

Site-Specific mRNA Cleavage for Selective and Quantitative Profiling of Alternative Splicing with Label-Free Optical Biosensors

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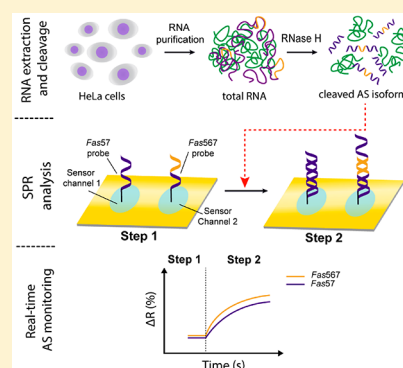
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Supporting Information

ABSTRACT: Alternative splicing of mRNA precursors is a key process in gene regulation, contributing to the diversity of proteomes by the alternative selection of exonic sequences. Alterations in this mechanism are associated with most cancers, enhancing their proliferation and survival, and can be employed as cancer biomarkers. Label-free optical biosensors are ideal tools for the highly sensitive and label-free analysis of nucleic acids. However, their application for alternative splicing analysis has been hampered due to the formation of complex and intricate long-range base-pairing interactions which make the direct detection in mRNA isoforms difficult. To solve this bottleneck, we introduce a methodology for the generation of length-controlled RNA fragments from purified total RNA, which can be easily detected by the biosensor. The methodology seizes RNase H enzyme activity to degrade the upstream and downstream RNA segments flanking the target sequence upon hybridization to specific DNA oligos. It allows the fast and direct monitoring of *Fas* gene alternative splicing in real time, employing a surface plasmon resonance biosensor. We demonstrate the selective and specific detection of mRNA fragments in the pM–nM concentration range, reducing quantification errors and showing 81% accuracy when compared to RT-qPCR. The site-specific cleavage outperformed random RNA hydrolysis by increasing the detection accuracy by 20%, making this methodology particularly appropriate for label-free quantification of alternative splicing events in complex samples.



Gene regulation pathways play important roles in every process of life, including cell differentiation, metabolism, cell cycle, and signal transduction. There are many regulatory mechanisms that serve the cells to quickly adapt to environmental changes by modulating gene expression levels, establishing a complex and highly structured gene regulatory network. Alternative splicing (AS) is a key post-transcriptional process in the gene expression regulatory pathway. Splicing of precursor mRNA (pre-mRNA) can generate different mature mRNAs that contain various combinations of exons, contributing to the diversity of proteomes. Alterations in AS processes can cause or modify the development of a variety of diseases including cancer.¹ Most cancers are associated with a switch in the splicing pattern of specific isoforms that provides them with proliferative capacity and survival properties.² In addition, splice variants diversify the repertoire of biomarkers and functionally contribute to drug resistance during chemotherapy.³ For example, Safikhani et al. demonstrated that

isoform analysis represents a promising brand-new class of biomarkers for patient stratification in cytotoxic and targeted anticancer therapies.⁴ Also, it has been demonstrated that RNA splicing events are crucial drivers of prostate cancer aggressiveness and therapeutic resistance in African American men.⁵ Therefore, the evaluation of splice variants holds great promise for improving disease diagnosis and, ultimately, patient care in the era of precision medicine.⁶ In fact, recent studies focusing on prostate cancer have demonstrated that mRNA analysis of circulating tumor cells could reveal substantial information on drug sensitivity and resistance,⁷ which can lead to the development of less invasive approaches to modulate and monitor this process in therapeutic settings.⁸

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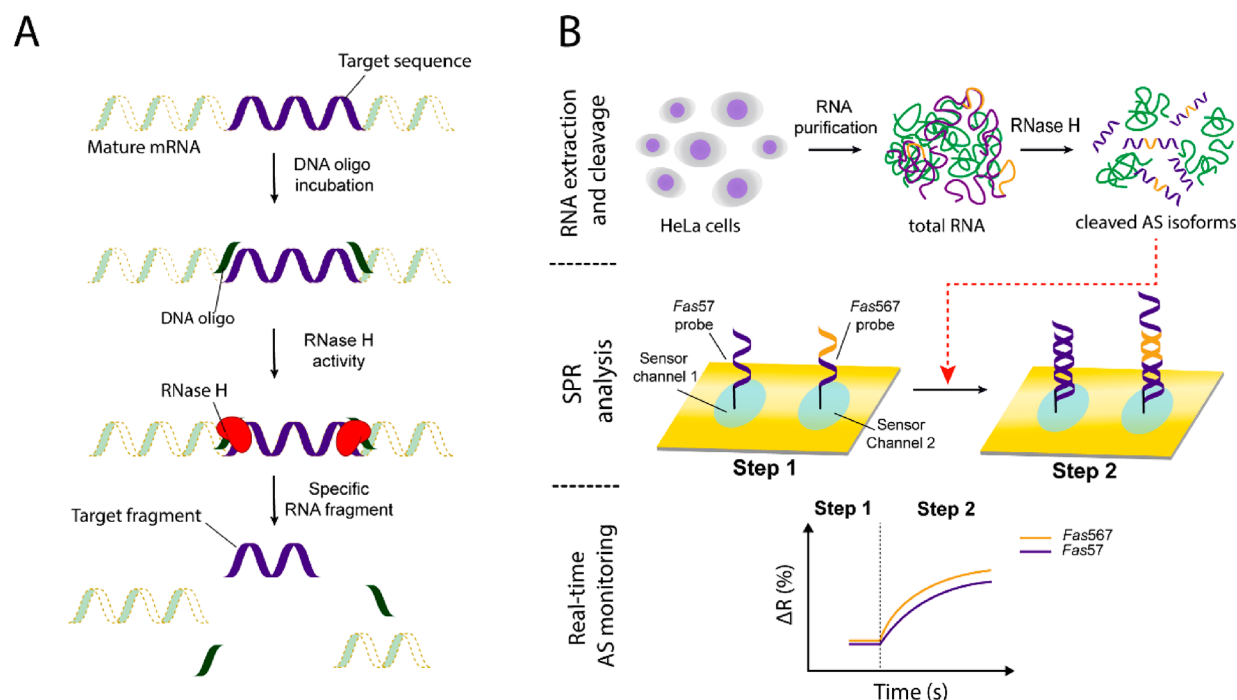


Figure 1. Scheme of the developed methodology: (A) RNase H-specific cleavage process; (B) SPR biosensor methodology for specific detection of AS isoforms in HeLa cells.

