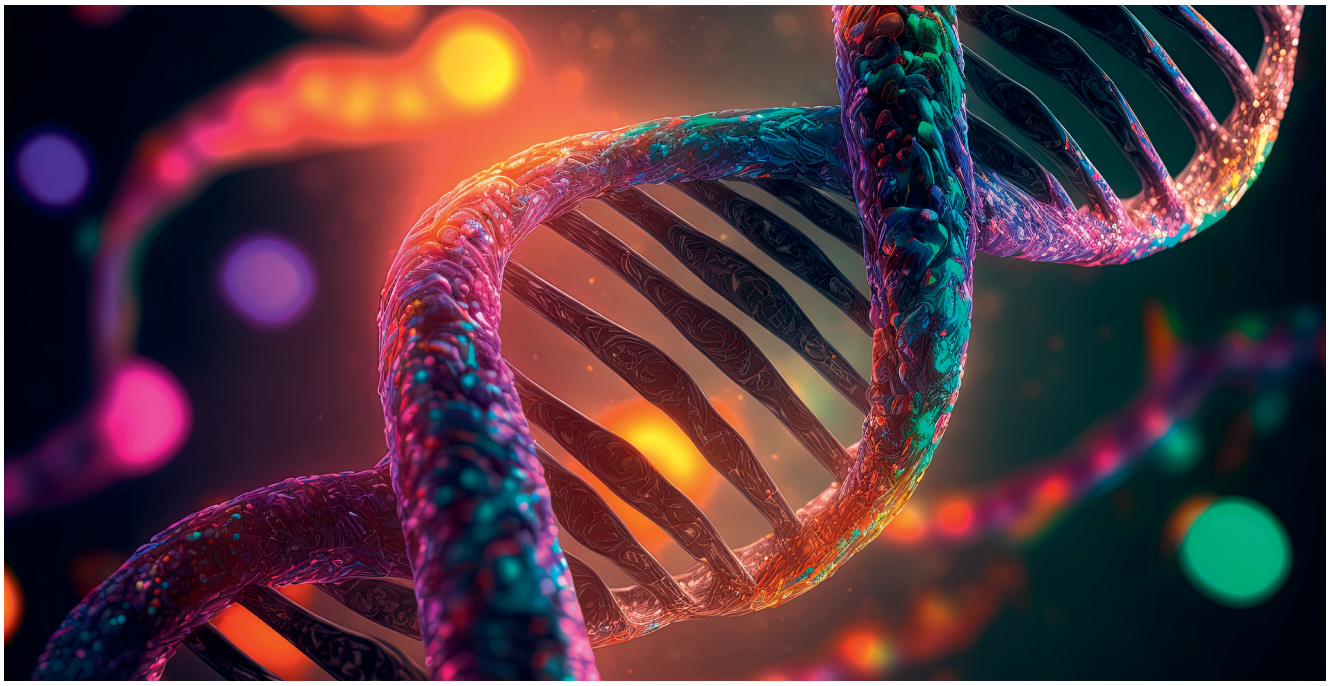
APPLICATION NOTE

Separation of siRNA and its single strands by SEC using YMC-Pack Diol-120

In the recent years the development of siRNA therapeutics for gene silencing has increased significantly. This has also raised the need for appropriate analytical methods such as high-performance liquid chromatography (HPLC). siRNA can be analysed as an intact duplex, consisting of a pair of complementary strands or as two separate single strands namely the sense and antisense strand. siRNA duplexes are frequently analysed by HPLC to estimate the amount of excess single strands, which is often associated with a reduction in therapeutic potency.



In contrast to other separation modes, size exclusion chromatography (SEC) enables the separation of molecules by their molecular weight. This allows the separation of different RNA species such as duplexes and single strands. In addition, no sample loss occurs due to minimal adsorption of the analytes by the stationary phase. This is critical when it comes to the analysis of siRNAs because of their electron rich backbone, which is prone to interact with other stationary phases. In this application note, a siRNA duplex and its single strands are separated using different YMC size exclusion columns in order to identify the best suitable SEC column.

Table 1: Chromatographic conditions.

Columns:	YMC-Pack Diol-60 (5 µm, 6 nm) 300 x 4.6 mm ID YMC-Pack Diol-120 (5 µm, 12 nm) 300 x 4.6 mm ID YMC-Pack Diol-200 (5 µm, 20 nm) 300 x 4.6 mm ID YMC-Pack Diol-300 (5 µm, 30 nm) 300 x 4.6 mm ID
Part Nos.:	DL06S05-3046WT DL12S05-3046WT DL20S05-3046WT DL30S05-3046WT
Eluent:	0.1 M KH_2PO_4-K_2HPO_4 (pH 7.0) containing 0.2 M NaCl
Flow rate:	0.17 mL/min
Detection:	UV at 260 nm
Temperature:	25 °C
Injection:	4.0 µL (each 5 nmol/mL)
Sample:	Sense strand Antisense strand siRNA duplex (Firefly luciferase GL2)

Table 2: Sequence of sense and antisense strands of the siRNA duplex.

