LEXOGEN CYTOSURGE®

From subcellular biopsies to transcriptomes:
FluidFM® OMNIUM and LUTHOR™ HD are streamlining Live-Seq

Abstract

The advent of single cell transcriptomics (scRNA-Seq) has revolutionized fundamental biological and biomedical research. In the field, droplet-based and combinatorial-barcoding technologies are the standard as they enable throughput by either capturing single cells into droplets or creating unique barcode combinations by cell splitting and pooling. Once each cell bears its own barcode, sequencing can occur, revealing the intricacy and abundance of different cell types and states within a population. Inherently to the library preparation process, low expressed genes and subtle signatures from rare cells are easily missed; moreover, any spatial or phenotypical information is lost in the process.

Chen *et. al.* (2022)[1] employed a cell biopsy technology that allows downstream single cell transcriptome profiling in live cells using a specific FluidFM® module coupled with an atomic force microscope. Here, we introduce a streamlined workflow to collect cytoplasmic biopsies from Panc-1 cells with the FluidFM OMNIUM platform and record a transcriptome from less-than-a-cell's worth of RNA using the commercially available ultra-low RNA input LUTHOR™ HD library preparation kit. We show that with LUTHOR HD, a transcriptome could be prepared and sequenced from as little as 0.15 pg of total RNA, the equivalent of <1% of a Panc-1 cell, allowing to record single-cell RNA profiles from Panc-1 cytoplasmic biopsies using the FluidFM OMNIUM platform.

Introduction

RNA sequencing has allowed researchers to define and investigate the cosmos of transcriptomics. Initially, transcriptomics studies were performed on RNA isolated from cell populations or whole tissue lysates. However, these experiments could not provide insights into subpopulations, rare cell types, and were lacking spatial information.

Droplet-based and combinatorial barcoding ("*split-pool*") technologies revolutionized the scRNA-seq field as they significantly increased throughput, revealing the intricacy and abundance of different cell types and states with single-cell resolution snapshots.[2–4]

Despite the continuous development of single-cell methods, thousands of cells need to be sequenced to compensate for sensitivity limits, where low expressed genes and signatures from rare cells are easy to miss. In addition, droplet-based or combinatorial split-pool solutions cannot provide any context to the local cell environment or correlate a single-cell

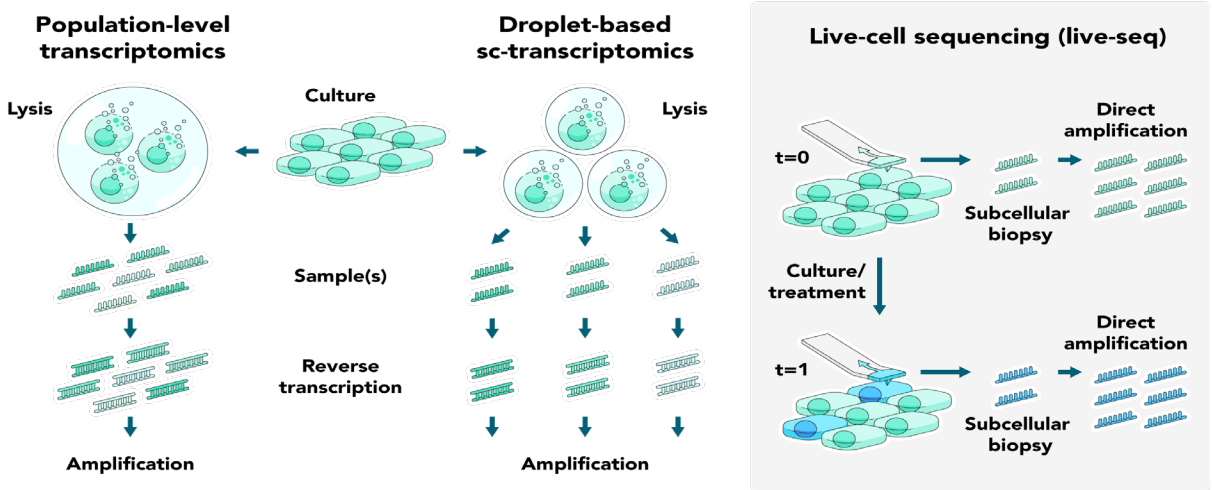

Figure 1: Evolution from population-level, over single-cell RNA-Seq, exemplified by droplet-based assays, to "*less-than-a-cell*" transcriptomics using live-cell sequencing.

phenotype directly to its expression profile. This does not restrict population stratification but does limit more in-depth studies into cell state transitions and minor cell clusters in heterogeneous cell populations (*see Figure 1*).