The knowledge of the mechanisms and dynamics of AS has grown substantially in recent years, confirming its critical role in human health and disease. The gold-standard approach to characterize AS is reverse transcription of mRNA followed by polymerase chain reaction amplification (RT-qPCR).⁹ Also, microarrays have been designed to sample AS events on a genome scale and successfully used to examine AS across tissues, cellular states, and species.¹⁰ RNA sequencing and its combination with computational tools have emerged as powerful tools for the examination of AS at a genome-wide level, providing opportunities to leverage AS to advanced diagnostics and prognostics tests for the delivery of effective medicines to patients. Recently, label-free optical biosensors have been pointed out as potential solutions for disease diagnostics and therapy follow-up by the analysis of nucleic acids.¹¹ These biosensors are highly sensitive analytical devices able to perform label-free assays in a timely and inexpensive manner, with minimal operator training and easy-to-perform procedures. The surface plasmon resonance (SPR) biosensor is considered the landmark in optical biosensors. SPR systems have been widely employed in the pharmaceutical industry and research laboratories for the study of numerous types of biomolecular interactions.¹² SPR biosensors can detect, monitor, and quantify molecules attaching to the sensor surface by measuring the change in the refractive index (RI) produced at its immediate vicinity, thus avoiding amplification steps or molecular labeling. However, the most challenging factor in label-free detection of mRNAs is the target length. Long single-stranded RNA sequences are prone to generate secondary and tertiary structures through complex base-pairing and backbone interactions facilitated by the intrinsic flexibility of RNA moieties. These complex structures can hide the target sequence and hinder their accessibility to the bioreceptor monolayer. In addition, the production of nucleic acid sequences by conventional oligonucleotide synthesis processes has some limitation due to chemical reaction efficiency. As a

result, the lengths of synthetic oligonucleotides typically do not exceed 150–200 nucleotides (nt),¹³ which impedes the precalibration of the biosensor by employing standards for target sequences over 200 nt. This is one possible reason why there is a shortage of publications related to the detection of long RNA sequences with label-free optical biosensors.¹¹ Our team previously demonstrated the capabilities of an SPR biosensor for the label-free analysis of AS events.¹⁴ In this study we showed that RNA fragmentation was necessary for the accurate assessment of AS events by the SPR biosensor, since the signal from these long sequences was almost negligible, probably due to the target sequences' low accessibility. To do that, a sample preprocessing step was applied for the fragmentation of purified total RNA. The strategy followed takes advantage of the susceptibility of phosphodiester bonds from the ribose sugar phosphate backbone of RNA to be hydrolyzed in alkaline medium,¹⁵ generating ~200 nt mRNA fragments, which can be directly analyzed by the SPR biosensor without PCR amplification. However, the incubation time is a crucial parameter in this approach since it promotes a random cleavage and is prone to lose sample input by disruption of the sequence of interest. This time will also vary depending on the sample quality. Poor-quality samples could be caused by RNA degradation. Thus, additional alkaline degradation is not advisable because it can foster the loss of target input and bias the final biosensor readout. In such cases, it is important to keep RNA integrity to ensure an accurate analysis. Therefore, other methodologies should be considered in order to achieve a more controlled cleavage of RNA molecules.

Whereas DNA molecules can be easily cut at specific sequence sites by restriction enzymes, there are no specific tools for selective RNA sequence fragmentation. This disadvantage makes it particularly difficult to generate RNA fragments of interest in a controlled manner. RNase H is an enzyme known to specifically degrade the RNA strand of

RNA–DNA hybrid duplexes.¹⁶ For example, it has been employed in the identification of a particular group of microbes based on the cleavage of a particular region in rRNAs (rRNAs).¹⁷ In this work, we introduce RNase H-mediated cleavage as a new methodology for the generation of specific RNA fragments of a length of interest and demonstrate its applicability for the real-time analysis of AS events using an SPR biosensor. The RNA site-specific cleavage employs two designed DNA oligonucleotides complementary to the upstream and downstream RNA segments flanking the specific RNA sequence of interest (Figure 1A). These DNA oligonucleotides, once hybridized to the endogenously transcribed AS isoforms, will promote the degradation of the hybridized RNA segment by RNase H activity, liberating the flanked sequence and making it available for its subsequent analysis by the SPR biosensor (Figure 1B). Compared to alkaline hydrolysis, which produces random RNA fragments, the RNase H method enables high-precision fragmentation, thus ensuring that target RNA sequence is released intact with high selectivity and efficiency. Further, it offers the advantage of analyzing the RNA fragments of interest directly in a PCR amplification-free mode and without risk of sample input loss, contrary to the alkaline hydrolysis approach. The methodology is implemented for the evaluation of AS events in *Fas* gene mRNA isoforms, which play antagonistic roles in programmed cell death and are relevant to cancer. AS of *Fas* gene generates two isoforms depending on the inclusion or skipping of its exon 6 (i.e., *FasS67* or *FasS7*, respectively). *FasS67* isoform encodes a full-length transmembrane protein receptor known as Fas/CD95 that binds to Fas ligand (FasL), activating the signaling cascade of the extrinsic apoptotic pathway. *FasS7* isoform lacks the trans-membrane domain, producing a soluble protein known as sFas that competes with the Fas receptor in the extracellular environment by binding to FasL. This version of Fas is overexpressed in cancer cells,¹⁸ and contributes to cancer aggressiveness.¹⁹ We have optimized the RNase H cleavage conditions in terms of buffer composition, enzyme concentration, and reaction temperature. Finally, the usefulness of the methodology has been demonstrated by the analysis of the endogenous expression of Fas gene alternative splicing isoforms in HeLa cells and its performance comparison with the gold-standard RT-qPCR technique for validation.

