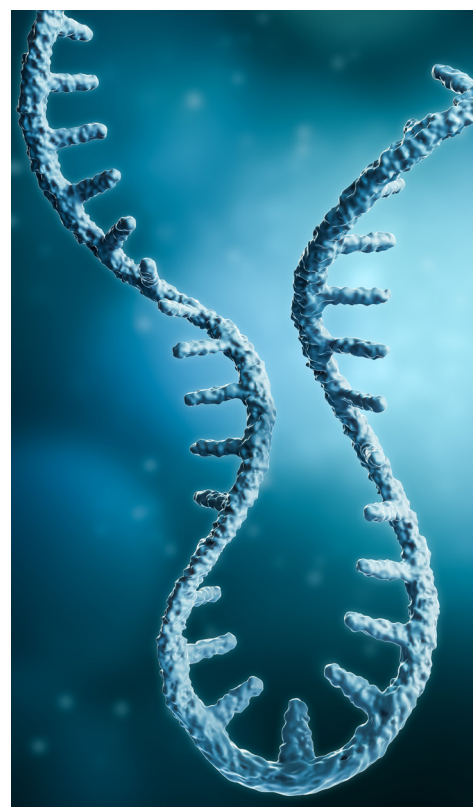


Automated hybridization assays for pharmacokinetic (PK) measurements of oligonucleotides

CASE STUDY

Core challenges include:

- Measuring ASO drug levels and their distribution in the body requires both high sensitivity and a broad concentration range
- Analysis in a variety of biological matrices is needed, including serum and tissue homogenates
- This case study describes ASO analysis using automated hybridization PK assays in biological samples



Automated hybridization assays for pharmacokinetic (PK) measurements of oligonucleotides

Cell and gene therapies represent a rapidly expanding area in drug development and with it a growing need for robust analysis tools to quantify oligonucleotide-based components in a variety of biological samples. Oligonucleotide medicines modulate gene expression (Figure 1) and provide opportunities to treat diseases that were previously considered “untreatable”¹. Oligonucleotide therapeutics include, for example antisense oligonucleotides (ASO), aptamers and siRNA (small inhibitory RNA). As of end of 2025, more than 25 oligonucleotide-based therapeutics have been approved, where ASO and siRNA represent the largest groups, and many more are being investigated in clinical trials^{2,3,4}. While

oligonucleotide drugs provide new opportunities they also come with challenges, for example with respect to delivery and safety¹ but also with requirements for suitable analytical methods.

This case study describes work to develop reliable immunoassays for the quantification of oligonucleotide therapeutics on the automated Gyrolab® system. Several automated hybridization assays were developed, using both dual hybridization and nuclease-dependent cutting assay formats. The assays were demonstrated to quantify ASO drug analogs with high sensitivity in plasma and tissue homogenates.

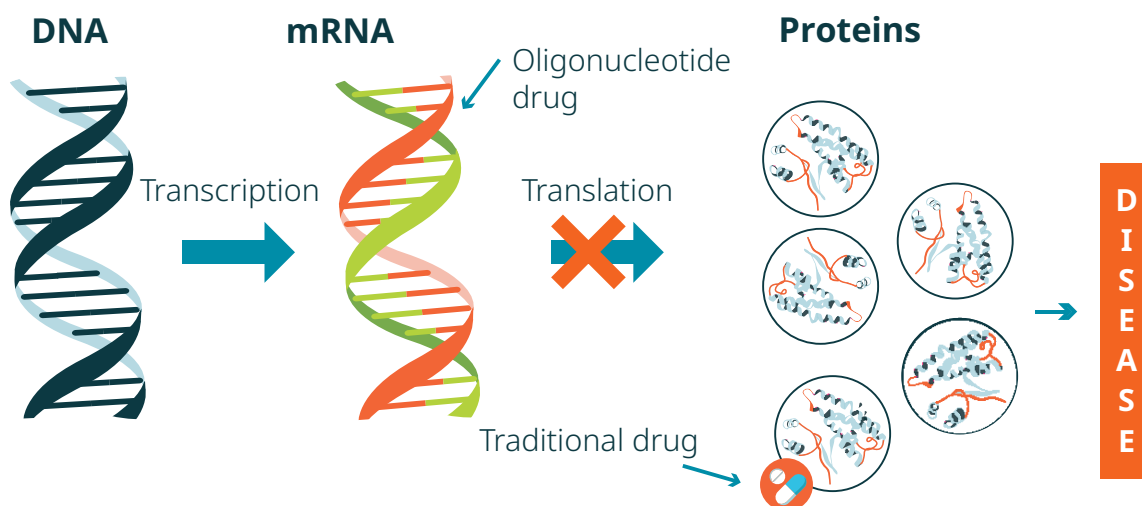


Figure 1: Oligonucleotide medicines modulate gene expression to treat disease

Antisense oligonucleotides (ASO)