In 2022, Chen *et. al.* showed for the first time that it is feasible to correlate the phenotype of a specific single cell to its downstream transcriptome and record cell state transitions of live cells.[1] They used the FluidFM technology to demonstrate that it is possible to take a cytoplasmic extract from a specifically selected cell, and then analyse the RNA from this subcellular fraction of cytoplasm, or "*biopsy*", using an adapted Smart-seq2 protocol (*see Figure 1*).[1,5]

This concept of "*less-than-a-cell*" sequencing is revolutionary as it provides direct read-outs from cells, to complement existing scRNA datasets. These workflows however require ultra-low input RNA protocols to process the samples and capture all RNAs expressed. The adapted Smart-seq2 protocol is not commercially available, making it challenging to onboard.

In this application note, we provide the proof of concept of a streamlined workflow to record single-cell biopsy transcriptomes from a few Panc-1 cells (*see box 1*) over the course of several days.

Box 1: Why using Panc-1 cells?

Establishing a correlation between the morphology of the tumor cell and his transcriptome is of fundamental importance in cancer research.

Pancreatic cancer is among the cancers with the highest mortality rates. This is caused on one hand by the difficulty in detection of primary tumors in the premetastatic stage, and on the other hand by its aggressiveness, intrinsic heterogeneity, and plasticity.[7,8]

We collaborate with the group of Dr. Carmelo Ferrai (Institute of Pathology, UMG Göttingen) to investigate morphological and transcriptional correlation using Live-seq.

The project lead by Dr. Ferrai aims to decipher how Panc-1 cells[9,10] respond to mechanotransductive stimuli. Establishing the transcriptional response to these stimuli will provide fundamental information about underlying drivers that trigger the *in vivo* aggressiveness of pancreatic cancer.

Box 2: LUTHOR HD and the THOR amplification method

The LUTHOR HD library preparation kit is based on the proprietary THOR amplification method.[11]

Traditional scRNA-seq library preparation methods are based on reverse-transcribing RNA first into cDNA followed by an exponential amplification by PCR. In contrast with traditional approaches, LUTHOR HD amplifies RNA copies directly from the mRNA templates that were isolated from the cell of interest. Through this approach, the amplification process is more linear and manages to capture low copy number transcripts more accurately.

The resulting high sensitivity makes LUTHOR HD an ideal library preparation kit for cytoplasmic biopsies.

Complementation of the biopsy collection workflow with a commercially available kit for transcriptome recording is essential to make "l*ess-than-a-cell*" RNA sequencing accessible and reliable. For this, we partnered with Lexogen, who recently launched the LUTHOR HD library preparation kit. LUTHOR HD is based on the THOR amplification method (*see box 2*) that is able to detect virtually every mRNA molecule in a single cell and has been previously proven to be able to record transcriptomes from as little as 1 pg RNA.[6] The workflow starts with collecting cytoplasmic biopsies from specific cells with the biopsy workflow on FluidFM OMNIUM. Then, by using the LUTHOR HD Kit, RNA-Seq libraries are obtained, precisely reflecting the transcriptomic complexity of each single cell for ultimate gene detection sensitivity and precision (*see also box 2*).

With LUTHOR HD, we were able to faithfully generate RNA libraries from as little as 0.15 pg of total RNA, corresponding to <1% of a regular Panc-1 cancer cell. Next, we showed that it is possible to retrieve a transcriptome from 7 out of 8 cytoplasmic biopsies with >2'800 genes detected per million reads in 50% of the samples.
Overall, this proof of concept validates that a subcellular biopsy containing less-than-a-cell's worth of RNA, can be analysed with LUTHOR HD. This streamlined workflow enables researchers to obtain transcriptomes from an individual cell, and link RNA profiles to their phenotypic appearance, possibly over the course of several days.

