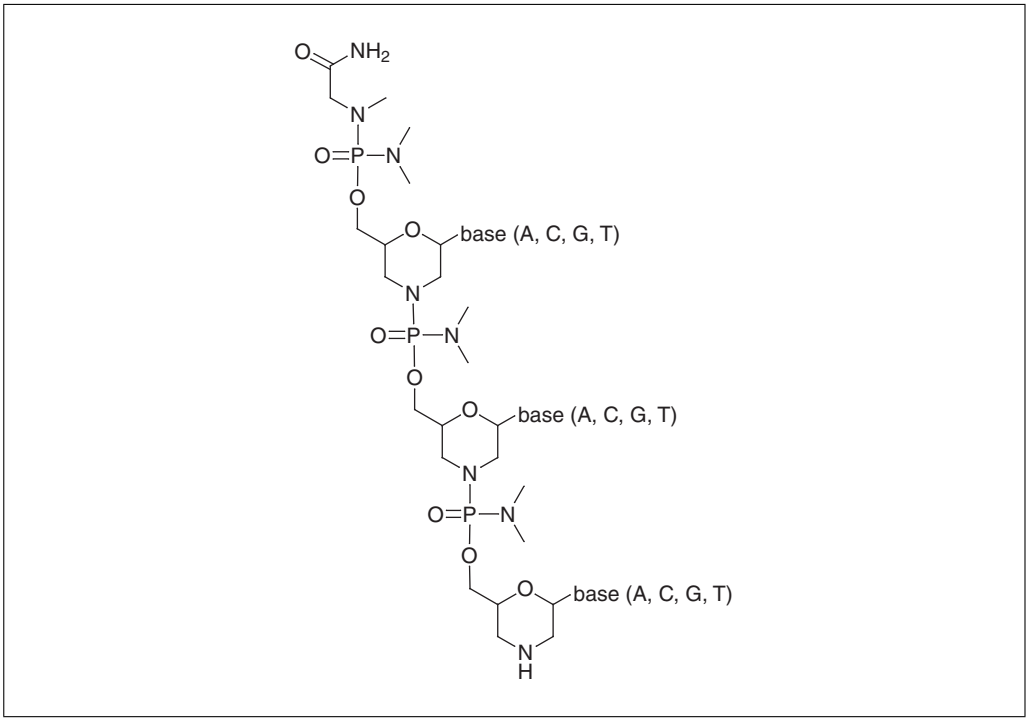

Figure 4.30.1 Structure of a Morpholino 3-mer.

oligonucleotides combine efficacy, specificity, stability, lack of non-antisense effects, and good water-solubility properties.

Morpholinos were originally conceived by James E. Summerton as a molecule intended for human therapeutics (Summerton, 2016). The first company he founded, now called Sarepta Therapeutics Inc., has successfully brought the first approved Morpholino drug through clinical trials, receiving accelerated approval from the U.S. FDA to market that drug (FDA, 2016). The drug, called eteplirsen or EXONDYS 51, is a Morpholino oligo targeting exon 51 of the most common human dystrophin transcript, the 79-exon isoform. For some people with Duchenne muscular dystrophy (DMD) who have frameshift mutations, causing exon 51 to be removed from mature mRNA ("skipping" the exon) can restore the reading frame of dystrophin so that an internally truncated protein is produced. This is predicted to benefit about 13% of the DMD population (Mendell et al., 2013). Small amounts of standard Morpholino oligos can enter dystrophic muscle, but do not usefully enter wild-type muscle; this shows that, for standard Morpholino oligos, DMD is especially amenable for treatment and delivery barriers will need to be overcome, perhaps by conjugation of the Morpholino to a delivery moiety, to apply the oligos effectively for treatment of many other diseases (Moulton and Moulton, 2010).

This unit presents three protocols: design of a knockdown experiment using Morpholinos (Basic Protocol 1), preparation of Morpholino solutions (Basic Protocol 2), and introduction of Morpholinos into cells by endocytosis in the presence of an amphiphilic peptide (Basic Protocol 3). The Commentary provides a thorough discussion of conditions and considerations for the application of Morpholinos.

BASIC PROTOCOL 1

Using Morpholinos to Control Gene Expression

DESIGN OF A MORPHOLINO KNOCKDOWN EXPERIMENT

This protocol outlines the choices commonly encountered while designing a Morpholino knockdown experiment. Considerations for the steps are addressed in the Commentary.

1. Choose the target gene.

2. Choose the cells or organism into which the oligo will be delivered.
3. Choose the sort of target process, typically blocking translation or modifying splicing. Other target processes may be chosen, such as inhibiting miRNA maturation, blocking poly(A) tailing, or blocking RNA translocation. The choice of target process will determine the molecular assays available for measuring antisense activity.
4. Obtain the sequence of the target RNA. Use the mRNA 5′-UTR and the first 25 coding bases for translation blockers, or pre-mRNA with introns and exons defined for splice blockers.
5. Choose a delivery method.
6. Select control oligos.
7. Decide whether end-modification of any oligos is necessary.
8. For blocking splicing, select which pre-mRNA splice junction (intron-exon, exon-intron) to block. If you wish to cause a frameshift and possibly activate nonsense-mediated decay, this will influence your choice of splice junction.
9. Select the oligo target (following the targeting rules described in the Commentary) to produce an optimized Morpholino sequence (the inverse complement of the target).
10. Use a transcript database and a homology search tool such as BLAST to test the selected target for homologies with other RNAs.

    If the selected target is too homologous with a region of an off-target mRNA where binding a Morpholino might change gene expression, a partially complementary Morpholino might affect the expression of that mRNA. Another target on the desired mRNA should be selected to prevent the off-site Morpholino interaction.

11. Order the synthesis of the selected Morpholinos.

PREPARATION AND VERIFICATION OF MORPHOLINO STOCK SOLUTIONS

BASIC PROTOCOL 2

This protocol describes the preparation of stock aqueous solutions of Morpholinos at concentrations of 1 mM or 500 μM, if necessary.

Materials

Lyophilized Morpholino oligo (Gene Tools)
Distilled autoclaved water without DEPC, sterile
0.1 M HCl

Glass or polypropylene/polyethylene tubes with labels
Quartz spectrophotometer cell (1-cm path length)
Parafilm
Lint-free lab tissues
UV spectrophotometer (or UV colorimeter) capable of measurements at 265 nm
Morpholino product information sheet

Prepare Morpholino solution

1. Read the amount of Morpholino given on the vial label and, using sterile technique, add the appropriate volume of distilled sterile water to make a 1 mM stock solution (i.e., 0.1 mL water for a vial containing 100 nmol Morpholino).

   The aqueous solubility of Morpholinos is sequence-dependent, but most Morpholino sequences with G content below 36% will dissolve in water at the recommended stock concentration of 1 mM. Do not keep Morpholino solutions of <1 μM, because

submicromolar concentrations can lose significant activity by binding to glass and plastic surfaces.

It is strongly recommended that Morpholino stock solutions be made with distilled water, but isotonic buffers (e.g., Ringer's solution, Danieau buffer) can also be used. The use of distilled water facilitates the process of concentrating Morpholinos and measuring their mass by MALDI-TOF mass spectrometry, should either process be required.

If water must be treated with diethylpyrocarbonate (DEPC), it is very important to autoclave the treated water to destroy residual DEPC before using it to dissolve Morpholinos. Otherwise, DEPC reacts with adenines and compromises the ability of Morpholinos to bind to their targets (Henderson et al., 1973).

2. Cap the vial and shake it.
3. Autoclave the oligo solution using the autoclave's liquid setting. As soon as the pressure in the autoclave has returned to ambient pressure, remove the oligo solution to prevent evaporation.
4. If desired, dispense into several tubes. Label tube(s) with the concentration and oligo name, and store any tubes that will not be used immediately.

Scrupulously avoid microbial contamination of the stock solutions. Store fluorescent-tagged Morpholinos in a closed box so that light will not bleach fluorescent moieties.

Morpholinos are best stored in sealed tubes at room temperature in a humid chamber (such as a bell jar containing an open beaker of water). If activity drops during storage, autoclave the oligo solution to disrupt complexes.

Check Morpholino concentration by UV absorbance

5. Turn on the UV spectrophotometer and let it warm up for a few minutes. Set the spectrophotometer to report absorbance at 265 nm.
6. Clean the quartz spectrophotometer cell, if needed, and rinse the inside twice with 0.1 M HCl. Carefully shake excess liquid from cell.

Do not touch the outside of the quartz spectrophotometer cell on the surfaces where light will pass through, as skin oils can skew the measurements.

7. Pipet 995 μL of 0.1 M HCl into the quartz cell and place the cell in the spectrophotometer. Blank the spectrophotometer at 265 nm.
8. Remove the cell from the spectrophotometer and pipet 5 μL aqueous Morpholino solution into the quartz cell.

Like natural nucleic acids, the nucleobases of a Morpholino are stacked and produce a hypochromic effect. Without unstacking the bases, the use of the molar absorptivity of an individual nucleobase to calculate the concentration of the oligo would lead to an erroneously low value. Oligos with A, C, and G bases can be unstacked by dissolving the oligos in 0.1 M HCl. Under these conditions, A, C, and G bases are protonated and are out of the stacked state due to electrostatic repulsion. When the nucleobases of the oligo are unstacked, the molar absorptivity of each nucleobase can be applied to determine the concentration of the oligo.

9. Place a piece of Parafilm over the open end of the cell, placing a thumb over the Parafilm to seal the cell, and invert several times to mix.
10. Remove the Parafilm. Wipe the outside of the cell with a lint-free tissue, if needed.
11. Place the cell in the spectrophotometer and read the absorbance at 265 nm (A_{265}).

12. If the absorbance is outside the spectrophotometer's linear response range, dilute the solution and repeat the measurement, adjusting the dilution factor in step 13 to account for this change.

13. Calculate the molar concentration (C) of the original Morpholino solution as:

$$C = (A_{265} \times 200) / (\varepsilon\ b)$$

where 200 is the factor for dilution in HCl, ε is the molar absorptivity, and b is the path length of the cell (1 cm).

The molar absorptivity (ε) of the Morpholino is provided on the product information sheet. Alternatively, ε can be calculated by multiplying the molar absorptivity of each nucleobase (A, C, G, and T) by the number of instances that the nucleobase is present in the oligo, and adding these products.

For a 1-cm path-length spectrometer, this Beer's law calculation works when absorbance ≤2, where the relationship of absorbance to concentration is linear. If the measured absorbance is >2, the sample should be diluted and remeasured.

DELIVERY OF MORPHOLINOS INTO CELLS USING ENDO-PORTER

BASIC PROTOCOL 3

Endo-Porter is an amphiphilic peptide. After co-endocytosis with Morpholinos, Endo-Porter permeabilizes membranes of acidic endosomes, releasing the Morpholino from the endosomes to the cytosol (Summerton, 2005). Endo-Porter was optimized using a HeLa cell line. Because tolerance of other cell types toward Endo-Porter often varies, a range of Endo-Porter concentrations should be tested before beginning knockdown experiments. The DMSO formulation is typically several-fold more effective than the aqueous formulation.