ASO are single-strand oligonucleotides that can modify target RNA and protein expression through different mechanisms across a range of disease areas. The ASO can silence protein expression by cleaving the RNA or modulate the expression by blocking sites on the target RNA^{1,5}. ASO represent the largest portion of approved oligonucleotide drugs. Four of the ASO on the market, including the drug casimersen, enable exon-skipping as treatment for Duchenne muscular dystrophy^{2,3}.

Common mechanisms are:

1. RNase H1-dependent degradation – the ASO hybridizes with target mRNA. The resulting hybrid is recognized and cleaved by RNase H1, leading to mRNA degradation and gene silencing
2. Splicing modulation – ASOs bind to specific RNA regions, and change the splicing patterns or protein translation through steric hindrance

While ASO drugs provide revolutionary opportunities to treat disease, they also come with a range of challenges, including pharmacokinetic (PK) properties. Chemical modifications to the nucleotide backbone to improve the stability and PK profile of the ASO. For example, modifications of the ribose portion of the nucleotides, such as 2'-O-methoxyethyl (MOE) or phosphorodiamidate morpholino oligomer (PMO), are frequently employed to improve the ASO properties^{2,3}.

ASO drug development also comes with new requirements with respect to PK analysis. Measuring ASO drugs and mapping their distribution in the body may be challenging, requiring both high sensitivity and a broad concentration range. In addition, the analysis methods need to be suited for a variety of biological samples including tissue homogenates. The PK analyses of ASO frequently employ the use of hybridization assays and these need to be run with a throughput that match the drug discovery timelines.

Hybridization assays

Standard immunoassays, like ELISA, utilize antibodies for capture and detection of analytes. For hybridization assays, instead, complementary oligonucleotide sequences are used as capture and detection reagents (Figure 2). To be suited for use on Gyrolab, the capture oligo is biotinylated and the detection oligo is fluorophore labeled.

1. Dual hybridization assays (DHA) - employ one capture and one detection oligo. If not held in place by the analyte, the detection oligo is washed away, and no signal is detected.
2. Nuclease-dependent cutting assay (NCA) – the dual-purpose probe bears a biotinylation for capture and a fluorophore for detection and binds to the full length of the analyte. Unpaired probes are cleaved by a nuclease and the detection molecule is washed away resulting in loss of signal. The use of NCA improves selectivity for the parent oligonucleotide.