MATERIALS AND METHODS

Chemical Reagents and Buffers. All chemical reagents, solvents, and buffer solutions employed in this work are listed in the Supporting Information.

SPR Biosensor. The SPR biosensor device is based on Kretschmann configuration and monitors refractive index (RI) changes in real time.²⁰ A schematic illustration and the working principle of the SPR biosensor is in the Supporting Information (Figure S1).

DNA Sequences. All DNA probes and synthetic targets were purchased from IBA GmbH (Goettingen, Germany) and are summarized in Table S1. DNA-probe sequences incorporate a thiol group at the 5'-end to enable chemical coupling with the gold sensor surface. In addition, a spacing region, which consists of a 15-thymine sequence (polyT₁₅) is placed between the thiol group and the matching region. This sequence will not participate in the hybridization event, but, instead, it acts as a vertical spacer due to the low affinity of thymine bases for gold surfaces.

The length of this polyT₁₅ has been demonstrated to outperform the biosensor performance compared to other lengths.²¹ mRNA isoform sequences were obtained from Ensembl genome browser (<http://www.ensembl.org/index.html>). DNA/RNA sequences employed are summarized in Table S1.

DNA-Probe Immobilization. Prior to DNA-probe immobilization, gold sensor chips were cleaned by consecutive sonication cycles (1 min) with solvents of increasing polarity (i.e., acetone, ethanol, and dH₂O) previously heated to their boiling point. Then, substrates were dried under nitrogen flux and placed in a UV/O₃ generator (BioForce Nanosciences, USA) for 20 min. After that, the gold sensor chips were subsequently rinsed with ethanol and water and dried under nitrogen flux. A gold sensor chip was then placed into the SPR device.

For RNase H activity optimization, formation of a self-assembled monolayer (SAM) of the DNA₁ probe was carried out in situ on the gold sensor chip by flowing 250 mL of a solution containing 1 μ M DNA₁ probe in 1 $\times$ PB solution at a 12 mL/min rate. For *Fas* gene isoform detection, formation of mixed SAMs of either *FasS6*-probe or *FasS7*-probe/MCH (20:1, 1 μ M) was carried out in situ on the gold sensor chip by flowing 250 mL of the mix in 1 $\times$ PB solution at a 12 mL/min rate. We immobilized each isoform probe in independent SPR channels.

Alkaline Hydrolysis of Purified Total RNA from HeLa Cells. Total RNA from human cervical carcinoma HeLa cells (approximately 1.5 million cells) was isolated using the RNeasy kit (Qiagen, Germany). For alkaline hydrolysis, samples were preheated at 95 °C for 15 s and mixed with preheated 1 $\times$ alkaline hydrolysis buffer (50 mM carbonate/bicarbonate, 1 mM EDTA, pH 9.2) to start the hydrolysis reaction. Total RNA samples were exposed to the alkaline buffer for 3 min. After each incubation time, samples were placed on ice and reaction was stopped with RNA fragmentation stop solution (3 M sodium acetate, pH 5.2). After applying the fragmentation protocol, endogenous levels of *Fas* isoforms were checked using the SPR biosensor.

RNase H Cleavage of Purified Total RNA from HeLa Cells. Total RNA from human cervical carcinoma HeLa cells (approximately 1.5 million cells) was isolated using the RNeasy kit (Qiagen, Germany). They were transfected at 50% confluency with 0.4 μ g of *Fas* minigenes²² and 10 μ g of plasmid DNA expressing either β -galactosidase or PTB, per culture plate of 10 cm. When 6-well plates were used, 50 ng of minigene and 3 μ g of expression plasmid DNA were transfected per well. When minigene and protein expression vectors were used, cells were collected 48 h post-transfection. Total RNA was isolated using the RNeasy kit (Qiagen), and the pattern of alternative splicing was analyzed by RT-PCR. Reverse transcription was carried out using AMV-Reverse Transcriptase (Promega) and specific reverse primers.²² Polymerase chain reaction was carried out using GoTaq (Promega) and forward and reverse primers corresponding to the exonic regions flanking the alternatively spliced sequences.²³ For SPR biosensor analysis, two DNA-oligos were designed, complementary to the sequences flanking exon 5 (3'-end of exon 4) and exon 7 (5'-end of exon 8) (see Table S2). These sequences, once hybridized to the endogenously transcribed isoforms, will promote the degradation of the RNA sequence in contact, producing fragments of approximately 200 nt (Table S2). We incubated total RNA with a 10