Results

Recording transcriptional profiles from "*less-than-a-cell*" worth of RNA input

We set out to test the sensitivity and reproducibility of the LUTHOR HD kit starting from a total of 2 million Panc-1 cells. The Panc-1 cells were lysed and 50.5 µg of total RNA was obtained, an equivalent of approximately 25.2 pg of total RNA per individual cell.
A dilution series was generated from 80 pg to 1.25 pg of extracted RNA, which corresponds to RNA quantities obtained from 3 whole cells down to 1/20th of a cell, respectively (1.25 pg).

Taking these numbers into consideration, we used LUTHOR HD to generate the library of two replicates per RNA input, followed by sequencing. In addition to the RNA samples, a no-RNA input negative control was used to determine the background.

In the first quality control experiment at a read depth of 1 million, LUTHOR HD detected between 13'000 (80 pg, *data not shown*) and 8'000 (1.25 pg) genes in the RNA samples compared to only 250 in the negative control, demonstrating a high sensitivity for all RNA samples and a clear distinction to the negative control. Moreover, library saturation (detected genes) was reached between 0.25 and 0.75 million reads. To assess reproducibility between technical replicates of the LUTHOR HD libraries, correlation analysis was performed. Results showed that R^2 values ranged from 0.97 (80 pg) to 0.84 (1.25 pg), which indicates robust and reproducible library generation, even with as little as 1.25 pg of total input RNA (*data not shown*).

To further test the sensitivity boundaries, another dilution series was prepared with Panc-1 RNA ranging from 10 pg (2/5th of a cell) down to only 0.15 pg of total RNA (1/160th of a cell). Sequencing libraries were prepared again following the LUTHOR HD protocol, including technical replicates of each input (*Figure 2*). Sequencing results show that at 1 million reads, RNA quantities of 10 pg and 1.25 pg enabled the detection of 12'000 and 7'000 genes, respectively (*Figure 2A*).

Excitedly, the LUTHOR HD kit also faithfully detected approximately 2'800 genes at 1 million reads from only 0.15 pg of total RNA. This extremely low amount of total RNA corresponds to as little as 1/160th of a cell (*Figure 2B*). Reproducibility between technical replicates ranged from R^2=0.91 (10 pg) to R^2=0.67 (0.15 pg) (*Figure 2C*).

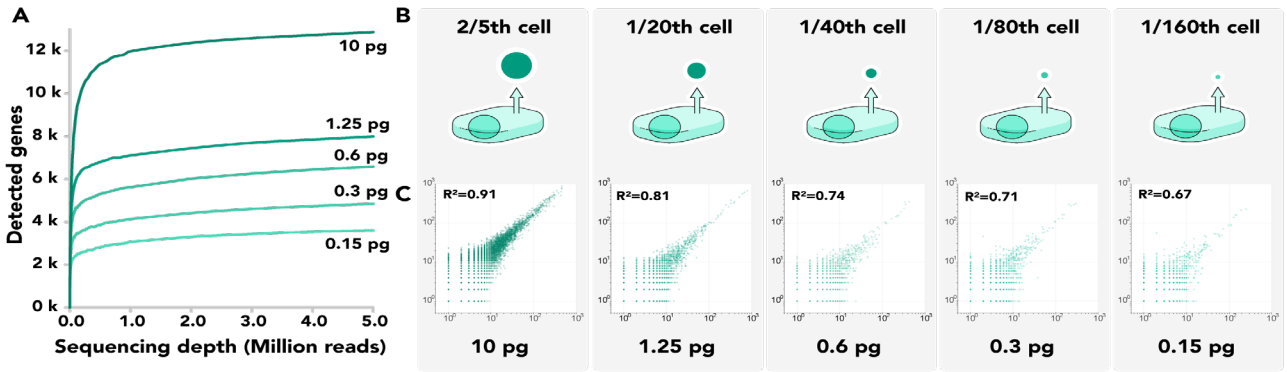

Figure 2: Sequencing single-cell cytoplasmic biopsies with LUTHOR HD.

A: Number of detected genes from different concentrations of total RNA from Panc-1 cells. The detected genes (in thousands (k)) were plotted against the sequencing depth.

B: The quantity of RNA in average Panc-1 cell equivalents. 50.5 µg of total RNA was obtained from 2 million Panc-1 cells, hence one cell contains approximately 25.2 pg of total RNA.

C. Correlation between technical replicates. The R^2 -values are calculated using orthogonal regression of gene-based collapsed and mapped reads (downsampled to 1 million reads).