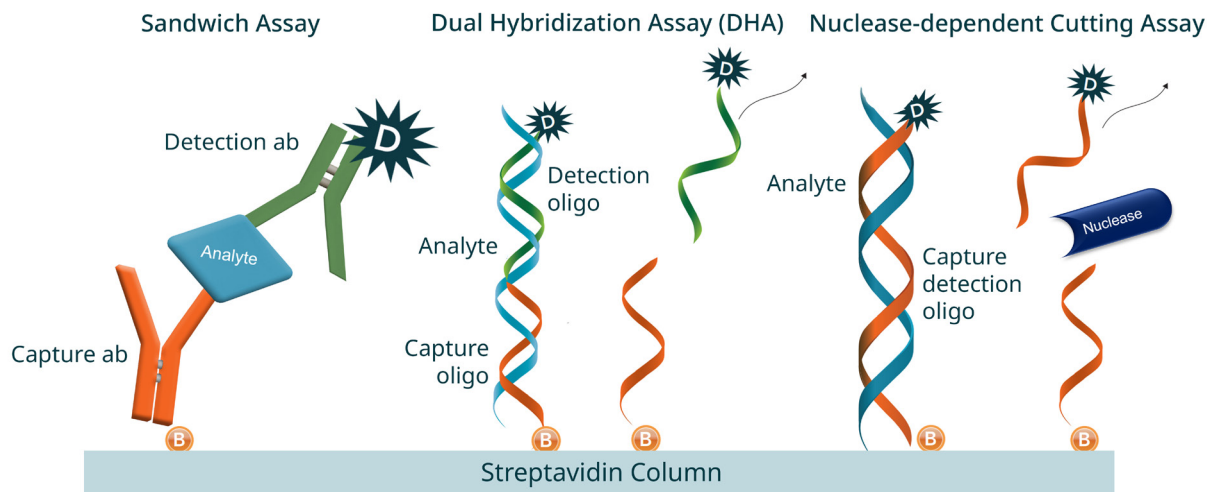
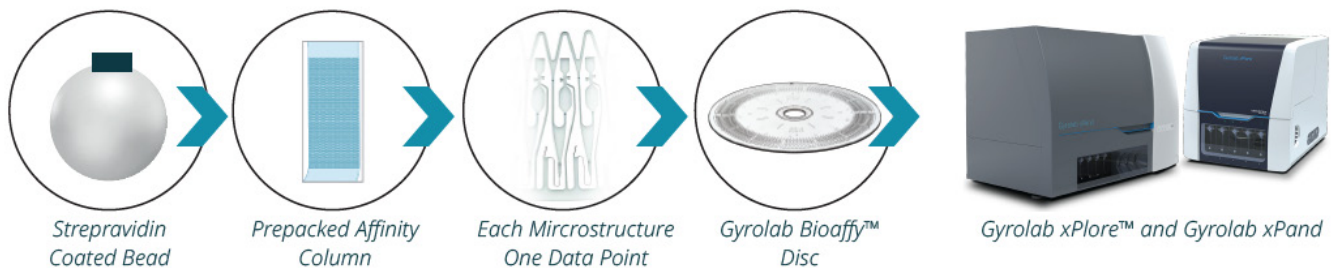


Figure 2: Standard immunoassays typically use antibodies for capture and detection while hybridization assays, like dual hybridization assay (DHA) or nuclease-dependent cutting assays (NCA), make use of oligos for capture and detection.

The Gyrolab technology

The Gyrolab platform runs automated immunoassays using a flow-through microfluidic technology. Automated delivery of samples and reagents is achieved by centrifugal force and capillary action. Analytes are captured by biotinylated reagents on streptavidin beads in flow-through 15 nL-columns on discs and detected by Alexa Fluor™ 647 labeled detection reagents. This automated platform enables walk-away execution of immunoassays. The flow-through format uses small sample volumes and has a high tolerance towards biological matrices.



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Results:

DHA with automated pre-incubation using Gyrolab Mixing CD developed to quantify ASO drug analog

The Gyrolab system enables automated walk-away running of the immunoassays, but in the case of hybridization assays, a mixing and pre-incubation step is needed before loading into the instrument. We developed a dual hybridization assay (DHA) for measuring levels of an ASO drug analog based on the sequence of the drug casimersen⁶. We also evaluated the use of the Gyrolab Mixing CD 96 to enable a fully automated immunoassay (Figure 3), including the pre-incubation step. As different nucleotide modifications are used to increase the stability,

for example PMO, a couple of different backbone-modified variants of the casimersen sequence were tested with similar results.

The assays showed excellent performance with both manual mixing and automated mixing using the Gyrolab Mixing CD 96 (Figures 3 and 4). Different incubation times were assayed using the automated mixing and showed that two 30-minute incubations for capture and detection worked well, but shortening the incubation time was unfavorable and reduced the signal-to-background ratio (Figure 3).