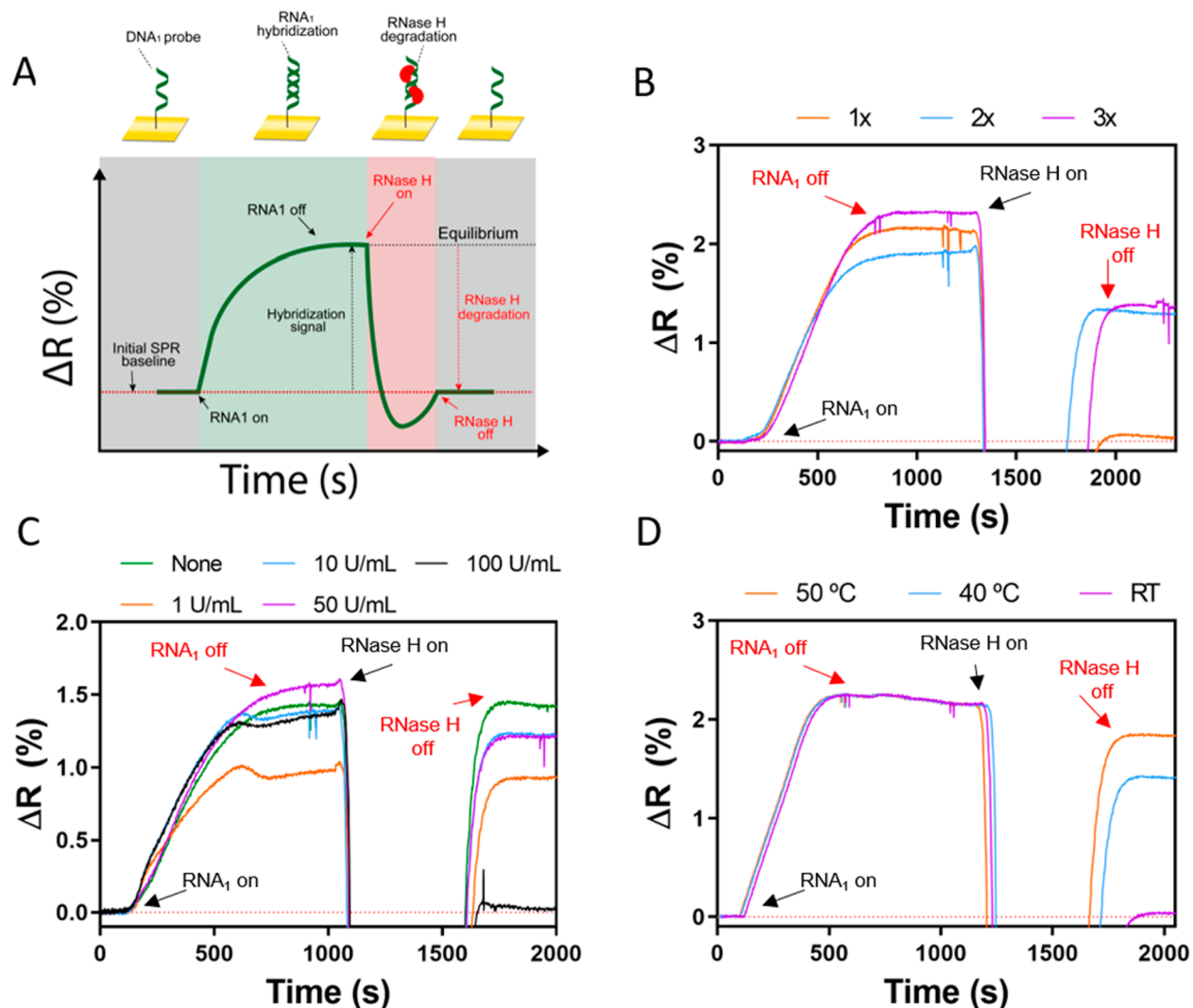


Figure 2. Optimization of RNase H conditions for *Fas* gene alternative splicing analysis through SPR biosensor: (A) scheme of the strategy followed for the optimization of the RNase H activity by SPR analysis; (B) RNase H buffer concentration optimization; (C) RNase H concentration optimization; (D) Temperature optimization for RNase H activity optimization (RT, room temperature).

μ M concentration of each DNA probe at 95 °C for 2 min and cooled it on ice for 5 min for probe-annealing. After annealing, RNA samples were incubated with 100 U/mL RNase H in 1× RNase H buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT; pH 8.3) at room temperature (RT) for 60 min. RNase H was later inactivated by incubating at 65 °C for 15 min. After applying the fragmentation protocol, endogenous levels of *Fas* isoforms were evaluated using the SPR biosensor.

Isoform Hybridization and Biosensor Regeneration. Splicing variants' detection was performed by injection of the target splicing variant samples into the biosensors at a 15 μ L/min rate and subsequent hybridization with their complementary DNA probes immobilized on the sensor surface. These samples were dissolved in the hybridization buffer (3× SSC—0.45 M NaCl, 0.045 M sodium citrate—with 45% formamide). Isoform-probe interactions were disrupted by using 50% formamide in aqueous solution. Calibration curves were obtained for each DNA probe by triplicate measurements of the DNA *Fas* gene AS isoforms' synthetic standards at different concentrations (2.5, 5, 10, 20, 30, and 50 nM). The mean and the standard deviation (SD) of each concentration were plotted versus the target concentration and fitted to a

curve. Fragmented and nonfragmented total RNA samples from HeLa cells were diluted in hybridization buffer to 20 ng/ μ L and analyzed by SPR biosensor at the same conditions used for the standards. In addition, purified total RNA from bacteria (XLI Blue) was used as a negative control.