Sense strand	5'-CGUACGCGGAAUACUUCGAdTdT-3
Antisense strand	3'-dTdTGCAUGCGCCUUAUGAAGCU-5

Due to their similar length, separation of an siRNA duplex and its single strands is challenging. Only the difference in the hydrodynamic radii of the molecules enables their separation by SEC. Therefore, the choice of an optimal pore size is crucial. The best resolution of the siRNA as well as sense and antisense strand was achieved by using the YMC-Pack Diol-120 column, which has a pore size of 12 nm (figure 1). The YMC-Pack Diol-200 column (20 nm pore size) also attained the separation of the siRNA duplex and its single strands but with higher retention times. This, in contrast, makes the YMC-Pack Diol-120 more efficient. The use of YMC-Pack Diol-60 (60 nm pore size) and YMC-Pack Diol-300 columns (30 nm pore size) did not lead to sufficient separation.

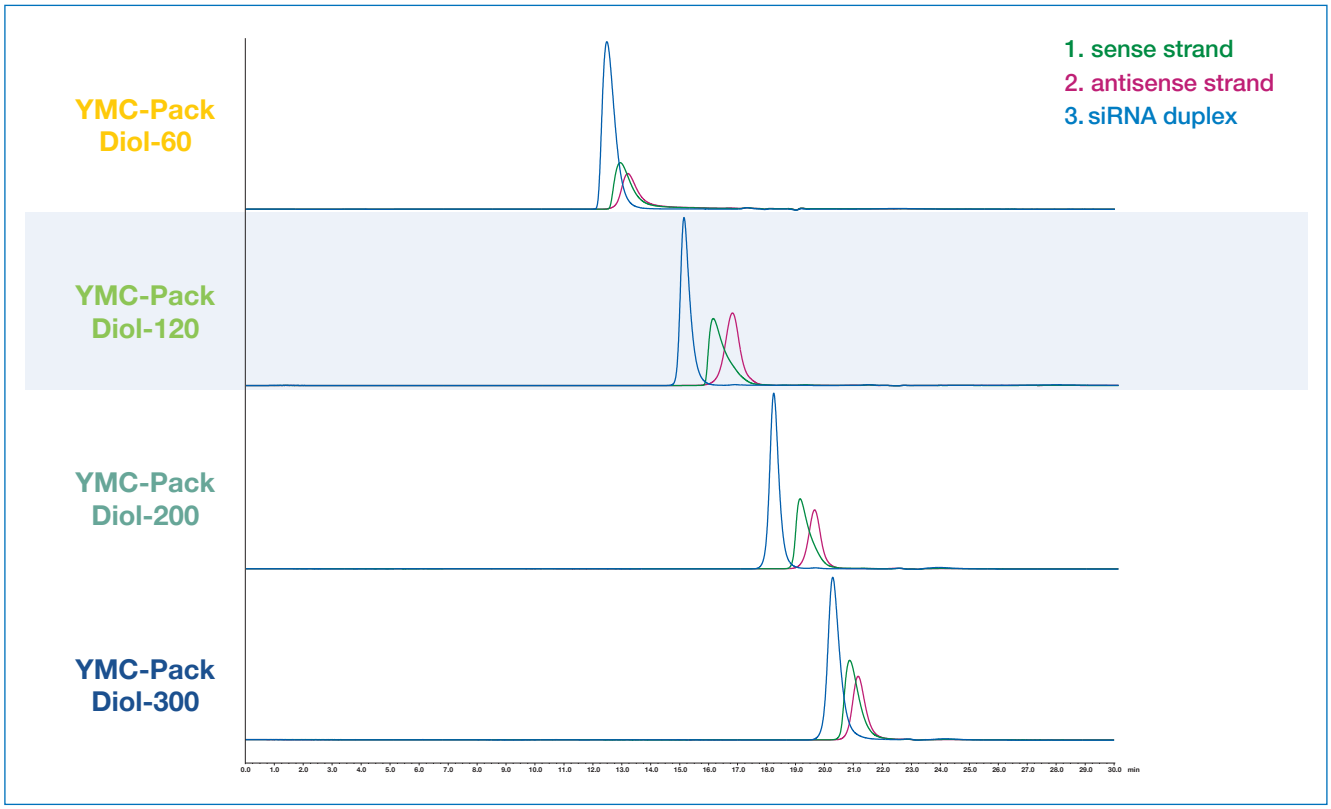


Figure 1: Effect of pore sizes on the separation of sense strand, antisense strand and siRNA duplex using different YMC-Pack Diol SEC columns.