Materials

1 mM Endo-Porter solution (aqueous or DMSO formulation; Gene Tools)
Cell cultures in plates or flasks at 80% to 100% confluence
1 mM Morpholino stock solution (Gene Tools)
1 mM fluoresceinated dextran, 10 kDa
Cell culture medium with 10% or less serum
Fluorescence microscope

Select amount of Endo-Porter for cell type

1. Prepare concentrations of 2, 4, 6, and 8 μM Endo-Porter by pipetting 2, 4, 6, and 8 μL of a 1 mM Endo-Porter solution into 1-mL aliquots of cell culture.

2. Add 10 μM fluorescently labeled Morpholino (10 μL of 1 mM stock per 990 μL cell culture) or 10 kDa fluoresceinated dextran (10 μL of 1 mM stock per 990 μL cell culture). Swirl well to mix.

3. Allow endocytotic uptake to proceed over a period of 24 hr.

4. Observe intracellular fluorescence using a fluorescence microscope.

   See discussion on assessing delivery in the Commentary section.

5. Observe cells 72 hr after delivery to determine any cellular toxicity. For adherent cells, look for morphology changes such as drawing in of extended processes causing the cells to become rounder—for many cell types, such a change in shape indicates toxicity.

For subsequent Morpholino delivery to the selected cell type, use the concentration of Endo-Porter that gave the best delivery without toxicity.

Deliver Morpholinos to cells

6. Using a cell culture not previously exposed to Endo-Porter, replace spent culture medium with fresh medium (with up to 10% serum).

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

   For functional experiments (e.g., gene knockdown, splice blocking), Morpholinos are typically effective at concentrations as low as 1 μM. However, it is recommended that a range of concentrations be tested (such as 1, 4, and 10 μM Morpholino) to define optimal conditions.

8. Add Endo-Porter to produce the optimized concentration for the cell type and immediately swirl to mix.

   Endo-Porter forms complexes in aqueous solutions; when adding the DMSO formulation of Endo-Porter to aqueous solution, a rapid swirling of the mixture helps keep the complexes small and abundant, improving delivery.

9. Place the plates or flasks in the incubator. Wait at least 16 hr before assessing uptake by fluorescence, and at least 24 hr before measuring knockdowns by molecular assays.

   The delay needed to assay a knockdown depends on the stability of any pre-existing protein encoded by the targeted mRNA; a protein with a long half-life will take longer to disappear from the cells. If needed for a very stable protein, cells can be re-dosed with Morpholinos using Endo-Porter, typically after 4 days. Endo-Porter sticks onto cell membranes through cell washes, so it might be necessary to decrease the Endo-Porter concentration during re-delivery to avoid toxicity.

COMMENTARY

Background Information

The morpholino phosphorodiamidate backbone of a morpholino oligo has no significant ionic charge at neutral pH, in contrast with the polyanionic phosphodiester backbone of a natural nucleic acid. This favors the interaction of Morpholinos with nucleic acids, since there is no repulsion between anionic backbones as there is in duplexes of natural nucleic acids. Dissolved in pure water, nucleic acids lose their ability to form stable Watson-Crick bonds due to anionic repulsion between strands, whereas Morpholinos will still bind to complementary sequences (Summerton, 2004). Because Morpholinos are uncharged, they have no strong electrostatic interactions with proteins. Unmodified Morpholinos have little or no affinity for bovine or human serum albumin when assessed by surface plasmon resonance spectroscopy (H.M. Moulton, unpub. observ.). In contrast, interactions of anionic phosphorothioate oligos with proteins cause multiple physiological, non-antisense effects (Lebedeva and Stein, 2001). Proteins that bind nucleic acids generally interact electrostatically with the anionic phosphates of nucleic acids, stabilizing binding. Morpholinos appear to have little or no interaction with nucleic acid–binding proteins (Hudziak et al., 1996). The synthesis of their subunits and assembly on a solid-phase resin has been described (Summerton and Weller, 1997), and modified synthesis protocols offering alterative protecting group and activation schemes and have been published (Bhadra et al., 2015).

Morpholinos are very stable to nucleolytic enzymes. There are no known enzymes that degrade Morpholinos. Specifically, Morpholinos have been exposed to a range of nucleases (e.g., DNase I, DNase II, Benzonase, S1 nuclease, mung bean nuclease, *Bal* 31 nuclease, RNase A, RNase T1, phosphodiesterase I, and phosphodiesterase II) and proteases (e.g., pronase E, proteinase K, and pig liver esterase) under conditions where lytic enzymes would degrade their substrates. In no case was degradation of the Morpholinos detected (Hudziak et al., 1996). Morpholinos were incubated in serum and in liver homogenate without

Table 4.30.1 RNA Binding Affinity of Various Oligo Types Ranked by Dissociation Temperature in Physiological Isotonic Buffers

Affinity	Type of oligo
Strongest	RNA:RNA, PNA:RNA, 2′-*O*-methyl-RNA:RNA (all very similar)
Strong	Morpholino:RNA
Medium	DNA:RNA
Weakest	Phosphorothioate:RNA

degradation (Summerton and Weller, 1997). When peptide-Morpholino conjugates were extracted from cells and analyzed by MALDI-TOF mass spectrometry, the Morpholino oligo component was not degraded in the cells, although a range of degradation products from the peptide moiety were detected (Youngblood et al., 2007).

No crystal structure or high-resolution NMR structural analysis of phosphorodiamidate Morpholinos has been published. However, the study of a Morpholino phosphorodiamidate ApA dimer using circular dichroic spectroscopy showed stacking of bases in aqueous phosphate buffer (Kang et al., 1992). On the basis of molecular modeling, the bases of Morpholinos should stack in a fashion analogous to those of natural nucleic acids, allowing strong interactions with complementary nucleic acid sequences by Watson-Crick base pairing. A 400 MHz ^{1}H NMR analysis of a carbamate-linked nucleic acid analog using morpholine rings instead of sugars found the morpholine ring in the chair conformation (Stirchak et al., 1989). Molecular modeling of a Morpholino with the morpholine rings in the chair conformation suggests that a Morpholino and an RNA form an A-form heteroduplex with a helical pitch similar to that of an A-form RNA-RNA duplex (J.E. Summerton, unpub. observ.).

Various types of antisense oligos are ranked by their affinity for binding to single strands of sense RNA based on their dissociation temperatures in physiological salt buffers (Table 4.30.1; Stein et al., 1997). The affinity of RNA for RNA is greater than the affinity of Morpholinos for RNA. However, single strands of mRNA folded into secondary structures contain single-stranded regions, such as the loops of stem-loops, with which Morpholinos can readily hybridize. Given that double-stranded regions of most RNA secondary structures are shorter than 25 base pairs, the overall binding affinity of Morpholinos for RNA is sufficient to invade and displace those short double-stranded regions (Summerton, 1999).

Antisense oligos such as DNA and phosphorothioate (*S*-DNA) oligos recruit RNase H to degrade their mRNA targets (Summerton, 1999). RNAi and siRNA also employ an antisense mechanism to recognize a sense mRNA through interaction with RISC, which leads to enzymatic degradation of complementary mRNA and translational blocking of partially complementary mRNA (Scacheri et al., 2004). In contrast, instead of degrading mRNA, antisense Morpholinos were designed to block the translation of mRNA into protein (Summerton and Weller, 1997). Figure 4.30.2 compares steric blocking, RNase H–dependent, and RISC-dependent oligos.

When comparing an RNase H–dependent oligo (a methylphosphonate diester/phosphodiester chimera) with a Morpholino, a CpGNNN motif was shown to induce apoptosis and cell cycle arrest when present in the RNase H–dependent oligo but not when present in the Morpholino (Tidd et al., 2001). There have been no reports of Morpholinos inducing either interferon production or induction of NF-κB mediated inflammation, and Morpholinos containing CpG motifs do not stimulate immune responses (J.E. Summerton and A. Krieg, unpub. observ.), suggesting that Morpholino-RNA heteroduplexes do not stimulate Toll-like receptors.

Morpholinos complementary to sequences in the 5′-UTR and the first 25 coding bases of an mRNA can halt the progression of the initiation complex toward the start codon, preventing assembly of the entire ribosome. This inhibits the translation of the mRNA sequence into a polypeptide. Morpholinos targeted entirely downstream of the start codon are usually ineffective for blocking translation (Summerton, 1999).

In addition to their application to knock down gene expression, because steric-blocking oligos do not trigger degradation of RNA, Morpholinos are also widely used to block splicing of pre-mRNA. Splicing in eukaryotes is directed by snRNPs that bind to introns and mark the intron-exon boundaries. Morpholinos targeted to these snRNP-binding sites can modify splicing (Sazani et al., 2001), either preventing splicing and causing an intron inclusion (Giles et al., 1999) or redirecting splicing and causing an exon excision (Draper et al., 2001). Blocking a splice site can cause

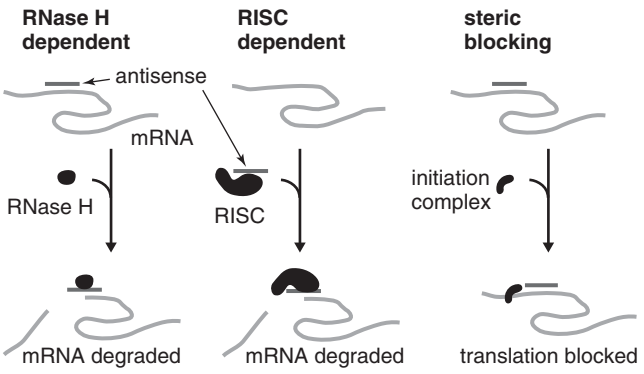

Figure 4.30.2 Comparison of RNase H–dependent, RISC-dependent, and steric-blocking oligos.

activation of a cryptic splice site, complicating interpretation of the splice modification by producing partial deletions of exons (Draper et al., 2001) or partial inclusions of introns. In some cases, a single Morpholino can cause skipping of several adjacent exons.

Morpholinos stimulate site-specific ribosome frameshifting when bound just downstream of a shift site on an mRNA, and they do so with far higher efficiency than RNA, phosphorothioate oligos, or 2′-*O*-methyl RNA oligos (Howard et al., 2004).

Although Morpholinos are most often used to block the translation initiation complex or the snRNPs that direct splicing, there are other mRNA sequences that are attractive targets for steric blocking. Specifically, Morpholinos can block miRNA activity by binding to the pre/pri-miRNA and inhibiting its maturation, binding to the miRNA and preventing it from binding its mRNA target (Kloosterman et al., 2004), or binding to the site on the mRNA where the miRNA would otherwise bind (Choi et al., 2007). Along similar lines, Morpholinos targeted across the cleavage site of a hammerhead ribozyme inhibited auto-cleavage, leading to a greater than two orders of magnitude increase in the expression of a downstream reporter gene (Yen et al., 2004). Morpholinos have also been shown to block intronic splice silencers (Bruno et al., 2004) and exonic splice enhancers (McClorey et al., 2006), redirecting splicing. Morpholinos binding poly(A) signal sequences can inhibit poly(A) tailing (Wada et al., 2012). Morpholinos binding translocation sequences can inhibit directed movement of RNA within cells (Arthur et al., 2009).