Data Analysis. The data were collected and analyzed using Origin 8.0 software (OriginLab, Northampton, MA, USA). Graphical representation and calibration curve interpolation were performed in Graphpad prism 8 (Graphpad Software, San Diego, CA, USA, -EEUU-) The experimental detection limit (LOD) was defined as the target concentration providing a ΔR (%) in the hybridization signal at least three times higher than that of the standard deviation of the control signal. The coefficients of variation were obtained as the ratio of the standard deviation to the mean, expressed in percentages (% CV). Agreement between RT-qPCR and SPR was assessed by applying the Bland-Altman model for replicate measurements.

RESULTS AND DISCUSSION

Specific RNA Cleavage Optimization. The assessment of the RNase H activity was carried out using a portable custom-designed SPR biosensor.²⁰ This SPR biosensor is

based on Kretschmann configuration and monitors refractive index changes at a fixed angle of incidence. It enables analysis in real time, giving us the opportunity to demonstrate the full degradation of the hybridized RNA while it happens. The biosensor response is represented in sensograms that reproduce the binding event by monitoring the increase (binding events) or decrease (unbinding events) of the intensity of the reflected light (Δ reflectivity, ΔR (%)) versus time (s). This change of the intensity of the reflected light is directly related to the mass changes on the gold surface, which correspond to the concentration of the hybridized RNA. Therefore, effective cleavage would only be considered if the sensor baseline returned to its initial state prior to RNA hybridization. The complexity of these types of experiments increases in the case of PCR experiments, since the presence of the RNase H primers could provoke cross-reactivity during amplification. We assessed and optimized RNase H activity exploring different parameters of the reaction, including the following: (i) the reaction buffer concentration, (ii) the enzyme concentration, and (iii) the reaction temperature. We employed a short RNA sequence (RNA₁) (Table S1) for the evaluation of the activity of the enzyme. We immobilized a specific complementary DNA probe (DNA₁ probe Table S1) for the specific capture of RNA₁ and, subsequently, monitored the RNA hybridization and RNase H activity in real time (Figure 2A). A change in the refractive index will be observed during the specific hybridization of the complementary RNA₁ target, which will be directly related to its concentration in the analyzed sample. Then, the interaction will find its equilibrium state and will reach a plateau in the SPR sensor signal. The net change in the baseline, expressed as variation of reflectivity (ΔR (%)), will be directly proportional to the concentration of the hybridized target sequence. RNase H can be employed during the examination of RNA sequences in SPR biosensors for the regeneration of the sensor surface. The regeneration is underpinned by the RNase H-induced degradation of the RNA sequences previously hybridized to the immobilized DNA probes, rendering them available for further analyses. Thus, eventually, the hybridization will be interrupted by the RNase H solution, degrading RNA₁ sequence, and returning the baseline to its original level.

First, we evaluated effect of the reaction buffer on RNase H activity. The buffer composition, pH, and ionic strength of the solution are known to influence enzymatic cleavage efficiency. The optimum buffer composition for RNase H activity has been established and reported elsewhere (i.e., 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, pH 8.3).¹⁷ However, the concentration of salts must be tested for specific applications. The study was carried out as depicted in Figure 2A. DNA₁ probe was immobilized in situ on the gold sensor chip and continuous 5× saline—sodium citrate (5× SSC) buffer at pH 7 was supplied over the functionalized sensor surface until a baseline was reached. Then, RNA₁ was hybridized at a 50 nM concentration in 5× SSC buffer with its specific receptor attached to the biosensor surface. Once we reached the equilibrium of the interaction, we flowed 250 U/mL enzyme at different RNase H buffer concentrations (1×, 2×, and 3×) at RT (i.e., 25 °C). The capability of the enzyme at each buffer concentration to return the sensor signal to the initial level due to the cleavage was evaluated. As can be observed in Figure 2B, the SPR signal dropped drastically below the initial baseline during the RNase H activity (RNase on). This dip in the signal was mainly caused by the difference

in the RNase H buffer composition compared to the continuous buffer, producing a drastic drop in the refractive index of the medium and obscuring the monitoring of the degradation process. After RNase H treatment, the sensor surface was washed again with the running buffer and the net change between the sensor signal at the equilibrium of interaction and the new baseline provided the approximate percentage of degraded RNA by the enzyme's activity. At a 2× buffer concentration, the baseline did not return to its initial level, indicating that only 48% of the hybridized RNA was degraded. A similar result was observed at 3× buffer concentration. On the contrary, the baseline completely returned to the initial level at a 1× buffer concentration, confirming the highest RNase H activity at this buffer concentration. Once 1× was established as the proper buffer concentration, we tested the optimal enzyme concentration needed for the reaction. We injected four different concentrations of RNase H enzyme: none, 1, 10, 50, and 100 U/mL. As depicted in Figure 2C, the latter cleaved the hybridized RNA almost completely and achieved the same results as when using 250 U/mL, while lower concentration failed in the full degradation of the RNA, indicating that a concentration of 100 U/mL RNase H was sufficient to carry out the complete cleavage of the sequence. Finally, we explored the enzyme activity at different reaction temperatures. We tested room temperature (RT, 25 °C) and 40 and 50 °C by preheating the RNase H solution before flowing it in the SPR biosensor. At 40 and 50 °C, the enzyme was not fully effective, cleaving less than half of the hybridized RNA (Figure 2D).

On the contrary, RT conditions achieved the best results, returning the sensor signal to the initial levels. According to these results, we considered that optimal conditions for RNase H activity were 100 U/mL enzyme concentration diluted in a 1× reaction buffer at RT.