Brief conclusion:
Overall, our experiments showed that LUTHOR HD is highly sensitive and can faithfully produce libraries from subcellular amounts of total RNA. Our experiments showed gene detection rates of 7'000 - 8'000 per 1 million reads and a good correlation between replicates from samples derived from as little as 1.25 pg of total RNA. Even smaller amounts, down to 0.15 pg, generated libraries containing close to 3'000 genes. These findings confirm that LUTHOR HD can provide high quality transcriptomes from subcellular amounts of total RNA and could be suitable for the analysis of cytoplasmic biopsies.

Using cytoplasmic biopsies to generate high-definition transcriptomes from single cells.

After establishing that the LUTHOR HD workflow is suitable for subcellular amounts of total RNA, we set out to validate whether transcriptomic data could also be obtained from cytoplasmic biopsies collected with the FluidFM OMNIUM platform.

Extraction of cytoplasmic biopsies from Panc-1 cells using the FluidFM Biopsy workflow: The workflow used to collect cytoplasmic biopsies, as published by Chen *et al.*[1] was adapted to a semi-automated workflow on the FluidFM OMNIUM platform (*Figure 3*).

Panc-1 cells were maintained in DMEM supplemented with 10% fetal bovine serum and L-Glutamine. Confluent Panc-1 cells were distributed on IBIDI dishes at a dilution of 1:10 and grown for four days. Prior to the extraction, the cell culture medium was refreshed, and the extraction point on individual cells was manually selected in the FluidFM Ayra software using the bright field microscope function integrated into the FluidFM OMNIUM platform (*Figure 3*).

Next, the FluidFM microfluidic Nanosyringe was preloaded with 1-2 pl of LUTHOR CLB buffer supplemented with 0.2% Triton™ prior to biopsy collection, and automatically directed to the extraction point marked. Cytoplasm was then sampled from each Panc-1 cell by gentle insertion into the Panc-1 cell membrane with a force of 500 nN and applying negative pressure of 700 mbar through the Nanosyringe.

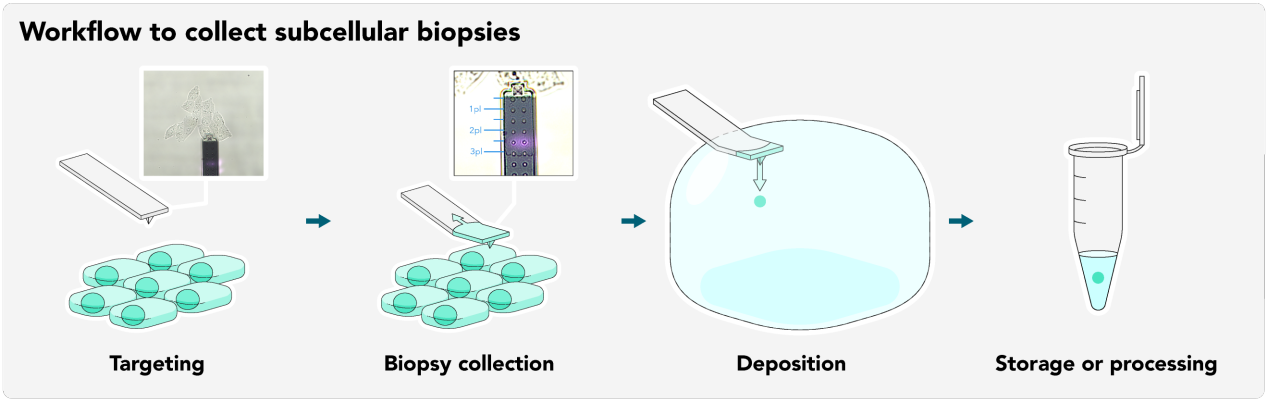

Figure 3: Schematic representation of the cytoplasmic extraction workflow.

Targeting: the targeted cell for extraction can be manually selected in the FluidFM Ayra software using the brightfield microscope function integrated in the FluidFM OMNIUM platform. The brightfield image shows a (cluster of) Panc-1 cell(s) before initiation of the extraction. Of each targeted cell, the extraction point is selected.

Biopsy collection: The cytoplasmic membrane of the target cell is gently penetrated by the pyramidal tip of the FluidFM Nanosyringe and 0.5-2 pl of cytoplasm is extracted by applying negative pressure. The above brightfield image shows the same Panc-1 cell after the biopsy was collected. Inside the Nanosyringe the collected cytoplasm mixed with the extraction buffer is visible. Due to the defined geometry of the microchannel, an estimation of the extracted volume can be determined.