Morpholinos are commonly microinjected into embryos at the single-cell or few-cell stages to block genes involved in development (Heasman et al., 2000; Nasevicius and Ekker, 2000; Nutt et al., 2001). Morpholinos are also commonly used in cell cultures (Tyson-Capper and Europe-Finner, 2006). Applications in intact adult organisms have until recently been limited by poor in vivo delivery into the cytosol of cells (Summerton, 1999; Sazani et al., 2002). However, advances in conjugating Morpholinos to cell-penetrating peptides (Nelson et al., 2005) or to octa-guanidinium dendrimers to form Vivo-Morpholinos (Li and Morcos, 2008) now allow effective systemic delivery into adult organisms (Alonso et al., 2005; Kinney et al., 2005; Neuman et al., 2005; Enterlein et al., 2006). Combinations of several oligonucleotide sequences can bind to several different RNA targets simultaneously if introduced together into embryos (Ekker, 2000) or cell cultures (Summerton, 2005), allowing multiple knockdowns or synergistic targeting of a single messenger.

Targeting of viral RNA with Morpholinos has been reported for hepatitis C (Jubin et al., 2000; McCaffrey et al., 2003), dengue virus (Kinney et al., 2005), Ebola virus (Enterlein et al., 2006; Warfield et al., 2006), SARS virus (Neuman et al., 2005), West Nile virus (Deas et al., 2005), equine arterivirus (van den Born et al., 2005), mouse hepatitis virus (Neuman et al., 2004), novirhabdovirus (Alonso et al., 2005), and vesivirus (Stein et al., 2001). In addition to translation start sites, successful targets for inhibition of viral replication include cyclization sequences (Deas et al., 2005), terminal stem loops (Deas et al., 2005), and internal ribosomal entry sites (IRES; Jubin et al., 2000).

Radioisotope delivery into organisms can be pretargeted using Morpholinos (Mang'era et al., 2001). Practitioners of nuclear medicine strive to minimize radiation exposure of a patient while delivering radionuclides to target tissues for imaging or for therapeutic applications. By attaching radioisotopes to

antibodies that are specific for target tissues, the antibodies can anchor isotopes on these tissues. Because the large antibody molecules diffuse slowly, the isotopes must be maintained in the plasma at high concentrations or for long durations to achieve good delivery of radioisotope-linked antibodies to their targets. Pretargeting with Morpholinos involves introducing an antibody-Morpholino conjugate into the bloodstream; this can be done using high concentrations or re-dosing to saturate the target without exposing the patient to radiation during this pretargeting stage. Next, a conjugate of a radioisotope (possibly chelated) with a complementary Morpholino is added to the blood. Because the Morpholino has a much smaller molecular mass than an antibody, the radionuclide-Morpholino conjugate diffuses relatively quickly and is captured at the target tissue more rapidly through Morpholino-Morpholino pairing. Unbound radionuclide-Morpholino conjugate is rapidly eliminated through the kidneys. This technique allows delivery of radioisotopes to the targeted tissue while exposing the organism to lower doses of radiation away from the targeted region. In the process of developing these techniques, pharmacokinetics of Morpholino-radionuclide conjugates have been studied in vivo (Liu et al., 2002a,b; He et al., 2003). In a recent modification, signals are amplified by binding a polymer bearing many complementary Morpholinos to each Morpholino-conjugated antibody fragment, followed by delivering radioisotope-labeled Morpholino complementary to the polymer-linked Morpholinos (He et al., 2003, 2004). Cell-surface membrane proteins can be concentrated in a small region of the plasma membrane by binding them with Morpholino-conjugated antibodies and then exposing the cell to polymer particles bearing the complementary Morpholinos (Chu et al., 2015).

Critical Parameters

Choosing Morpholino sequences

The parameters considered when selecting oligonucleotide target sequences include CG%, G%, self-complementarity, tetra-G moieties, length of the oligo, and the intended temperature at which the oligo will be used. The targeting recommendations are summarized below and in Table 4.30.2.

CG range

A range of 40% to 60% CG is considered ideal for 25-base Morpholinos in 37°C systems. Oligos with <40% CG may lack the affinity needed for effective steric blocking, while oligos with >60% CG are more likely to interact with off-target messengers through high-affinity subsequences. However, T_m prediction is a more reliable predictor of Morpholino activity and has mostly supplanted using CG content as a design criterion.

Predicted T_m

In a collaboration between DNA Software and Gene Tools LLC, complementary Morpholino-RNA heteroduplex melting parameters were measured and an algorithm developed for calculating predicted T_m of the duplexes based on Morpholino sequence. Hypotheses were tested in an iterative process on new oligo sequences until T_m could be predicted with acceptable accuracy. DNA Software makes this algorithm available in some of their oligo design software packages. Gene Tools typically prefers oligos with predicted T_m between 80°C and 100°C at 10 μM oligo.

G content

G content affects aqueous solubility of an oligo, with higher G contents being less soluble, particularly when the oligo is dissolved in isotonic salt solutions. Morpholinos with G contents up to 36% should be soluble in the millimolar range in pure water or aqueous buffer. However, chilling or especially freeze-thaw cycles can cause high-G oligos to precipitate or associate with their container walls, from which recovery of oligo into the solution can be difficult to impossible (see Basic Protocol 2).

Self-complementarity

Self-complementary sequences can cause either intramolecular interactions, forming stem-loops, or intermolecular interactions, forming dimeric Morpholinos. When a short sequence of one part of an oligo is complementary to another short sequence separated by an intervening sequence, stem-loops can form. If small self-complementary sequences are separated by zero to a few bases, formation of a stable stem-loop is unlikely because a hairpin with a small loop is not energetically favored. To prevent loss of oligo activity through competition between self-pairing and target binding, it is prudent to limit self-complementary sequences in oligo designs to 16 contiguous hydrogen bonds or less, where CG pairs contribute three hydrogen bonds and AT pairs contribute two hydrogen bonds. For

Table 4.30.2 Summary of Morpholino Targeting Recommendations for 37°C Systems

Parameter	Recommendation	Comments
T_m range	80°C to 100°C at 10 μM oligo	At lower T_m, affinity may be too low to block processes; higher T_m favors nonspecific binding of subsequences
G content	Up to 36% G	Higher G causes loss of water solubility; avoid upper end of acceptable range, if possible
Self-complementarity	16 contiguous H-bonds maximum	For intermolecular (complementary palindrome) and intramolecular (stem loop) binding. Example: AGCGCT has 16 H-bonds ($2 + 3 + 3 + 3 + 3 + 2 = 16$). Check for non-Watson-Crick G-T pairing, which can participate in self-complementarities.
Consecutive G	3 consecutive Gs maximum	Runs of ≥4 G can associate through Hoogsteen bonding to form oligo tetramers
Oligo length	25 bases or shorter by only a few bases	Using shorter oligos can decrease the chance of off-target interaction for high CG oligos

instance, the short sequences ATGGC and GCCAT can form 13 contiguous hydrogen bonds ($2 + 2 + 3 + 3 + 3 = 13$). When analyzing sequences for self-complementarity, check for both Watson-Crick base-pairing and for GT base-pairing. Like an AT pair, a GT pair also forms two hydrogen bonds. However, because the overall stability of the GT pair is far lower than an AT pair, a GT pair can be scored as a single hydrogen bond when calculating its contribution to the stability of a self-complementary moiety (Aboul-ela et al., 1985).

An oligo containing a self-complementary sequence can form dimers. To prevent loss of oligo activity through competition between dimer formation and target binding, it is prudent to limit complementary palindromes to 16 contiguous hydrogen bonds or less. For instance, if two oligos bearing the self-complementary sequence ATGCATGCGT encounter each other, they can form 22 contiguous hydrogen bonds ($2 + 1 + 3 + 3 + 2 + 2 + 3 + 3 + 1 + 2 = 22$, taking into account the GT pairs) and would likely have poor antisense activity.

G tetrads

Nucleic acids containing GGGG moieties can interact through Hoogsteen bonding to form oligo tetramers (Cheong and Moore, 1992). Morpholinos containing G tetrads have reduced activity, likely through the same mechanism. Because of this, contiguous stretches of four or more G bases should be avoided when designing Morpholinos.

MIL and oligo length

The minimum inhibitory length (MIL) of an antisense oligo is the length needed to achieve 50% reduction in translation of a targeted gene at a concentration typically achieved in cells. The MIL of Morpholinos varies somewhat between targets, but averages about 14 bases for 37°C cell cultures (Summerton, 1999). To ensure good affinity between Morpholinos and their RNA targets, the oligos are usually synthesized as 25-mers. The T_m can influence the MIL of an oligo, with a higher-T_m oligo having a shorter MIL. Oligos with high T_m might interact with off-target RNA; these oligos can be shortened by a few bases to lessen the likelihood of off-target interactions (decreasing the T_m in the process). The marginal loss of affinity resulting from shortening a high-T_m oligo to produce an acceptable T_m will not ruin activity but will slightly improve specificity. A more effective way to improve specificity is to choose a 25-base target that produces an oligo with an acceptable T_m, though this will not be possible for all transcripts.

Temperature and oligo selection

The targeting guidelines were developed for oligos to be used at 37°C. Many embryos are grown at lower temperatures. When temperatures are decreased appreciably, stability of base-pairing increases. The ideal T_m for oligos designed for use at lower temperatures would be expected to be lower than the 80° to 100°C at 10 μM oligo that is recommended for 37°C systems. In practice, however, the same T_m range has performed well for colder systems (e.g., zebrafish and frogs). The allowable number of base pairs in self-complementary sequences might prudently be reduced for colder systems. Solubility is also decreased at lower temperatures, so it is best to select oligos with lower G contents for use in colder systems.

Targetable region for translation blockers

To block translation, a 25-mer Morpholino can target anywhere between the 5′ cap to 25 nucleotides into the coding sequence. The target can extend downstream into the coding sequence as long as the translational start codon is covered. In the first steps of translation, the initiation complex forms at the 5′ cap and then scans through the UTR to the start codon (Fig. 4.30.3A). At the start codon, the large ribosomal subunit binds, initiation factors dissociate, and translation proceeds through the coding region. If a Morpholino gets in the way of the initiation complex before the initiation complex reaches the start codon, it prevents assembly of the ribosome and translation of the mRNA. Nonetheless, it is preferable to target the start codon instead of upstream for two reasons. First, the quality of sequence deposited in public databases can be poor in the UTR, especially for older sequence records. Second, though rare in vertebrate genomes, internal ribosome entry sites (IRES) do exist and can allow a ribosome to enter and assemble downstream of a Morpholino bound in the 5′-UTR.