Comparative Study of mRNA Fragmentation Procedures for *Fas* Gene AS Evaluation. The optimized procedure for specific mRNA fragmentation by the RNase H enzyme was applied and evaluated for selective biosensing of endogenous levels of *Fas* gene AS isoforms directly from HeLa cells samples. We also compared its performance with nonfragmented and with alkaline-hydrolyzed samples. RNase H cleavage was performed off-sensor on purified RNA from HeLa cells to assess the expression levels of *Fas* gene mRNA isoforms by following the experimental procedure depicted in Figure 1. In order to obtain *Fas* gene isoform fragments of interest, we designed 25 nt DNA sequences complementary to the sequences flanking exon 5 (3'-end of exon 4) and exon 7 (5'-end of exon 8). These sequences, once hybridized to the endogenously transcribed isoforms, should promote the degradation of the hybridized RNA sequence, producing fragments of approximately 200 nt (Table S2). For comparison, alkaline hydrolysis was performed in parallel as previously described.¹⁴ RNA samples were preheated at 95 °C for 15 s and mixed with preheated 1× alkaline hydrolysis buffer, and the mix was incubated for 3.5 min (incubation time was previously optimized in order to obtain RNA fragments of an average of 200 nt) (Figure S2). To stop RNA degradation, samples were placed on ice and mixed with sodium acetate (pH 5.2). The time of incubation is crucial and will strongly depend on the concentration and quality of the samples. Lower RNA concentrations will be degraded faster, leading to shorter fragments for similar incubation times. Likewise, poor-quality samples will already present some level of degradation and

could lead to sample input losses. Therefore, it should be carefully optimized in each case. After each fragmentation process, samples were diluted in the hybridization buffer to 20 ng/ μ L purified total RNA. In order to detect the *Fas* gene AS isoforms through SPR, a clean gold sensor chip was activated and placed into the SPR device. Immobilization of *Fas*S7 and *Fas*S6 probes was carried out in independent microfluidic cells.

Mixed SAMs of either *Fas*S67 or *Fas*S7 probes and alkanethiol 6-mercapto-1-hexanol (MCH) at a ratio of 20:1 were prepared and flowed over two different SPR sensor channels. MCH is a commonly used backfiller that acts as a lateral spacer, improving the target accessibility by minimizing steric hindrance forces created between the DNA probes in densely packed monolayers. After that, the mRNA samples obtained from HeLa cells after fragmentation are flowed over the functionalized sensor surface, and the specific *Fas*S7 and *Fas*S67 are captured and detected in real time. We monitored the SPR response recorded for each isoform in both SPR sensor channels. Results obtained using RNase H cleavage (SPR^{H}) and alkaline hydrolysis protocol (SPR^{Δ}) were compared with nonfragmented samples (SPR^0). Panels A and B of Figure 3 show the SPR responses obtained after injection of HeLa cell total RNA samples for each receptor. SPR sensograms showed a significant increment of the sensor signal after sample injection time (≈ 100 s) due to differences in the refractive index of the hybridization buffer. After sample

interaction, we washed the sensor surface with the continuous running buffer and analyzed the net changes in the baseline levels, which will correspond to the quantity of target hybridized to the biosensor. As can be seen in Figure 3, endogenous RNAs without any cleavage generated SPR responses of 0.02% and 0.09% ΔR for *Fas*S7 and *Fas*S67, respectively. In contrast, samples treated with RNase H produced ΔR of 0.12% and 0.57% for *Fas*S7 and *Fas*S67, respectively. Alkaline hydrolyzed samples showed increments in the SPR reflectivity of 0.09% (*Fas*S7) and 0.49% (*Fas*S67). Negative control RNA purified from bacteria produced negligible SPR sensor responses, returning to the baseline level and confirming the specificity of the detection. Therefore, the application of either of the fragmentation protocols resulted in an increment in the SPR sensor responses for both isoforms. These results confirm our hypothesis that RNA fragmentation increases the accessibility of the target to the monolayer and, therefore, enhances the SPR detection response.

Validation of the Measurement Results Using RT-qPCR. In order to obtain quantitative data of *Fas* AS isoform levels, we carried out calibration curves using synthetic DNA standard sequences (*Fas*S67 and *Fas*S7 isoforms, Table S1) corresponding to the exonic regions 567 and 57 of *Fas* gene AS isoforms (Figure 4A,B). These DNA sequences were designed with lengths of 150–200 nt to simulate the fragment lengths obtained by the fragmentation processes. We analyzed different concentrations (ranging from 0 to 50 nM) for both sequences diluted in the hybridization buffer. By applying these conditions, we achieved limits of detection of 387 and 438 pM for *Fas*S7 and *Fas*S67 isoforms, respectively. Such detection limits prove the excellent sensitivity of our methodology for the direct and label-free assay of nucleic acids, which is usually in the nM–pM range.²⁴ SPR quantitative data were obtained by interpolation of the SPR response in the calibration curve equation specific for each isoform. Figure 4C shows the quantification of the two isoforms under each condition. Quantitative data obtained from nonfragmented samples (SPR^0) were 24.6/5.5 nM for *Fas*S67/*Fas*S7.

In contrast, the quantities detected increased by a factor of 10 after the application of either fragmentation protocol (262/12 nM for SPR^{Δ} and 295/13.1 nM for SPR^{H}).

To validate the results and the methodology introduced in this work for site-specific mRNA cleavage, we carried out parallel analysis of HeLa RNA samples with RT-qPCR. Table 1 shows the comparison between the quantification performed by RT-qPCR and the three different approaches carried out using the SPR biosensor. According to RT-qPCR data (365/20.3 nM), SPR results from nonfragmented samples heavily underestimated RNA abundance (accuracy lower than 30%), confirming the need for a fragmentation process.