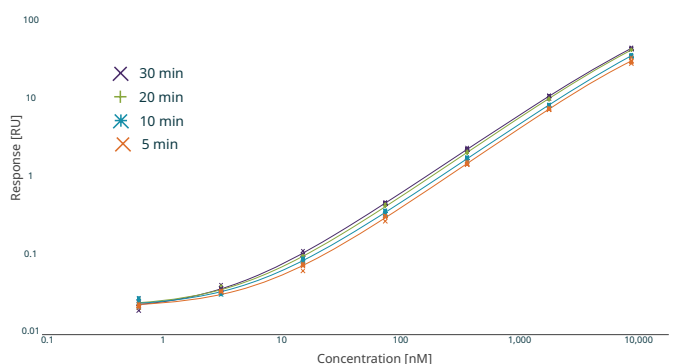
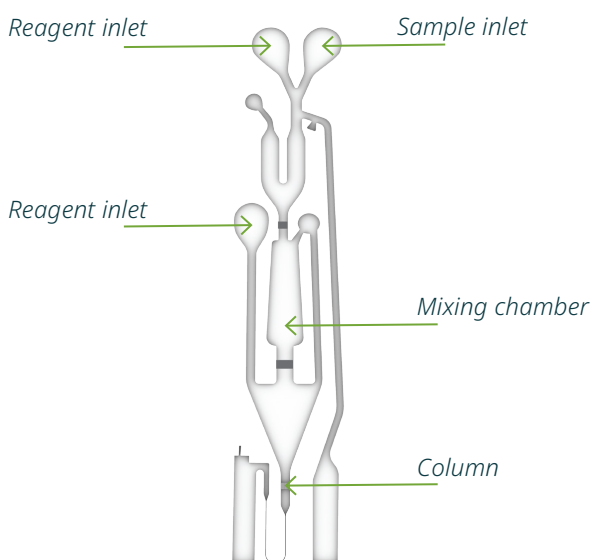


Figure 3: Gyrolab Mixing CD 96 worked well for automated incubation and was used to assess the impact of incubation time. The results showed that shortening the incubation times reduced the signal-to-background.

Fully automated DHA quantified ASO analog with high sensitivity in biological matrix

The levels of casimersen analog were measured in 50% human serum using the DHA developed for Gyrolab. The standard curve was prepared in 50% serum and the quality control samples were prepared in neat serum and diluted 1:2. The results from the serum samples showed the assay to be sensitive, with a lower limit of quantification (LLOQ) of 2 pM, and an excellent coefficient of variation (%CV<5, Figure 4). These results show that hybridization assays can be run in an automated fashion on Gyrolab instruments. The dual hybridization assay worked well both with manual and automated mixing and displayed high tolerance towards the biological matrix.

Gyrolab DHA developed for different size ASO

Pre-clinical and clinical PK measurements of oligonucleotide drugs can be challenging, as hybridization efficiency strongly depends on the sequence. Certain sequence features promote the formation of secondary structures or self-dimerization, that can interfere with probe binding in hybridization assays.

We developed DHA for three ASO drug analogs of different size (22-30 bases) and sequence composition on the Gyrolab platform. All three ASOs could be reliably measured in serum with a broad dynamic range, also when analyzed by different operators (Figure 4).

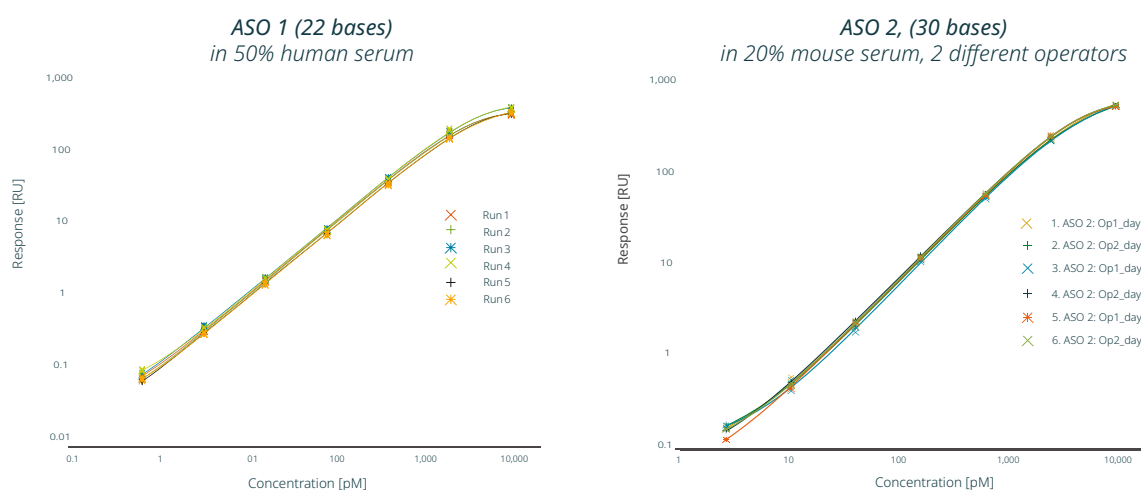


Figure 4: *Left panel*, DHA with automated pre-mixing using Gyrolab Mixing CD 96 was used to quantify a casimersen analog (ASO1, 22 bases) in neat serum diluted 1:2. The lower limit of quantification (LLOQ) was 2 pM, very similar to the results in buffer. The reproducibility was good with a very low coefficient of variation (%CV <5). *Right panel*, Automated DHA with a manual mixing step before loading on the Gyrolab instrument was used to quantify an ASO drug analog (30 bases) in 20% mouse serum. The assay was run by two different operators on three different days and showed excellent reproducibility.