Deposition followed by storage or processing: by applying gentle positive pressure to the Nanosyringe, the cytoplasmic biopsy is deposited into a 1 µl buffer droplet containing the reagents for the library preparation. Repeated aspiration and release of 3-5 pl of buffer ensures that the contents of the biopsy are transferred efficiently to the buffer droplet. Lastly, the buffer droplet is transferred followed by direct downstream processing or snap freezing.

During the extraction process, that lasts between 6-10 min per sample, the volume extracted from the cytoplasm was carefully monitored and the extraction volume determined using the defined dimensions on the Nanosyringe (*Figure 3*).

In the next step, the extracted cytoplasm was subsequently deposited from the Nanosyringe into a 1 µl LUTHOR CLB buffer droplet by applying a positive pressure of 50 mbar. Residual material was then flushed out. Subsequently, the buffer droplet containing the cytoplasmic biopsy was transferred to a reaction tube (containing additional CLB buffer), ready for downstream processing and library generation (*Figure 3*).

The process was repeated with 8 individual cells, of which the extracts collected ranged between 1.3 to 1.8 pl (*Table 1*).

Library preparation from biopsies and initial QC: The cytoplasmic biopsies were subjected to library generation using the LUTHOR HD kit as described in Survila *et al.*[11].

In addition to the 8 cytoplasmic extracts, positive and negative controls were included:

- Positive control: libraries from 10 pg of total RNA isolated from Panc-1 cells (as described earlier).
- Negative control: No input control (NIC) consisting of extraction buffer that was loaded into the Nanosyringe and released into the 1 µl transfer buffer droplet, and a PCR control.

Prior to sequencing a quantitative assessment was done to determine the library quality. The cDNA was run through a Bioanalyzer 2100 (Agilent) to determine the library shape and yield, followed by qPCR to assess optimal amplification cycles for the final PCR step of the library preparation protocol.
Overall, 7 of the 8 cytoplasmic biopsies collected showed lower threshold (Ct) values compared to the negative control suggesting successful library generation for 7 biopsy samples (*data not shown*).

Table 1: Overview of samples, controls, detected genes during sequencing and extraction volume.

Grey denotes the different controls, red indicates samples that did not pass initial quality control based on the Bioanalyzer trace and qPCR test, and yellow indicates cell biopsies that were removed from further analysis due to low gene count.

Sample	Genes detected per million reads	Volume extracted (pl)
Positive controls		
10 pg Panc-1 RNA (1)	9'326	-
10 pg Panc-1 RNA (2)	9'712	-
Negative control		
No input control (NIC)	20	-
Cell biopsies		
Cell 1	2'824	1.3 pl
Cell 2	3'501	1.7 pl
Cell 3	1'131	1.3 pl
Cell 4	274	1.4 pl
Cell 5	486	1.4 pl
Cell 6	5'051	1.8 pl
Cell 7	7'242	1.5 pl
Cell 8	Not sequenced	1.3 pl

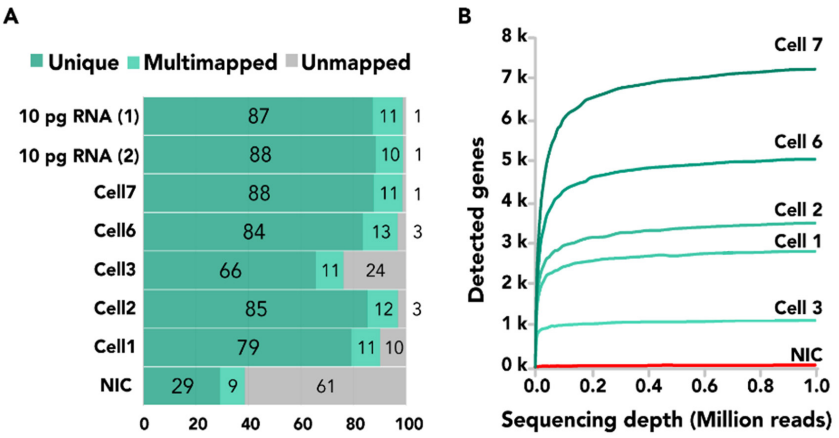

Figure 4: Overview of the biopsy sequencing results.