RNase H cleavage results showed 81% accuracy for *Fas*S67 and 65% for *Fas*S7 compared to RT-qPCR, whereas alkaline hydrolysis showed accuracies of 65% for *Fas*S67 and 59% for *Fas*S7. Thus, RNase H outperformed alkaline hydrolysis up to 20% for the detection of the different isoforms, leveraging this approach for a more accurate detection of AS events. The slight underestimation of isoform concentrations by SPR^{H} compared to the results from the RT-qPCR could be due to limitations in the sensitivity of the SPR biosensor, since analyses are performed in an amplification-free and label-free scheme. Finally, to further confirm the advantages of the

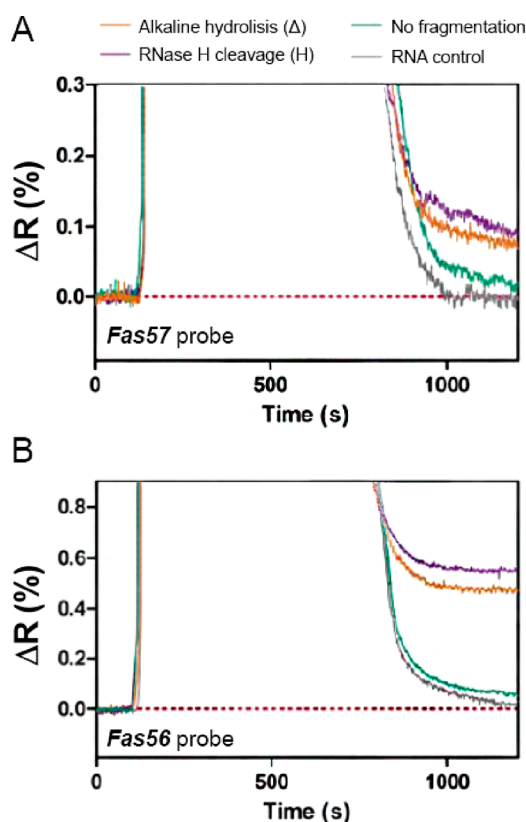


Figure 3. SPR sensograms of the detection of 20 ng/ μ L total HeLa cell RNA for the endogenous expression of both (A) *Fas*S67 isoform and (B) *Fas*S7 isoform after applying RNase H cleavage (H) (purple line) and the alkaline hydrolysis (Δ , orange line) and without any sample fragmentation (0, green line). The gray line represents the signal given by the RNA control sample. Adapted with permission from ref 14. Copyright 2016 Elsevier.

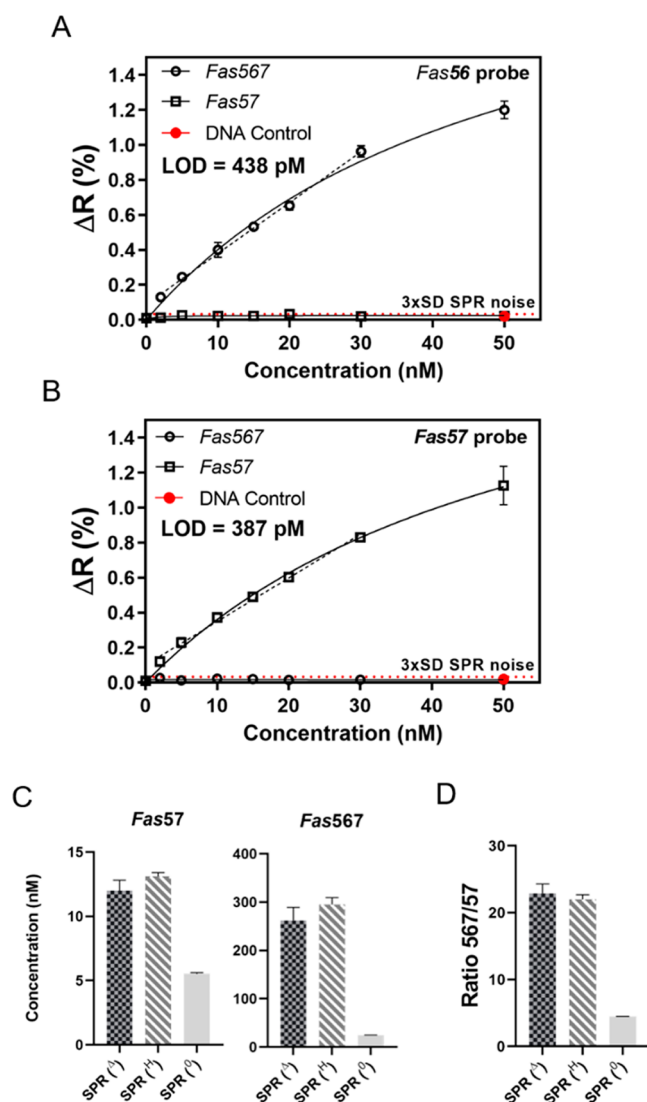


Figure 4. Quantitative analysis of mRNA *Fas* gene isoforms by SPR. SPR calibration plots for *Fas57* (A; $R^2 = 0.99$) and *Fas56* (B; $R^2 = 0.99$) probes using the optimized hybridization and detection conditions. Solid lines correspond to the nonlinear fit of the calibration curves. Red dashed lines correspond to 3xSD SPR noise. (C) Histograms comparing the analytical quantification of *Fas57* (left) and *Fas567* (right) isoforms. (D) Histogram comparing the 567/57 ratio after each condition tested by SPR. All data show mean $\pm$ SD of duplicate measurements. Panels A and B are adapted with permission from ref 14. Copyright 2016 Elsevier.

RNase H fragmentation, we applied the procedure to quantify *Fas*(567/57) isoform ratios in a highly accurate and selective manner (Figure 4D). Isoform ratio is a key feature to predict the aberrant regulation of AS events. Therefore, it is the most important parameter in order to test the efficiency of AS event analysis.