Although the sense and antisense strand have the same molecular weight, their retention time and peak shape vary slightly. This is probably caused by minor secondary interactions with the stationary phase. If the goal is the separation of siRNA duplex from single strands, these interactions can be overcome by addition of an organic solvent to the mobile phase. By addition of 30% acetonitrile to the eluent, both single strands elute at the same time and their peak shape is improved (figure 2). Addition of an organic solvent can therefore improve the separation behaviour when analysing siRNA.

A further improvement in resolution can also be achieved by the use of smaller particles, such as 3 µm in the case of YMC-Pack Diol-120.

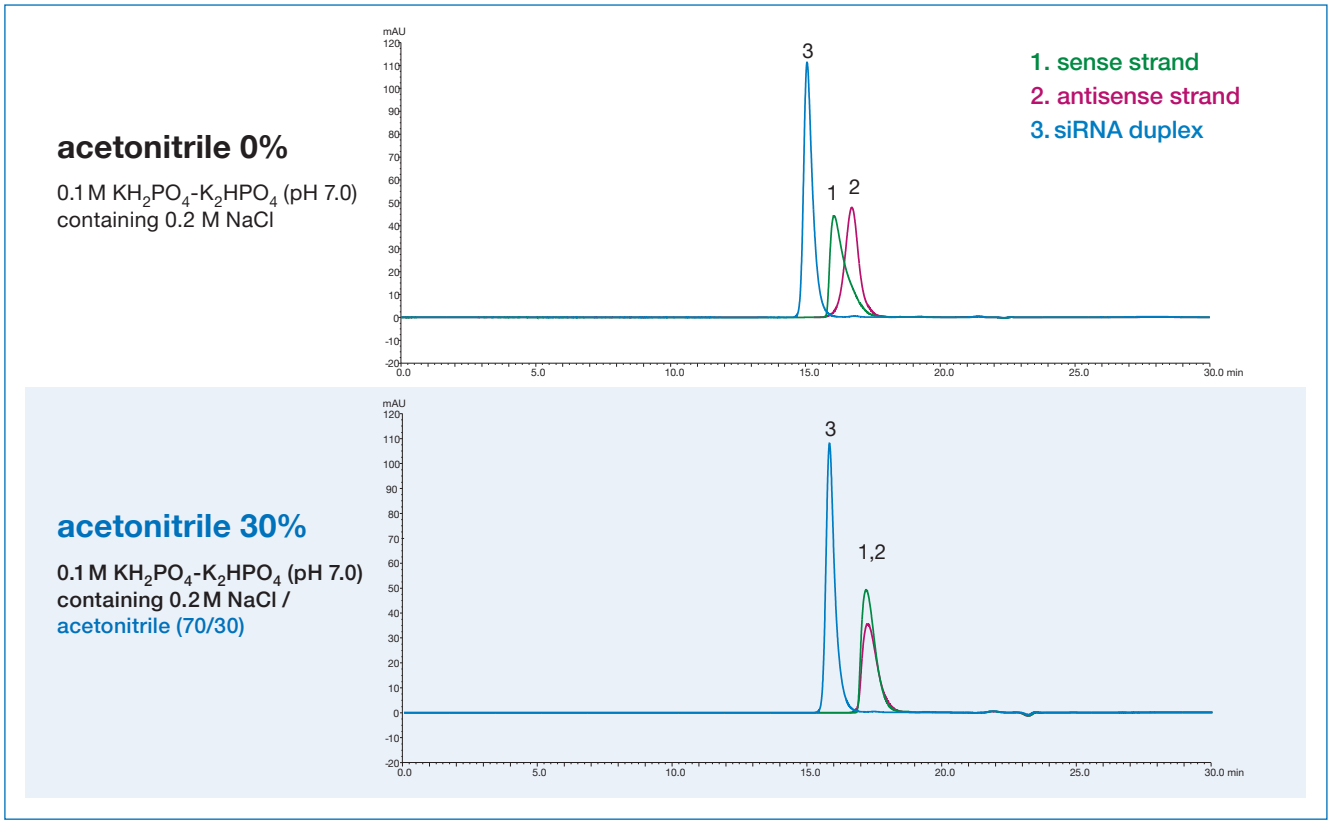


Figure 2: Effect of acetonitrile on separation of sense strand, antisense strand and siRNA duplex by SEC using YMC-Pack Diol-120.