DHAs for ASO quantification in tissue homogenates

To assess the distribution of an ASO drug in different organs, PK studies include analysis of different types of biological samples, ranging from serum and spinal fluid to tissue homogenates. Most of the approved ASO therapeutics are targeting the liver, for example through lipid nanoparticle (LNP) delivery or GalNAC-conjugations to target liver cells³. The Gyrolab method is generally tolerant to biological matrices and to assess the DHA performance in tissue we measured the levels of ASO in mouse liver homogenates. All three ASOs could be readily quantified in the liver extracts after dilution (Figure 5, example shown for ASO2).

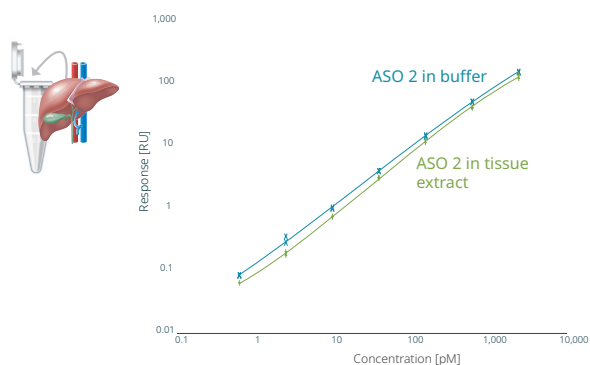


Figure 5: The DHA assays could quantify the three ASO drug analogs in liver homogenates with results comparable to those in buffer.

NCA on the Gyrolab platform - novel ultra-slow spin method

Nuclease-dependent cutting assays (NCA) were assessed as an alternative hybridization method for ASO detection. NCA typically provide better selectivity for the parent oligonucleotide and are therefore an attractive alternative, but initial attempts to run NCA on Gyrolab were unsuccessful. To optimize assay performance, we developed a novel ultra-slow spin method on Gyrolab to increase the effectiveness of the enzyme, by allowing more time for the nuclease to cleave non-hybridized oligonucleotide on the microfluidic column. This ultra-slow spin NCA method enabled successful quantification of the ASO analogs. The sensitivity could be further increased by lowering the probe concentration and repeat addition of enzyme. These modifications resulted in lower background and increased sensitivity (Figure 6).

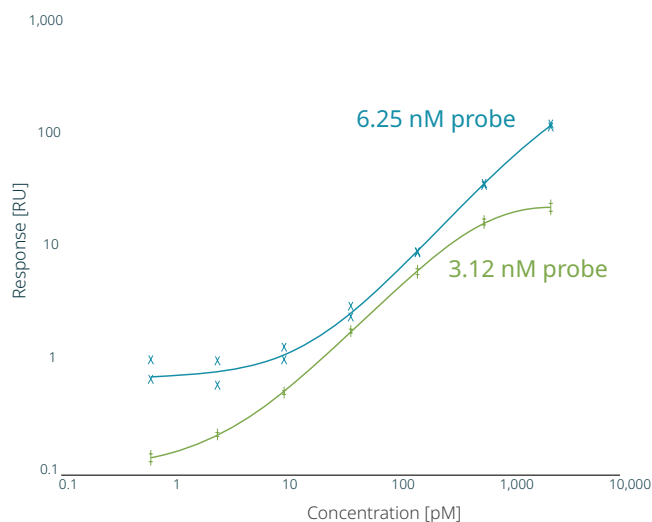


Figure 6: A novel microfluidic method to increase the time on column to boost nuclease cleavage was used to quantify ASO analogs. The sensitivity could be improved further by lowering the probe concentration and double the addition nuclease.

Taken together, these results show that the Gyrolab platform is well suited for the automated running of hybridization assays, both DHA and NCA, for reliable quantification of ASO in different biological samples with high sensitivity.