As can be appreciated in the figure, the ratio between isoforms is very different in the absence of fragmentation. According to SPR^H results, *Fas567* isoform had increased expression compared to *Fas57* isoform, leading to an isoform ratio (567/57) of 22.0 ± 0.7 . These results show a good correlation with the RT-qPCR (567/57, 17.9 ± 0.4). Also, in order to test the agreement between the two techniques, different RNA samples presenting different isoform ratios were analyzed and compared by SPR^H and RT-qPCR. To induce

Table 1. RT-qPCR and SPR Comparison of the Isoform Content Analysis from HeLa Cells' Endogenous Expression^a

<i>Fas</i> isoform	concentration (nM)		
	RT-qPCR ^b	SPR ^b	fragmentation
567	365 ± 17^a	24.6 ± 0.40	no fragmentation
		262 ± 27	alkaline hydrolysis
		295 ± 14	RNaseH cleavage
57	20.3 ± 0.4^a	5.5 ± 0.1	no fragmentation
		12.0 ± 0.8	alkaline hydrolysis
		13.1 ± 0.3	RNaseH cleavage

^aIsoform concentration in nM for SPR was calculated through the calibration curves obtained for each receptor according to their ΔR (%). ^bMean $\pm$ SD of endogenous concentration of HeLa cells' total RNA calculated from two different samples in each experiment.

different isoform ratios, HeLa cells were transfected with a minigene expressing *Fas* genomic sequences. Then, total RNA was purified either alone or co-transfected with a vector expressing the polypyrimidine-tract-binding protein (PTB). PTB induces skipping of *Fas* exon 6 acting through an exonic silencer sequence.²³ While expression of the minigene alone leads to preferential accumulation of exon 6—including transcripts, coexpression of *Fas567* minigene with PTB leads to higher accumulation of transcripts that skip exon 6.²² Each condition was analyzed by SPR^H and RT-qPCR and isoform ratios were calculated. All samples tested (6/6) were found within the limits of agreement (Figure S3). In fact, the agreement between SPR measurements and RT-PCR is higher for RNase H digestion compared to alkaline hydrolysis performed in our previous work by 20% (83% agreement), confirming that RNase H cleavage is a more accurate approach for the analysis of AS isoforms in an amplification-free and label-free detection system.

CONCLUSION

Alternative splicing can contribute to the development and progression of human malignancies, and it has been highlighted as a hallmark of cancer. The use of alternatively spliced isoforms as biomarkers will likely increase in the near future. Evaluating the impact of AS variants in cancer could be adopted as an integral part of clinical diagnosis. In addition, modulation of isoform ratios poses great promise in future precision medicine for personalized therapies. Despite the significant advances in optical biosensors and their potential application for rapid diagnostics tests, there is a lack of applications related to long RNA sequences, such as mRNA isoforms, due to the difficult access of these sequences to their specific DNA probes in the biosensor bioreceptor monolayer.

We show that this biosensing approach can be greatly enhanced by previous sample preprocessing steps to generate targets with the desired length that will not display the limitations associated with long RNAs.

We have adopted a methodology for the site-specific cleavage of mRNA sequences in order to obtain fragments with a precise sequence and length of interest. It has been employed for the fast and direct monitoring of alternative splicing events in real time using an SPR biosensor. Our methodology involves the design of specific DNA oligonucleotides that hybridize the flanking segments of the RNA fragment of interest, promoting their degradation by RNase H activity. This approach permits the generation of mRNA fragments

with the appropriate length with high selectivity, ensuring their accurate quantification via optical biosensors. The feasibility of the methodology was tested in complex RNA mixtures purified from HeLa cells expressing endogenous *Fas* gene mRNA transcripts.

The results strongly correlated with RT-qPCR data, with a detection accuracy up to 81%. In addition, they showed higher correlation between the RNase H cleavage and RT-qPCR compared with the conventional fragmentation process based on alkaline hydrolysis by 20%. These results confirm that site-specific RNA fragmentation reduces errors in isoform quantification, probably due to a lower loss of sample input than when applying random fragmentation. A slight underestimation of the isoform concentrations was observed in the SPR analysis, which could be probably due to limitations in the sensor sensitivity. One solution could be the implementation of this methodology in other label-free optical biosensors. For example, bimodal waveguide interferometers (BiMW) have been demonstrated to outperform SPR sensitivities up to 3 orders of magnitude in the detection of AS events.²⁵ Our methodology, along with the label-free detection and the minimized sample manipulation, provides an advance in the real-time monitoring of AS events. In our view, these results open the door for the routine use of alternatively spliced isoforms as biomarkers for diagnosis and patient follow-ups during therapy. In addition, it tackles the challenges encountered in the analysis of long RNA sequences with label-free optical biosensors, enabling one to exploit the potential of these biosensors for highly sensitive, selective, fast, and user-friendly genomic and epigenomic analyses. Moreover, it can be implemented in more integrated and sensitive platforms that allow for multiplexed analysis of multiple AS events. This work provides a significant step forward for the development of sensitive diagnostic tools based on nucleic acid biomarker detection for cancer research.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.analchem.9b03898](https://doi.org/10.1021/acs.analchem.9b03898).

Details of reagents and buffers, the SPR system employed, DNA/RNA sequences, and additional results for the validation of the methodology (PDF)

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Author Contributions

C.S.H., J.V., and L.M.L. devised the idea and designed the experiments. C.S.H. performed the SPR experiments. S.B. performed the RT-qPCR validation experiments and provided the HeLa cell samples. C.S.H., S.B., M.S., J.V., and L.M.L. analyzed the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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