Targetable region for splice blockers

To block splicing, Morpholinos are typically targeted to pre-mRNA across or adjacent to the boundaries between exons and introns. A pre-mRNA that undergoes splicing has two flanking exons (the first and last exon) and an arbitrary number of internal exons. The first exon has a single splice site, a splice donor, where it contacts intron 1. The internal exons have two junctions each, a splice acceptor at the upstream end and a splice donor at the downstream end. The last exon has only a splice acceptor at its upstream end. Targeting the splice sites of the internal exons usually causes exon excision, resulting in an mRNA missing the exon with the blocked splice site (Fig. 4.30.3B). Targeting splice sites of the flanking (first or last) exons usually causes intron inclusion, resulting in an mRNA containing the first or last intron. Intron inclusion can also occur when targeting internal exons, though it is less frequently observed; if it does occur, it is the intron into which the oligo binds that is included in the mature mRNA. Sometimes blocking a splice site activates a cryptic splice site or triggers a double exon skip, resulting in an mRNA with an unexpected mass.

The snRNPs that direct splicing bind at the intronic sides of the splice junctions, so Morpholinos are chosen that are complementary to more intronic sequence than exonic sequence. Morpholinos can have good activity if targeted entirely to intronic sequence near the splice junction, but activity decreases as the target is moved farther into the intron (Morcos, 2007).

Splicing can also be modified by preventing excision of an arbitrary intron by blocking the nucleophilic adenosine that closes the splicing lariat (P.A. Morcos, unpub. observ.) or by targeting splice-regulatory sequences.

It is often the goal of a splice-blocking experiment to eliminate activity of a protein. A useful technique is to eliminate an upstream exon that has a number of bases not evenly divisible by three, causing downstream translation to be frameshifted. If the active site of the protein is known, a straightforward strategy is to target a Morpholino to the exon encoding the active site, causing the loss of that exon and of the active site. Another is to trigger inclusion of the first intron, especially useful if it contains an in-frame stop codon or if its number of nucleotides is not evenly divisible by three. Sometimes, causing a random exon exclusion or intron inclusion is sufficient to eliminate activity of a protein, perhaps due to a resulting change in the protein's tertiary structure.

Quality of sequence

Since a few mismatches can seriously decrease the activity of a Morpholino, the quality of the target sequence is an important consideration when designing Morpholinos. There are sometimes errors in sequence database files. Variations in sequence between strains of an organism can also present a problem. The most definitive way to ensure the correct target sequence is to sequence the targeted gene in the strain that will be used in the experiments.

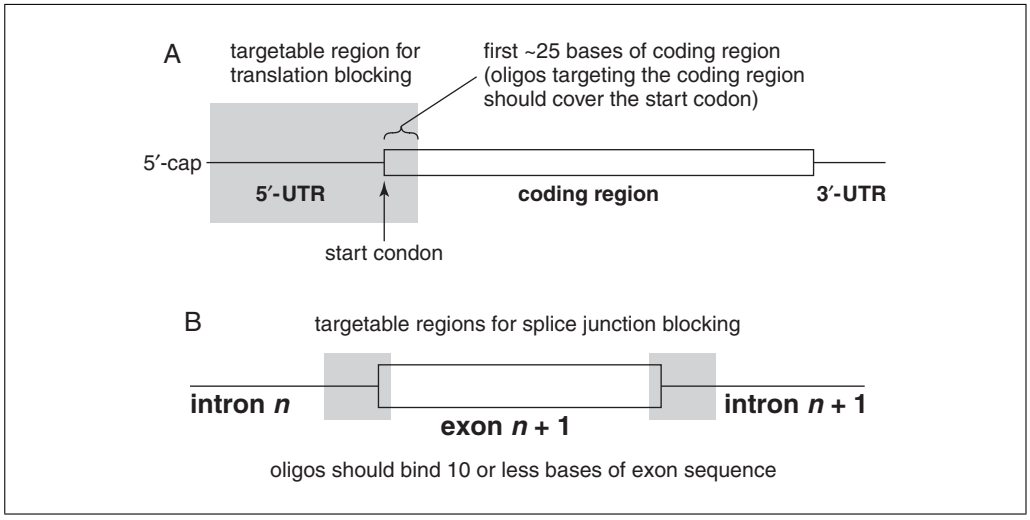

Figure 4.30.3 Targetable regions for translation blocking (**A**) and splice junction blocking (**B**).

Mismatched unintentional targets and Morpholinos

When a 25-base Morpholino is used near its lowest effective concentration, its effects are very specific compared to other knockdown technologies. Under such conditions, the oligo can also interact with sequences containing one or two mismatches when compared to the oligo's perfectly complementary target, although even a single mismatch can decrease activity (Khokha et al., 2002). However, few to no such sequences are expected to occur randomly in a base pool the size of the Morpholino-targetable sites in the human transcriptome (Summerton, 1999).

Effect of concentration on specificity

When the concentration of any antisense oligo is increased well above its minimum effective concentration, it can interact with targets containing more mismatches; at some concentration a Morpholino will begin altering expression of off-target mRNAs. Therefore, it is important that the oligo concentration be kept as low as practicable while still eliciting the desired change in gene expression. The concentration at which off-target effects occur, the concentration at which targeted knockdown occurs, and the ratio of these concentrations are all sequence-specific and unknown for each new oligo sequence. In most cases, an effective and specific concentration window exists such that, for complementary mRNA and off-target mispaired mRNA at similar concentrations, the onset of the targeted knockdown will occur at a lower concentration than the onset of the off-target knockdown. However, knocking down high-copy-number mRNAs requires higher oligo concentrations, increasing the probability of knocking down low-copy-number off-target mRNAs; such a situation can narrow or even close the effective and specific concentration window.

Acceptable off-target homology

A single mismatch in a Morpholino 25-mer may cause a significant decrease in antisense activity (Khokha et al., 2002), though many single-mismatched oligos have retained good activity. When used near the concentration at which a perfectly complementary Morpholino elicits a knockdown, five mismatches distributed throughout a 25-mer usually decreases activity of the mismatched oligo to near undetectable levels (S.T. Knuth, unpub. observ.).

It is prudent to check the target sequence of a proposed oligo against a nucleotide sequence database in order to identify regions where the Morpholino might bind to off-target mRNA. When searching for homologous targets, keep in mind that 25-base Morpholinos will only block translation when targeted to the 5′-UTR and first 25 bases of coding sequence. Morpholinos can modify splicing if targeted in introns near intron-exon boundaries. If the Morpholino has homology to an off-target mRNA outside of these limited regions, binding of the oligo to the mRNA is not likely to affect expression of the off-target mRNA (though blocking miRNA targets or regulatory sequences such as exonic splice enhancers may affect expression).

When comparing a 25-base Morpholino against a nearly complementary off-target RNA sequence in a region where a Morpholino might have a biological effect, the fraction of homologous bases should always be

below 80%. However, that percentage ignores important considerations about the distribution of the mismatches throughout the oligo. About 14 contiguous bases of homology is the minimum inactivating length for a Morpholino (Summerton, 1999). However, if 10 bases of perfect homology are flanked with a mismatch at either side and some runs of homologous bases are just beyond the flanking single mismatches, the oligo may still bind sufficiently to block translation or splicing. High CG content can make shorter homologous sequences active, since CG pairs are more stable than AT pairs. Distributing five mismatches throughout a 25-mer almost always results in loss of knockdown at low concentrations, so 5-mispair oligos are sometimes used as specificity controls. If all five mismatches are at one end of the oligo, there are still 20 contiguous complementary bases in a 25-mer, and those 20 bases would retain considerable antisense activity. When checking a Morpholino target against a sequence database and finding a partially homologous region, following a rule of thumb like "<80% homology won't cause off-target knockdown" can lead to trouble; it is important to consider the distribution of the mismatches.

Additional factors to consider when analyzing partially homologous targets are that losing a CG pair due to a mismatch impacts the oligo activity more than losing an AT pair (three H-bonds compared to two), and that mismatches sometimes form GT pairs, which contribute about half the stability of an AT pair (Aboulela et al., 1985).

Delivery of Morpholinos to the cytosol/nuclear compartment of cells

Unmodified Morpholinos

Since unmodified Morpholinos diffuse between the cytosol and the nucleus, delivery of Morpholinos to the cytosol is sufficient to ensure entry into the nucleus (Morcos, 2001). However, unmodified Morpholinos do not readily diffuse across the plasma membrane of most cell types. If unmodified Morpholinos are added to cell cultures without delivery reagents, high concentrations and long exposure times must be used to achieve minimal delivery (Sazani et al., 2001). Further demonstrating plasma membrane impermeability, when Morpholinos are microinjected into one blastomere of a *Xenopus laevis* embryo at the two-cell stage, daughter cells of the injected cell will contain Morpholino activity while daughter cells of the uninjected cell contain no detectable Morpholino activity (Nutt et al., 2001). There have been some reports of particular cell types in tissue explants that take up experimentally useful concentrations of unmodified Morpholinos. These cell types include epithelial cells in mouse embryo pancreatic explant cultures during E11 through 13 (Prasadan et al., 2002) and liver cells in mouse embryo E10 liver explants (Monga et al., 2003). Using an engineered mouse with a stably integrated green fluorescent protein (GFP) up-regulation splice-correction reporter system (see Up-regulation system, below), Sazani showed that there is scant uptake of unmodified Morpholinos into most tissues from the blood of adult mice (Sazani et al., 2002).

Scrape loading

Scrape loading of Morpholinos into adherent cell cultures was an early method for introducing Morpholinos into cultured cells (Partridge et al., 1996). When adherent cells are gently lifted from the bottom of a well using a soft rubber scraper, the cells become transiently permeable, allowing Morpholinos to diffuse into the cytosol from the medium. This technique will not deliver oligos to all cells in a culture, and reproducibility depends on the technique of the experimenter. This method has fallen out of favor as more reproducible techniques producing more homogeneous delivery have been developed.

Microinjection

Microinjection of Morpholinos into early embryos is a widely used technique for knocking down gene expression. Microinjection introduces Morpholinos directly into the cytosol. As the cytoplasm is apportioned into daughter cells at cell division, both daughter cells will contain Morpholinos. Some embryos, such as *Xenopus* sp., have strong permeability barriers that prevent appreciable leakage to the daughters of uninjected cells (Nutt et al., 2001). Other embryos such as the zebrafish, *Danio rerio*, allow diffusion of Morpholinos between cells through the first few cell divisions (for a good model of Morpholino diffusion in zebrafish embryos, see Kimmel and Law, 1985a,b).

Electroporation

Electroporation has become a standard method for delivery of Morpholinos into chick embryos (Kos et al., 2003), especially for studies of neural tube development (Tucker, 2004). Electroporation has also been used to

deliver Morpholinos into other embryos including mice (Mellitzer et al., 2002), into brains of developing rats (Takahashi et al., 2002), into zebrafish (Cerda et al., 2006), into clipped fins of zebrafish (Thummel et al., 2006), and into cell cultures (Jubin, 2005). Uncharged Morpholinos can be electroporated; the electroporation procedure makes cells transiently permeable so that Morpholinos can diffuse across the plasma membrane. Most investigators prefer to use Morpholinos conjugated to an ionic fluorescent tag for electroporation.