Conclusions:

- Automated DHA were successfully developed for the Gyrolab platform to quantify three ASO drug analogs of different size and sequence in serum and tissue extracts with high sensitivity
- DHA pre-incubation step could be performed on the automated Gyrolab platform by using a Gyrolab Mixing CD
- NCA to detect the ASO analogs were developed using a novel ultra-slow spin method to increase the on-column cleavage time
- These results suggest that the Gyrolab platform can support PK quantification with high sensitivity and throughput of a broad range of ASOs in preclinical and clinical samples

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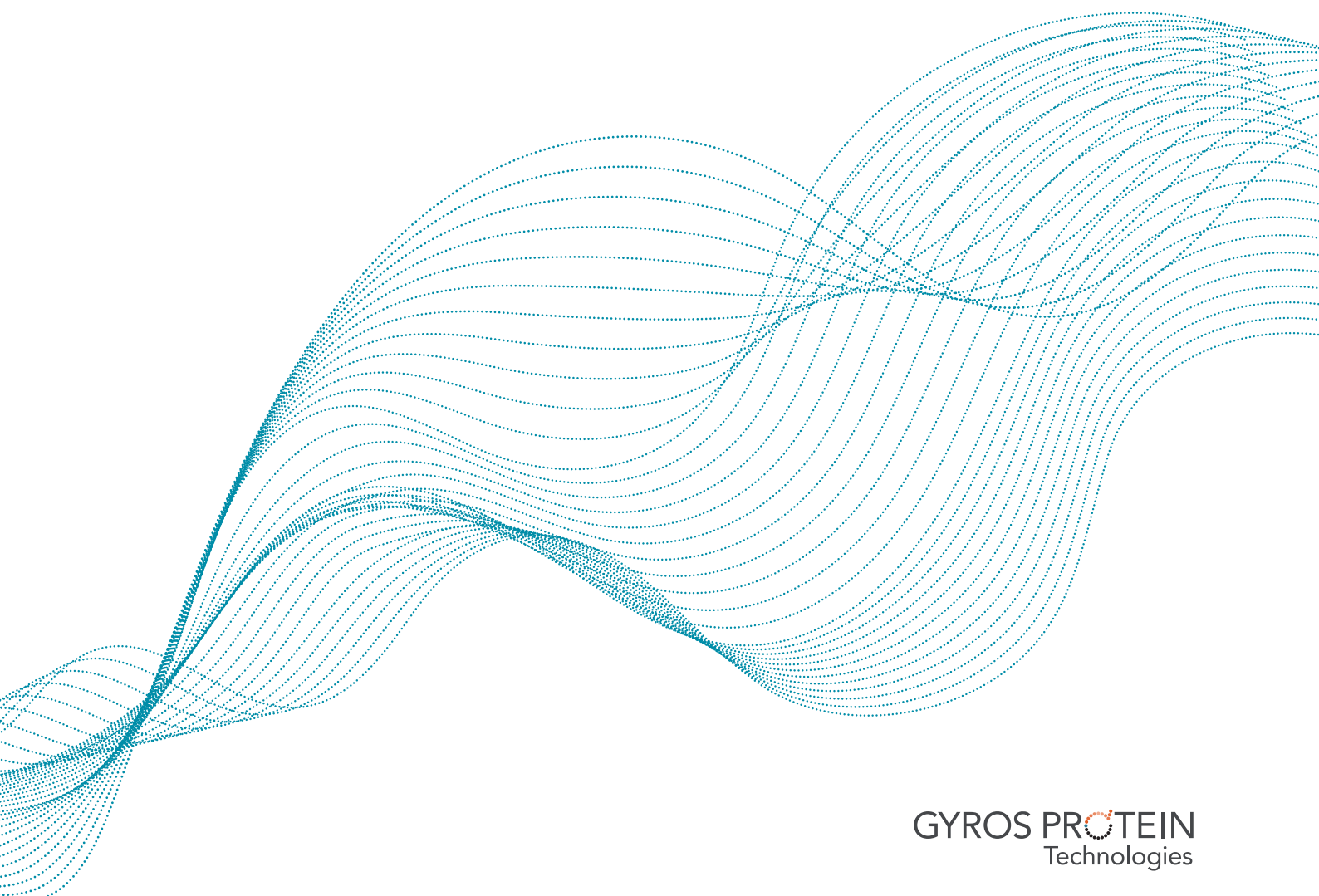
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