A: Overview of the genes detected in the control and biopsy samples mapped against the human genome (GRCh38). The graph shows all unique, multimapped, and unmapped reads.

B: Detection of the number of genes at a sequencing depth of 1 million reads for each of the biopsy samples that passed the quality threshold.

Sequencing: Next, the libraries were subjected to sequencing and the obtained reads were mapped to the human genome (GRCh38).
A respective 88% and 87% of the reads from the positive controls could be uniquely mapped. Interestingly, the reads obtained from the biopsies revealed a degree of heterogeneity with a range of 42 - 88% of uniquely mapped reads. Cell biopsies 4 and 5 showed less than 500 detected genes per million reads and were excluded from further analysis (*Figure 4A*).

At a read depth of 1 million, the cytoplasmic biopsies varied between 274 and 7'242 genes detected: in comparison, the positive controls (10 pg of total RNA) detected 9'712 / 9'326 genes while the negative control only 20 genes were mapped at a read depth of 1 million reads per sample (*Table 1, Figure 4*).

Brief conclusion:
Overall, the proof of concept data presented here show that we have defined a streamlined workflow that is able to successfully collect cytoplasmic biopsies from single Panc-1 cells from which LUTHOR HD is able to generate representable transcriptomes. Taken together, this work shows that it is possible to sequence a specific single cell fraction.

Discussion

In this application note, we show a proof of concept of the full workflow for collecting transcriptomes from a fraction of the cytoplasm of single cells. The workflow starts with the collection of cytoplasmic biopsies with the FluidFM OMNIUM platform, followed by downstream analysis of the collected subcellular amounts of RNA with LUTHOR HD.

In a first step, we defined the detection limits of LUTHOR HD from different concentrations of purified RNA extracted from Panc-1 cells. This validated that at the expected RNA content of a single Panc-1 cell, ca. 25 pg, as many as 12'500 genes could be detected, representing a very high sensitivity according to scRNA-Seq standards.[12]

This notion was further strengthened by the high correlations found between the technical replicates. At RNA concentrations of 10 pg, correlation was consistently above R^2 of 0.9, and even down to 0.15 pg, correlation remained close to R^2 of 0.7, indicating a consistent representation of the transcriptome even at RNA concentrations corresponding to <1% of a cell. In addition, sub-picogram amounts of RNA still generated libraries with thousands of detected genes, with a gradual decrease in line with the smaller input amounts, but without any drop-outs. This shows that the LUTHOR HD library preparation kit is suitable for the detection of subcellular amounts of total RNA, and that the protocol is not failing below a certain threshold, but systematically captures the full diversity of molecules present in the sample.

Subsequently, we showed that from 5 out of 8 biopsies collected, we were able to generate sequencing data with detection rates ranging between 1'000 and 7'000 genes per million reads. In an additional 2 biopsy samples, RNA was present, but only 274 and 480 genes per million reads could be detected.
In comparison, Chen *et. al.*[1] reported recording of single cell transcriptomes from approximately 40% of their biopsies. This result is encouraging and a further step towards streamlined live-cell RNA sequencing. This new field will help understanding many unknowns, such as intracellular RNA distribution[13,14], cellular biomechanics, or mechanotransduction.[15]

Outlook

This work marks the first proof of concept of a streamlined, commercial solution to record transcriptomes from a cytoplasmic biopsy containing less-than-a-cell's worth of RNA. In the future, Cytosurge and Lexogen will continue to work together and to extend the number of biopsies analysed from different cell types. In addition, we aim to further validate the workflow to support live-cell sequencing at different time-points, thereby streamlining time-resolved transcriptomics on living cells.

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About Cytosurge AG

Cytosurge develops innovative research solutions based on the FluidFM® technology for high precision single cell manipulation.

FluidFM combines microfluidics and force microscopy to enable gentle interactions with cells. With the capabilities of FluidFM, we seek to advance the comprehension of intricate cellular processes, thus fostering new dimensions in cellular analysis.

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About Lexogen GmbH

Lexogen is a leading company in RNA sequencing, providing innovative solutions for transcriptome analysis and RNA research.

Our products and services are designed to meet the evolving needs of the scientific community, delivering end-to-end solutions for high-quality, reliable results.

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