Endo-Porter

Endo-Porter is a reagent developed to deliver Morpholino oligos conveniently and reproducibly to the cytosol of cultured cells through an endocytotic pathway. Endo-Porter is an amphiphilic peptide that becomes cationic at low pH. In culture medium, Endo-Porter is uncharged but sticks to the surface of cells. Upon endocytosis, Endo-Porter is protonated in the acidic endosome and permeabilizes the endosomal membrane, releasing the endosomal contents into the cytoplasm. Morpholinos co-endocytosed with membrane-associated Endo-Porter are released into the cytoplasm when the endosome is permeabilized (Summerton, 2005). Endo-Porter allows simultaneous delivery of multiple Morpholinos. The concentration of Morpholinos can be varied independently of the Endo-Porter concentration, allowing dose-response antisense studies while holding the delivery reagent concentration constant. Cells treated with a 5 μM carboxyfluoresceinated Morpholino and 8 μM Endo-Porter gave transfection efficiencies of 82% for human amnion-derived WISH cells and 78% for human myometrial cells when assayed by confocal microscopy (Tyson-Capper and Europe-Finner, 2006), though concentrations too low to be detected by fluorescence might still be sufficient to have measurable antisense activity. Endo-Porter has been used successfully with traditionally hard-to-transfect cells such as cardiomyocytes (Masaki et al., 2005). It works well with unmodified Morpholinos or carboxyfluoresceinated Morpholinos, but best delivery is achieved with lissaminated Morpholinos (S.T. Knuth, unpub. observ.). Endo-Porter is commercially available in neat DMSO or in a less-effective aqueous formulation for cells sensitive to DMSO.

The recommended concentration of Endo-Porter is 6 μM, achieved by using 6 μL of a 1 mM Endo-Porter solution per milliliter of cell culture; this concentration gives good delivery without toxicity to many cell types. However, cell types vary in their tolerance to Endo-Porter, with some cells tolerating higher exposures while other cells are harmed by a 6 μM Endo-Porter solution. When trying Endo-Porter with a new cell type, it is prudent initially to test a range of Endo-Porter concentrations (e.g., 2, 4, 6, and 8 μM) to assess delivery and to check the tolerance of the cells for the reagent.

Special Delivery

Morpholinos are sometimes delivered using cationic delivery reagents, such as ethoxylated polyethylenimine (EPEI) or Lipofectamine. However, since Morpholinos are not charged, they will not form electrostatic complexes with cationic delivery reagents. Without such complexation, the Morpholinos are poorly delivered to the cytosol of treated cell cultures. To overcome this limitation, Morpholinos can be annealed to complementary or partially complementary strands of anionic nucleic acids. Special Delivery oligos are heteroduplexes of Morpholinos and partially complementary DNA, and are delivered after complexation with EPEI (Morcos, 2001). Special Delivery oligos were designed as a replacement for scrape loading of adherent cells, but can also be used with cells in suspension. Special Delivery oligos provide a more homogeneous delivery than scrape loading, and many studies have been published using them. However, several problems are inherent in the system: (1) EPEI is somewhat toxic to cells; (2) the concentration ratio of heteroduplex to EPEI is fixed; (3) only a single oligo sequence can be delivered at an effective concentration at any one time; and (4) the complexation procedure, which must be done prior to each delivery, adds complexity and variability to the experiment. While Special Delivery oligos can be made by following a fairly simple protocol, they are no longer available commercially as paired heteroduplexes. This approach has mostly been supplanted by Endo-Porter, which is simpler to use, more versatile, more effective, and less toxic in most cell types.

Peptide conjugates

Cell-penetrating peptides covalently conjugated to Morpholinos enhance cytosolic delivery of Morpholinos in cell culture (Neuman et al., 2005) and in vivo (Kinney et al., 2005; Neuman et al., 2005; Tripathi

et al., 2015). Most published research describing Morpholino-peptide conjugates has used arginine-rich peptides (Moulton et al., 2004; Neuman et al., 2004; Deas et al., 2005; Kinney et al., 2005; Nelson et al., 2005; McClorey et al., 2006). Conjugation with arginine-rich peptides alters the specificity, target affinity, and toxicity of Morpholinos (Nelson et al., 2005).

Due to the high density of cationic charges on the peptide moiety, Morpholinos conjugated with arginine-rich peptides associate with subcellular structures and with outer cell surfaces. This property might lead to false-positive artifacts when assessing delivery of arginine-conjugated peptides by fluorescence-based methods, such as fluorescence microscopy, fluorometry, or flow cytometry. To determine the concentration of an internalized conjugate using fluorescence-based methods, the membrane-associated conjugate should be removed in order to avoid overestimation. Trypsin treatment has been effective for eliminating binding of Morpholino-peptide conjugates to the outside of cells (Moulton and Moulton, 2003).

An example of a peptide-Morpholino conjugate is the pip6a-Morpholino targeting SMN2, used for treatment of a transgenic mouse model of spinal muscular atrophy carrying a human SMN2 gene. This uses a Morpholino targeting exon 7 of the human SMN2 gene, conjugated with an arginine-rich pip6a cell-penetrating peptide. Spinal muscular atrophy is a severe neuromuscular disease of humans caused by loss of functional SMN1 protein. Fortunately, the SMN1 gene is duplicated, forming the SMN2 gene. SMN2 has a mutant splice site preventing its expression, but, if splicing is redirected to the wild-spliced site by blocking a splice silencer regulatory motif in intron 7, the SMN2 protein is expressed and can compensate for the lack of SMN1. When 10.0 μg/g pip6a-Morpholino was administered once to SMA model mouse pups (postnatal day 0) by facial vein injection, median survival time increased to 167 days compared to 12 days for untreated and saline-treated mice. Muscle strength and the ability to respond to a head-down position on a slope by turning around (negative geotaxis assay) were both significantly improved by pip6a-Morpholino treatment compared to untreated or standard Morpholino-treated pups by day 12. With doses of pip6a-Morpholino given on postnatal day 0 and 2, survival improved to a median of 283 days for 5.0 μg/g doses and 457 days for 10.0 μg/g doses, with no detected toxicity. Two doses of a scrambled-sequence pip6a-Morpholino did not improve survival, while two doses of SMN2 Morpholino without a cell-penetrating peptide improved survival to a median 54 days for 10 μg/g doses. The mice given two doses of pip6a-Morpholino gained weight faster than untreated SMA mice, but not as fast as wild type. Hindlimb strength and performance in the negative geotaxis assay of the two-dose mice greatly improved over untreated SMA mice. Dose-dependent increases in the transgenic human SMA protein and the full-length transgene transcript were measured, and improvement in neural morphology was observed after treatment with SMN2 pip6a-Morpholino. Dosing adult mice carrying a human SMN2 transgene twice, 2 days apart, by tail-vein injection of 18 mg/kg pip6a-Morpholino resulted in significant up-regulation of the full-length human transgene, with up to three-fold more of the transgene product accumulating 7 days post-administration in some muscles of the pip6a-Morpholino treated mice relative to saline-treated controls (Hammond et al., 2016).

Conjugation of two Morpholino oligos targeting different transcripts to a single pip6a cell-penetrating peptide has successfully delivered both antisense activities into cell culture and the DMD model mdx mice after intramuscular injection. These bispecific conjugates are proposed as a therapeutic strategy to decrease toxicity due to the cell-penetrating peptide component of the conjugate by delivering more oligo per peptide (Shabanpoor et al., 2015).

Vivo-Morpholinos

Conjugation of the 3′ Morpholino nitrogen of a Morpholino oligo with an eight-tipped dendrimer with a guanidinium moiety at each tip produces a Vivo-Morpholino. These oligos were designed for enhanced in vivo delivery, and are based on the arginine-rich cell-penetrating peptides, which carry a guanidinium moiety on each arginine side chain (Li and Morcos, 2008). The dendrimeric delivery moiety is toxic, so the dose of a Vivo-Morpholino is limited in vivo by the amount tolerated by the animal. For i.v. administration, the LD_{50} in mice is about about 30 mg/kg (S. Jiang, pers. comm.), and in vivo doses for antisense work will rarely exceed 12.5 mg/kg. Toxicity in mice involves change in blood viscosity (Ferguson et al., 2014). Vivo-Morpholinos are effective in many tissues, including smooth and skeletal muscle, and are especially effective at entering liver and

kidney (Morcos et al., 2008); the moderate uptake as seen in cardiomyocytes can still be useful (Wu et al., 2009). Vivo-Morpholinos do not cross the blood-brain barrier effectively, but are effective in the brain if administered in the cerebrospinal fluid, for instance by intracerebroventricular infusion or injection [Reissner et al., 2012; the doses reported in this paper in nmol should have been reported in pmol, i.e., the are 1000× too high (K. Reissner, pers. comm.). Local injections can keep overall dose lower and produce antisense effects in particular tissues (Kumar et al., 2015). Vivo-Morpholinos are also effective in cell culture systems (Pérez et al., 2009).

Minimum effective Morpholino concentration

To avoid off-target knockdowns, the lowest concentration of Morpholino producing the desired knockdown should be determined. When delivering Morpholinos to cell cultures using Endo-Porter, starting with a 10 μM Morpholino concentration for both fluorescent delivery assays and functional experiments increases the chances that the fluorescence will be visible in the cytosol and that the first functional experiment will produce measurable results. Because a Morpholino concentration of 10 μM might cause nonspecific effects due to interaction with nontarget genes, functional assays should be performed using a range of Morpholino concentrations. Determining the lowest Morpholino concentration that produces measurable results allows one, subsequently, to minimize off-target knockdowns and to conserve oligo. Effective Morpholino concentrations in culture medium for knockdown experiments are typically in the 1 to 10 μM range.

Simultaneous oligo strategy

Oligos can sometimes be delivered together to enhance their effects. Pairs of nonoverlapping translation-blocking Morpholinos targeting the same mRNA are often dose-synergistic and can be used simultaneously in order to decrease the concentration required for a knockdown (Ekker and Larson, 2001). If the paired oligos are simultaneously introduced into the same cells, they are sometimes effective at much lower concentrations than for either oligo alone. If oligos are individually toxic in zebrafish, their use in combination at concentrations below their toxicity thresholds might elicit the desired phenotype without toxicity. Efficiency of splice-modification can be increased by blocking both donor and acceptor splice sites flanking a single exon (P.A. Morcos, unpub. observ.). Targeting several exons simultaneously is an effective way to deplete a wild-spliced mRNA (Draper et al., 2001). When designing oligos intended for co-delivery, check for complementarity between oligos that may cause them to form Morpholino heterodimers and lose activity (see Troubleshooting: Oligo activity decreases with pairs of oligos).

