Producing siRNA for *in vivo* applications

Introduction

The use of siRNA for functional genomics and target validation *in vitro* has seen tremendous growth in the past five years and is already impacting medical research. There is growing interest in performing *in vivo* studies, both for target validation and for development of novel siRNA therapeutics. Delivery technologies that were largely ignored in the past are now being actively pursued by a number of companies and may enable the systemic application of siRNA. The key component is the siRNA, with or without some form of chemical modification(s), to improve stability *in vivo* and/or minimize potential immunostimulatory effects. Although the siRNAs used in pilot *in vivo* studies do not need to be synthesized under full cGMP conditions, they do need to be manufactured and purified in environments that are clean and do not introduce potentially toxic substances that could lead to adverse events. What are the major issues and how do we as manufacturers of chemically synthesized siRNAs deal with them?

Potential issues

Organic chemical contaminants from the solid-phase synthesis are largely removed during the deprotection and purification steps and, in the case of cGMP, a residual solvent analysis is required. Traces of potentially toxic heavy metals are best minimized by the inclusion of a low concentration of a heavy metal chelating agent such as EDTA in the anion-exchange HPLC buffers. To avoid problems with non-biocompatible salts, this purification should be conducted using a sodium chloride gradient in the presence of sodium phosphate buffer. However, by far the greatest threat is from endotoxins. Even when present in very small amounts, endotoxins can cause severe inflammatory responses or even the death of laboratory animals.

The endotoxin problem and how to deal with it

Endotoxins are natural products found in some bacteria. The classic endotoxin is LPS (lipopolysaccharide) and is a normal component of the outer membrane of the cell wall of Gram negative bacteria. LPS has a molecular weight of about 10 kDa, comprised of a complicated sugar chain and a lipid component known as lipid A. These toxic molecules are liberated when bacteria are destroyed by lysis or other methods; most of the physiological effects are caused by the lipid A component. The interested reader is advised to refer to http://textbookofbacteriology.net/endotoxin.html for details on the structures of these components.

In mammals the LPS binds to serum proteins, the lipid binding protein (LBP), and the resultant complex interacts with the CD14 receptors on monocytes and macrophages and other receptors on endothelial cells. Further binding events lead to an association with Toll-like receptor 4 (TLR4), leading to several distinct cascade processes: (i) the production of cytokines, including IL-1, IL-6, IL-8, TNF and platelet activating factor, which in turn stimulate the production of prostaglandins and leukotrienes,

which lead to inflammation and septic shock; (ii) activation of the complement cascade, again leading to inflammation; (iii) activation of the coagulation cascade leading to inflammation, intravascular coagulation, hemorrhage and shock. It is clear that the presence of endotoxin contaminants will have dire consequences on the outcome of any *in vivo* siRNA experiments.

How do we detect and avoid such contamination? The gold standard is the Limulus (horse shoe crab) amebocyte lysate (LAL) test, which is an *in vitro* assay that can be performed on medical devices, solutions or solids, for the detection and quantification of bacterial endotoxins. Validated test methods are available at many laboratories and include the gel-clot technique as well as the kinetic turbidometric and colorimetric assays[1].

Working in very clean conditions helps to minimize bacterial and endotoxin contamination as well as potential contamination by RNases. Although sterile filtration removes bacteria and the cell-wall-associated endotoxins, processes that result in bacterial lysis liberate LPS into solution. Sterilized water is a perfect breeding ground for bacteria and should be replaced very frequently. A very well maintained water-purification system, comprised of distillation or reverse osmosis and ion-exchange plus filtration through activated carbon and a final hollow-fiber filtration (0.002 micron pore size), will remove bacteria and endotoxins. It should be noted that endotoxins are extremely resilient and remain viable even after steam sterilization and normal desiccation and are able to pass through filters.

To minimize endotoxin levels, IDT's "*in vivo* clean siRNAs" are purified by preparative HPLC using sterile buffers containing a trace of EDTA (removing heavy metals). Glassware is autoclaved and product fractions are collected in sterile Falcon tubes. Desalting and concentration of the purified siRNA is achieved by ultra/dia-filtration and prior to use the device and membranes are flushed with 0.1 M sodium hydroxide solution followed by sterile water. After annealing the two strands the siRNA duplex is lyophilized on a freeze-drier, and the vacuum is released using an in-line sterile air filter to prevent ingress of airborne bacteria. As part of the QC of this siRNA, an endotoxin test is carried out by an external laboratory using a validated assay. The amount of sample required for the kinetic turbidometric method is 20 mg. Our clients have set an upper limit for endotoxins of ≤ 2 EU/mg and the actual values we obtain lie mostly in the range from 0.01 to 0.1, sometimes even lower.

This attention to detail helps ensure the highest possible quality product and results in RNAs that can be trusted for use in critical applications such as *in vivo* target validation or pre-clinical studies.

References

1. Lindsay, G.K. et al., J. Clin. Microbiol., 1989, **27**, 947-951