Assessing oligo delivery

It is best to begin a set of Morpholino experiments in a cell line by confirming and optimizing delivery. Most experimental problems involving Morpholinos in cell culture are due to insufficient delivery of oligo and can be solved by optimizing delivery to the particular type of cells used. Checking whether good cytosolic delivery can be achieved using a fluorescent standard control oligo or fluorescent surrogate before starting to use custom-made Morpholinos is usually the most efficient use of time and resources.

By fluorescence

Fluorescence can be measured by fluorescence microscopy, flow cytometry, or fluorometry. Only fluorescence microscopy can distinguish cytosolic and nuclear fluorescence (indicating successful delivery of a fluorescent Morpholino) from endosomal or surface-bound fluorescence (which does not contribute to antisense activity). A fluorescence microscope and a fluorescently labeled marker such as a Morpholino or 10-kDa dextran are required for a reliable delivery assay. Using a 10-kDa fluoresceinated dextran or a carboxyfluoresceinated standard Morpholino control before using a more expensive, custom-made Morpholino produces reliable uptake assays at reduced cost. After delivery, live cells may be conveniently observed using an inverted epifluorescence microscope. Fixing cells can lead to false positives for delivery due to permeabilization of the plasma membrane and release of the oligo from endosomes during fixation. Using an objective with a higher numerical aperture increases the amount of light gathered from a cell and helps reveal dim fluorescence. If diffuse fluorescence is seen throughout the cytosol of the cells, the Morpholino has been delivered successfully. Bright punctate spots are likely labeled oligos trapped in endosomes. Punctate fluorescence does not indicate delivery, but it does not preclude it either; the diffuse fluorescence is the important signal for determining whether delivery has succeeded.

For delivery with Endo-Porter, start by assaying a range of Endo-Porter concentrations for delivery efficacy and cell tolerance (see Basic Protocol 3) or by trying a concentration of 6 μM Endo-Porter in the selected cell culture. After Endo-Porter delivery, antisense activity can be detected using as little as 1 μM Morpholino. However, although antisense activity can be achieved at Morpholino concentrations that do not produce detectable fluorescence, proof-of-delivery experiments using microscopy do require detectable fluorescence. To accumulate enough fluorescence for microscopy, a concentration of about 10 μM Morpholino is needed. The Endo-Porter and labeled Morpholino should be left on the cells overnight to allow time for endocytotic uptake and accumulation.

By measuring antisense activity

If delivery is successful and a Morpholino targeting translation or splicing works as designed, a decrease in protein concentration or a shift in RT-PCR product mass (respectively) can be measured. Successful delivery might also be indicated by phenotypic effects, such as a decrease in targeted enzyme activity (Hayashi et al., 2005) or a change in morphology (Ekker, 2000). However, assaying only for a phenotypic effect becomes problematic if the expected change in phenotype does not occur; if antisense activity is not separately assessed at the level of protein concentration or mRNA mass, the experimenter will not be able to discern whether (1) the oligo failed to reach and interact with its target mRNA to produce the knockdown or splice-block, or (2) the knockdown or splice-block was successful but did not cause the expected phenotypic change.

Assaying translation-blocking activity

Activity of translation-blocking Morpholinos can be assayed using immunoblots. However, while Morpholinos can halt new translation, they do not cause degradation of existing protein; it therefore takes some time after Morpholino treatment before immunoblots will show evidence of a knockdown. The time required will vary with the half-life of the protein as well as the efficiency of the knockdown.

If no antibody is available for the protein product when targeting an mRNA for translation blocking, then indirect assays such as the change in phenotype of an embryo must sometimes be used to assess the effectiveness of translation blocking. Morpholinos can phenocopy (mimic the phenotype of) many known mutations that affect morphology during development and sometimes reveal new phenotypes concealed by expression compensation in mutants; embryos with phenotypes modified by Morpholino treatment are known as morphants (Ekker, 2000).

In some cases, the enzymatic activity of a target protein can be assayed (Hayashi et al., 2005). An enzyme activity assay may serve as an assay for Morpholino activity, although there must be a delay between application of the Morpholino and the enzyme activity assay to allow for degradation of pre-existing protein (see also the discussion of complementation in Troubleshooting).

The effect of a Morpholino on target RNA stability varies with the sequence. Target mRNA concentrations in Morpholino-treated cells may be decreased, unchanged, or increased relative to untreated cells. Changes in mRNA concentrations might be due to changes in the secondary structure of the mRNA on binding a Morpholino, thereby altering the availability of the mRNA for nucleolytic degradation. Consequently, mRNA assays such as northern blots or quantitative PCR are not suitable for assaying the activity of a translation-blocking Morpholino.

Assaying splice blocking activity

Because blocking splicing changes the mass of the mRNA produced, RT-PCR with appropriate choice of primers followed by gel electrophoresis to determine fragment size is a good molecular method for detecting the activity of splice-blocking Morpholinos. However, it is important to keep in mind that it cannot be predicted with certainty whether a splice-blocking Morpholino will cause an exon deletion (most common), an intron insertion, activation of a cryptic splice site (which can cause a partial insertion or deletion), or a multiple exon skip. Because cryptic sites might redirect splicing of only a fraction of the targeted pre-mRNA population, splice blocking can produce a mixture of RT-PCR product masses (Draper et al., 2001). To detect most of these changes, it is best to use primers targeted to the two exons flanking and closest to, but not including, the exon abutting the Morpholino's splice junction (though revealing a multi-exon skip requires more widely-spaced primers). Targeting a splice junction on an internal exon (i.e., not the first or last) is most likely to cause exon deletion. Primers should be chosen so that, if an exon deletion occurs, the RT-PCR product will be large enough to detect easily on

a gel (one hundred to several hundred bases). That means for the system exon1–intron1–(splice blocker target)–exon2–intron2–exon3, the RT-PCR primers should be targeted to exon 1 and exon 3 in order to detect either intron 1 insertions (unusual) or exon 2 deletions (common). If a large intron is inserted into the mature mRNA, the PCR polymerase might not reliably progress all the way between the primer sites in the exons; in this case exchanging one of the exonic primers for an intronic primer can reveal the presence of the inserted exon.

If the first (most 5′) or last (most 3′) splice junction in an mRNA is targeted, the usual result is an intron insertion instead of an exon deletion. However, targeting the first splice junction might activate a cryptic splice site, resulting in deletion of the 3′ end of the first exon or inclusion of a 5′ fragment of the first intron. When targeting the last exon, an intron insertion is a more likely outcome. This is because consensus sequences of splice acceptors are more complex than those of splice donors, so it is less likely that the last intron or exon will contain a near-consensus cryptic splice acceptor.

When assaying the activity of a splice-blocking Morpholino at the molecular level using RT-PCR, it is important to compare the expected size of the RT-PCR product after the splice modification with the size of the RT-PCR product produced by an untreated cell or organism. For easiest detection, splice-blocked RT-PCR products would be about half or twice the size of the native-spliced product (for exon deletion or intron insertion, respectively). A real system usually won't allow such a tidy result, but it is necessary for the change in mass to be clearly visible on the gel (e.g., a 5% change in mass can be difficult to detect). Sequencing the splice-modified RNA can reveal small changes that are hard to resolve on a gel.

Fidelity of replication can be a problem in RT-PCR, so it is best to design shorter RT-PCR products if all else is equal. This means, for the knockdown of exon 2, targeting primers in exon 1 near the junction with intron 1 and in exon 3 near the junction with intron 2 would be the best choice. However, all else is not equal; since fragments should be large enough that they are clearly visible, it is prudent to move the primers further into the flanking exons. When possible, primers should amplify RT-PCR products with lengths of hundreds of bases to ensure full-length replication and visible bands.

Splice modifications can cause downstream frameshifts or inclusion of intronic sequence in the mature messenger. Either of these results can cause a range of complicating effects, including truncation of the protein product by appearance of in-frame stop codons, translation suppression by appearance of a miRNA target site, degradation or suppression of the messenger through siRNA or miRNA activity, and nonsense-mediated mRNA decay.

Splice modification may or may not cause a change detectable by an immunochemical assay such as an ELISA or immunoblot, since the conformation of the modified protein around the antigenic site may or may not be changed by a splice modification. A large insertion or deletion might result in loss of antibody binding or at least a significant shift in the band position on a western blot, but a small insertion or deletion could be difficult to detect. Targeting to cause a frameshift will usually facilitate detection of oligo activity by immunochemical methods, especially if nonsense-mediated decay decreases protein expression from the splice-modified RNA.

It is possible that inserting an intron or deleting an exon will cause the protein product to lose function, but this is far from certain. If the active site of the protein is known and the exon encoding the active site is targeted, a loss of function is likely. However, if the active site is not known, then splice-blocking might not change the protein's activity. A protein might be made that retains the conformation of its active site even though it has an inserted or deleted polypeptide moiety at a different part of the protein. This means that looking for a phenotypic change in an embryo or assaying enzyme activity is often inadequate for assessing the splice-blocking activity of a Morpholino. This also means that while RT-PCR is a useful tool to confirm splice blocking activity, one should independently assay for protein function before concluding that a targeted gene is not required for a biological process, because successful splice-blocking may not alter the activity of the protein in the process.

Up-regulation system

Assaying antisense activity by knocking down a protein can lead to false positives, because toxicity can cause a decrease in gene expression and this can be misinterpreted as targeted gene knockdown unless careful controls are used. To address this problem and to provide an increased signal-to-noise ratio for antisense activity assays, Ryzard Kole's group developed a set of signal up-regulation

reporter systems based on splice modification. These systems use a mutation in human β-globin that creates a new splice site and causes thalassemia. The splice-mutant has a stop codon in-frame in the mRNA as well as a frameshift in the downstream coding region; when the mutant site is blocked, the stop codon is spliced out and the correct reading frame is restored. Constructs coupling this mutation to luciferase or GFP have been engineered. Of particular interest are the pLuc705 HeLa cell line (Schmajuk et al., 1999), which expresses luciferase when the mutant splice site is splice-blocked with control oligo, and the Sazani mouse (Sazani et al., 2002), which expresses GFP when splice-blocked with the appropriate oligo.

Controls

When an oligo is used to target an mRNA, a parallel experiment should be done using a negative control oligo. Negative control oligos include the standard control oligo and an invert oligo. A 5-mispair specificity control oligo is also sometimes used as a negative control. The negative control shows that the effects observed during the antisense experiment are due to the sequence of the targeting oligo and not to the backbone chemistry of the Morpholino or the cytosolic delivery method used.

Standard control oligo

A standard control Morpholino with the sequence CCTCCTACCTCAGTTACAATTTATA has been used in many organisms as a negative control sequence without triggering off-target or non-antisense effects. This negative control produced no toxic or teratogenic effects even when administered at considerably higher concentrations than typically used for specific knockdown experiments. Any custom-sequence control oligo has some risk of interacting with off-target RNA; in contrast, the standard control has an established history of inactivity and is a reliable choice for a negative control oligo. The standard control Morpholino is designed to splice-block the mutant splice site used in the pLuc705 up-regulation reporter system.

Invert control

If a negative control oligo needs to be related to the custom-made targeting oligo in terms of base composition, the invert oligo is a good choice of sequence. The invert has the same base sequence as the targeting oligo, but the sequence is reversed in the 5′-to-3′ orientation (i.e., 5′-ACGGTGC would become 5′-CGTGGCA). The advantage of an invert over a scrambled sequence is that the invert sequence can be conveniently generated by a simple algorithm and will have the same CG content, G content, and self-complementarities as the targeting oligo. However, there is always a risk with any custom-made oligo that the oligo may interact with unintended RNAs.

Sense control

Sense Morpholinos have sometimes caused an increase in concentration of the mRNA targeted by an antisense oligo (P.A. Morcos, unpub. observ.). Thus, a sense sequence is not a good choice for a negative-control Morpholino.

5-Mispair oligo

Off-target knockdown by any antisense molecule increases with increasing concentration. A 5-mispair oligo is used to define the effective and specific concentration window for a targeting oligo. Using the targeting oligo within its effective and specific concentration range decreases the chance of causing experimental artifacts by interaction with off-target RNA. A 5-mispair control oligo has nearly the same sequence as a targeting oligo, but has five mismatched bases distributed through the sequence. The mismatches should be distributed fairly evenly through a 25-mer oligo, starting a few bases in from each end. Where possible, the mismatches should be formed by exchanging C for G and G for C, since these mismatches disrupt the formation of three hydrogen bonds per base pair. Ideally, when a targeting oligo is used near the lowest concentration that produces a discernable effect, the 5-mispair oligo used at the same concentration will not produce the effect. However, as with any custom-sequence control oligo, there is a possibility that the 5-mispair oligo will interact with an untargeted RNA, triggering an off-target effect. If an oligo targets a high-copy-number transcript, requiring relatively high Morpholino concentration for knockdown, and the mispair oligo interacts with a low-copy-number transcript, the mispair oligo might cause effects even at concentrations below the concentration at which the targeting oligo becomes effective.

The 5-mispair oligo can be used in an experiment that determines the effective and specific window of concentrations for a targeting oligo, which is the concentration range between the onset of measurable activity for the targeting

oligo and the onset of measurable activity for its 5-mispair oligo.

The definition of the effective and specific concentration range based on a 5-mispair Morpholino evolved through trial and error. Originally a 4-mispair specificity control was recommended, but a 4-mispair oligo sometimes measurably decreased the target protein concentration at concentrations low enough that the corresponding targeting oligo was just becoming effective, so there was not a wide enough effective and specific concentration range to be consistently useful.

Many investigators use the 5-mispair oligo as a negative control, but that was not its intended purpose. Assuming that adding the mismatches does not create too much complementarity to an important off-target mRNA and trigger an off-target knockdown, the 5-mispair oligo usually behaves as a negative control when used at concentrations low enough that the targeting oligo is just becoming effective. However, the 5-mispair oligo is intended as a specificity control that shows the targeting oligo is being used in its effective and specific range. To demonstrate specificity of the targeting oligo, the targeting oligo, a 5-mispair control oligo, and a true negative control oligo (such as the standard control) are used at the same concentration in parallel treatments. If the 5-mispair and negative control oligos produce the same results, and the targeting oligo produces a different result, the experiment indicates that the targeting oligo has been used within its effective and specific concentration range. Appearance of an effect due to interaction of a target RNA with the 5-mispair control oligo suggests that, at that same concentration, the targeting oligo might also interact with off-target RNAs. Because not all five-mispair oligos successfully define an effective-and-specific concentration range, the experiment with the two non-overlapping oligos is becoming the preferred specificity control.

Two nonoverlapping translation blockers

Another strategy for showing that the effect of a translation-blocking Morpholino is due to the knockdown of its targeted mRNA is to use a second oligo targeted to a different and nonoverlapping site in the 5′-UTR of the targeted mRNA. If the second oligo has the same effect on the cells (or organism) as the first, this supports the hypothesis that the effect observed is due to the knockdown of the targeted gene. Because different oligos will usually have different T_m values, a matching phenotype might require different doses of the two oligos. If one of the oligos elicits effects not copied by the other, this is likely due to off-target RNA interaction and an additional non-overlapping oligo might be needed to support specificity.

Two splice blockers targeting one internal exon

If a Morpholino targeting a splice donor site produces the same result as a Morpholino targeting the splice acceptor of the same exon, this supports the hypothesis that the effect observed is due to the excision of the targeted exon. However, failure of the two oligos to produce the same result may be due to activation of a cryptic splice site(s) or other unexpected splice outcome by one or both of the oligos; interference with translation or regulatory sites on an unexpected RNA is also a possible source of phenotypic change.

mRNA rescue

A very strong proof of specificity involves the use of a rescue mRNA. A rescue mRNA codes for the same protein targeted by the Morpholino knockdown, but has a modified 5′-UTR that is not targeted by the Morpholino. For this experiment, the rescue mRNA and Morpholino are delivered to the cytosol together. If the co-delivered rescue mRNA and Morpholino produce the same wild-type phenotype as untreated cells or organisms, this supports the hypothesis that the morphant phenotype elicited by the Morpholino alone is due to interaction with the targeted RNA. Unfortunately, the mRNA rescue experiment cannot work for some genes when used in embryos. The timing of the onset of translation of some genes is crucial for development, and the early onset of translation resulting from co-injection of Morpholino and rescue mRNA in the early zygote may alter the developmental process so that these embryos never recapitulate the wild-type phenotype. Furthermore, the location of gene expression is often crucial for development, but oocyte microinjection causes rescue mRNAs to be present in all cells of the embryo until degraded or diluted by growth. An expression plasmid carrying the cDNA under control of the endogenous promoter could avoid extopic expression of the rescue mRNA.

End modifications

Several optional modifications attached to the ends of Morpholinos are commercially

available. Carboxyfluorescein, lissamine, and primary amines are the most commonly used. Optional groups are usually added to the secondary amine on the 3′-end of the oligo, and are assumed to be 3′ modifications unless explicitly declared to be 5′ modifications. Fluorophores and biotin are attached to Morpholinos through flexible spacers. The length of the spacers was chosen based on antisense activity studies to ensure that the fluorophores would not interfere with binding of the Morpholinos to their target RNA sequences. The primary amine modification includes a short spacer of two methylenes.

Carboxyfluorescein

Carboxyfluorescein is a green-emitting fluorophore that was chosen from among the fluoresceins for its good chemical stability. While its photostability is better than that of many of the fluoresceins, all of the fluoresceins are subject to photobleaching, so carboxyfluorescein should not be exposed to intense light unnecessarily. The excitation wavelength of a carboxyfluoresceinated Morpholino in water is 502 nm and its emission wavelength is 525 nm. Carboxyfluorescein has two negative charges at neutral pH.

Lissamine

Lissamine is a red-emitting sulforhodamine B. The excitation wavelength of a lissaminated Morpholino in water is 575 nm, and the emission wavelength is 593 nm. Lissamine is a zwitterion at neutral pH, with one positive and one negative charge. Adding a lissamine to a Morpholino increases its delivery efficiency with Endo-Porter, but adding lissamine to a Morpholino can decrease its aqueous solubility. It is therefore recommended to use a carboxyfluorescein tag when a fluorochrome is needed, especially for Morpholino sequences with relatively high G contents (>30% G).

Gene Tools Blue

Gene Tools Blue is a blue-emitting fluorescent tag with a pyrene core and three Morpholinosulfamide groups. The excitation wavelength of this fluor on a Morpholino in aqueous solution is 433 nm and the emission is at 465 nm. It can be imaged with a DAPI filter set, but that set is not optimized for Gene Tools Blue, so the concentration threshold for detection will be relatively high. With an optimized filter set, the fluor is still less bright than carboxyfluorescein and lissamine.

Primary amine

Morpholinos may be modified with a primary amine to provide a reactive site for attachment of other moieties to the oligo. An unmodified Morpholino has a secondary amine on the 3′ end, the pK_a of which is 6.5. The primary amine, with its pK_a of 10.2, provides a more reactive site. When a primary amine is attached to the 3′ end of the oligo, this converts the 3′-secondary amine of the morpholine ring to a tertiary amine as a consequence of the attachment of a short spacer tethering the new primary amine. When a primary amine is attached to the 5′ end of the oligo, the 3′-secondary amine is acetylated so that a reagent added to react with the primary amine will not react with the 3′ end of the oligo. When reacting a primary amine with a derivatizing reagent, it is prudent to include an additional short spacer to prevent steric hindrance between the moiety being added and the Morpholino.

Morpholino stock solutions and reconcentrating Morpholinos

Morpholino stock solutions in distilled water should be kept sterile and can be autoclaved. Do not use water containing diethylpyrocarbonate (DEPC). Morpholino stock solutions can be dissolved in buffers such as Ringer's solution or Danieau buffer, but this can cause problems later if the stock solution must be reconcentrated, since lyophilization can be more difficult from a buffer. Also, Morpholinos are substantially more soluble in distilled water than in isotonic salt solutions, and it is easier to interpret the MALDI-TOF spectrum of a Morpholino if no salt is present.

A solution of Morpholino in water can be concentrated by lyophilization (freeze-drying). Lyophilizing Morpholinos from water produces a fluffy solid with tremendous surface area that dissolves fairly readily if the sequence has good solubility properties. Dissolution of Morpholinos concentrated with a Speedvac may be more difficult or impossible, at least requiring patience and autoclaving; using a Speedvac with Morpholinos is not recommended.

Temperature during handling

Morpholinos are not degraded by nucleolytic enzymes. Solutions of DNA and RNA are normally kept on ice during experiments to prevent nucleolytic degradation, but this is not a concern with Morpholinos. However, some Morpholino solutions have low enough solubility that icing a solution may cause a loss of activity, due to the oligo aggregating or coming

out of solution. Therefore, icing Morpholino solutions is not only unnecessary but can cause problems; Morpholinos should be kept at room temperature during experiments.

Material affinity

Morpholinos have some affinity for plastics, so passaging very dilute (submicromolar) solutions through plastic containers may cause appreciable decreases in activity. Similarly, filter sterilization may cause Morpholino solutions to lose some activity, as some oligo binds to the filter. When put through the same procedures with the same exposure to plastic surfaces, high-concentration Morpholino solutions have a smaller fractional decrease in concentration than low-concentration Morpholino solutions. Therefore, if exposure to plastic surfaces is required, it is best to do the procedures with Morpholinos in a relatively concentrated state (>1 μM). Similarly, if Morpholinos are to be stored in solution for more than a few days, it is best to store them at relatively high concentration (between 10 μM and 1 mM). Since solutions of Morpholinos at very low concentrations (<1 μM) may lose activity over a time scale of minutes to hours, dilutions should be made just before use. If Morpholino solutions of less than about 100 μM are filtered, the concentration may be affected appreciably given the large surface area of filters. As the oligo bound is proportional to the surface it is exposed to, a small-diameter filter should be used to minimize oligo losses. Pall Acrodisc HT Tuffryn 0.2-μm membrane filters were found to bind less Morpholino per area than other filters tested (J.E. Summerton, unpub. observ.). The concentration of oligo in a solution can be measured spectrophotometrically just before and after performing a filtration to determine the loss of oligo caused by the procedure.

Troubleshooting

Loss of antisense activity over time

Morpholinos are best stored at room temperature. Some Morpholino solutions lose activity when stored frozen, not due to degradation of the oligos but simply to aggregation or association with container surfaces. The activity can usually be recovered by autoclaving prior to use.

Loss of fluorescence over time

Fluorescent tags can be photobleached by exposure to bright light or prolonged exposure to dim light. Always store fluorescent materials in the dark. Wrapping aluminum foil around tubes containing fluorescent materials is an easy and prudent method for protecting fluorophores. Fluorescent materials can autoquench at high concentrations and decrease their light emission, so measurements of fluorescence at very high concentration might lead to deceiving concentration calculations. Labeled Morpholinos, as 10 μM solutions, are well below the concentration at which their fluorophores autoquench.

No apparent activity

If a Morpholino does not produce the anticipated result, there are several possibilities to consider. Is the oligo in solution as a single-stranded molecule? Check concentration by UV spectrometry and autoclave the stock to disrupt aggregates. Has delivery been confirmed? If the oligo is not reaching the cytosol of the cells, no antisense activity will be observed. Is the activity checked by a molecular assay? If the selected activity assay determines a phenotype, such as a change in embryo morphology or in enzyme activity, the oligo may be successfully knocking down translation or modifying splicing, but a second protein may be complementing the lost activity of the target protein, thereby confounding the assay. Assaying translation blocking by immunoblot and splice blocking by RT-PCR can help determine whether the oligo is not interacting with its target or has not been delivered, or suggest there is a more subtle reason for the failure to produce the expected phenotype, such as complementation by another protein. Feedback up-regulation can also cause a knockdown to fail; greatly increased transcription of the targeted mRNA in response to an attempted knockdown can overwhelm the ability of the oligo to block all of the targeted messengers.

Oligo activity decreases with pairs of oligos

When two or more oligos are together in a cell, they may hybridize with each other if they share complementary sequences. If a pair of oligos has less activity than each individual oligo, check the sequences for complementarities. Sixteen contiguous hydrogen bonds of complementarity is the maximum recommended for oligos used together in cells or organisms at 37°C.

Clogging microinjectors

If a Morpholino solution causes a microinjector to clog, one can: (1) heat the solution to disrupt tiny clumps (65°C for 10 min or

autoclaving), (2) filter-sterilize the solution (although some oligo may be lost on the filter), or (3) try injecting a higher volume of a less concentrated solution.

Anticipated Results

Translation blockers

If a translation-blocking Morpholino knocks down expression of a protein, this activity can be revealed by a delayed decrease in the protein signal on an immunoblot using an antibody to the protein. The successful knockdown should also decrease the activity of the targeted protein, though an assay for the activity of that protein can be confounded by complementation by another protein.

Splice blockers

If a splice-blocking Morpholino changes the mass of an mRNA, this activity can be revealed soon after delivery by a change in the mass of an RT-PCR product produced using appropriately chosen primers and assessed on an electrophoretic gel. A successful exon excision should also result in a delayed decrease of the activity encoded by the deleted exon as pre-existing protein degrades. Splice blocking may also decrease activities encoded on untargeted exons of a target RNA due to frameshifts, inserted stop codons, or changes in tertiary structure.

Mutant and Morpholino phenotype comparisons

In an assessment of differences in phenotypes when comparing mutant zebrafish embryos with Morpholino knockdown of the same genes in wild-type zebrafish embryos, a lab study combined with a meta-analysis of published reports found less than 30% of mutants and Morphlino knockdowns shared phenotype changes from wild-type. The phenotype comparison led to a paper asserting that differences in the phenotypes may be due to off-target effects of the Morpholinos and that Morpholinos can only be considered to give valid data if they phenocopy mutants; the authors concluded "we would suggest broader communitywide and editorial guidelines that require an observed MO-induced phenotype to be validated in embryos bearing mutations in the same gene, after which a MO could then be reliably applied for subsequent functional studies." (Kok et al., 2015). Rigorous proof of specificity of the Morpholinos was only provided in about half of the papers used for the meta-analysis, and many of the mutants in those papers are hypomorphs (Blum et al., 2015). In one case, a megamind-targeted Morpholino was found to give a phenotype even in a TALEN-engineered fish line with the Morpholino binding sequence removed from the DNA at the megamind locus. However, this Morpholino was used at the excessive dose of 20 ng per embryo (Kok et al., 2015, figure 5; Morcos et al., 2015). Nevertheless, the call to verify morphant phenotypes with mutants has led to demands by reviewers for mutant confirmation of Morpholino studies, slowing publication of data and adding considerable expense to the release of new findings. Is this appropriate?

Soon after the Kok et al. (2015) paper, TALEN-induced egfl7 mutants were compared with egfl7 morphants, and gene expression changes in other genes were found in the mutants and not the morphants, revealing likely compensatory gene expression concealing protein function in the mutants but not the knockdowns (Rossi et al., 2015). The Morpholino gave rise to a severe vascular tube formation phenotype not seen in the mutant, which was shown to carry a severe or null mutation. Fish with egfl7 mutated were injected with the egfl7 Morpholino and surprisingly these fish had a reduced incidence of the vascular phenotype compared to wild-type fish injected with the same Morpholino, indicating that the mutant fish were less sensitive to the Morpholino than the wild-type fish; this was the first hint that a change in the mutant fish might conceal the phenotype the Morpholino induced in wild-type fish. The vascular defect phenotype associated with the Morpholino was recapitulated in experiments using CRISPR interference (CRISPRi) to inhibit egfl7 expression. Comparison of over 6000 protein concentrations by 4-hr 'single shot' liquid chromatography–tandem mass spectrometry (LC–MS/MS) found one protein that was strongly up-regulated ($>5\times$) in the mutants compared to the morphants; this protein, Emilin3a, shares an important functional structure, the EMI domain, with Egfl7, and might be compensating for the loss of egfl7 in the mutants. RNA sequencing and qPCR found emilin3a, emilin3b, and emilin2a up-regulated in the egfl7 mutants but not in embryos with egfl7 knocked down by Morpholinos or CRISPRi. When co-injected with the egfl7 Morpholino, mRNAs for Emilin2 or Emilin3 were able to rescue the circulatory phenotype. Similarly, up-regulation of vegfab was found for vegfaa mutants but not vegfaa morphants. Instead of Morpholino off-target

effects, compensation for loss of a transcript in a mutant may underlie many mutant-morphant phenotype discrepancies; indeed, "for egfl7, one can identify a dose of MO that has no effect in most egfl7 mutant embryos but causes clear vascular defects in WT, indicating that these morphant phenotypes are not due to off-target effects." (Rossi et al., 2015). If compensation in some mutants conceals the function of some genes, but these functions can be revealed by knockdown technologies, it is not reasonable to insist that Morpholino phenotypes must be copied by mutants for publication. To "require an observed MO-induced phenotype to be validated in embryos bearing mutations in the same gene" can exclude valuable insights from the literature. The mistrust of Morpholino data engendered by the Kok paper may even lead to grant-review committees inappropriately blocking consideration of well-crafted studies relying primarily on knockdown technologies to reveal gene function. Morpholinos act on RNA, while induced mutation directly alters DNA, and the loss of gene function occurs over different time scales, so differences in outcome should be expected and the strengths of each approach exploited.

Time Considerations

After delivery, wait for antisense effects to be measurable

When a Morpholino is delivered into the cytosol of a cell, pre-existing protein is not altered by the Morpholino. For a translation-blocking oligo, this means that even if translation of a protein is immediately and completely halted on Morpholino delivery, an assay for protein concentration will not immediately reveal a successful knockdown. At least part of the existing protein must be degraded before the knockdown will be evident on an immunoblot. Similarly, although a splice-blocking oligo may cause a rapid change in the mass of an RT-PCR product, the protein produced prior to splice blocking will persist in the cell until degraded.

Delivery systems using endocytotic uptake, such as Endo-Porter or Special Delivery, increase the lag between the start of delivery and the appearance of a knockdown or splice-block signal. An overnight wait is generally sufficient to allow for endocytotic uptake.

For embryonic studies, the presence of maternal transcripts in a zygote may delay the loss of protein activity when splice-blocking Morpholinos are used. Although a splice blocker can modify splicing of pre-mRNA transcribed in the zygote, maternal transcripts are already spliced before the onset of zygotic transcription and will be expressed in their unmodified form. Translation-blockers can block both maternal and zygotic transcripts, and thus might provide a more rapid knockdown than splice blockers.

Redelivery

When translation of a protein is blocked by a Morpholino, existing protein in the cell persists until broken down. After delivery, as cells grow and divide, the concentration of morpholino oligos in their cytoplasm decreases due to dilution. As a Morpholino binds its RNA target, it protects the binding site from nucleases, and the single-stranded antisense is only slowly released from its RNA footprint. Because of these processes, when Morpholinos are used to knock down genes that are highly transcribed or that code for unusually abundant or stable proteins, redelivery of the oligos may be required before a significant decrease in protein levels can be detected by immunoblotting. Knockdowns of proteins with rapid turn-over, such as transcription factors, are easily measured at 24 or 48 hr. Stable proteins can require longer treatment to see a knockdown. After an initial treatment with a Morpholino at the start of day 1, redelivery on day 4 usually suffices to produce a clear knockdown of stable proteins by day 6. However, attempts to block translation of actin have so far failed to produce a decrease in actin levels on immunoblots, suggesting that Morpholinos cannot knock down some very abundant proteins (P.A. Morcos, unpub. observ.).

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Key References

Draper et al., 2001. See above.

First description of splice blocking in a zebrafish, including an analysis of a cryptic splice site.

Nelson et al., 2005. See above.

Description of peptide-Morpholino conjugates now in use for in vivo experiments.

Summerton, 1999. See above.

Review article presenting data determining the effective region for targeting translation blocking oligos and presenting a detailed discussion of Morpholino specificity and minimum inhibitory length.

Summerton and Weller, 1997. See above.

Structure and early synthetic scheme for morpholino oligos.

Internet Resources

http://www.gene-tools.com

Commercial source for Morpholinos.

http://pubs.gene-tools.com

Morpholino publication database. As of late 2016, >8000 publications have reported experiments with morpholino oligos in a broad range of systems. Citations and many abstracts are searchable here.

http://www.zfin.org

Zebrafish Information Network. References related to Morpholino use in zebrafish are searchable in an annotated database.

http://zfin.org/cgi-bin/webdriver?MIval=aa-newmrkrselect.apg

Annotated database of zebrafish Morpholino sequences by gene name.

http://www.sarepta.com/

Sarepta Therapeutics, Inc., Morpholino therapeutics company.