MicroRNA In Vitro Diagnostics Using Immunoassay Analyzers

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BACKGROUND: The implementation of new biomarkers into clinical practice is one of the most important areas in medical research. Besides their clinical impact, novel in vitro diagnostic markers promise to have a substantial effect on healthcare costs. Although numerous publications report the discovery of biomarkers, only a fraction of those markers are routinely used. One key challenge is a measurement system that is compatible with clinical workflows.

METHODS: We designed a new immunoassay for microRNA (miRNA) quantification. The assay combines streptavidin-linked microparticles, a biotinylated catcher oligonucleotide complementary to a single miRNA species, and finally, a monoclonal antibody to DNA/RNA heterohybrids labeled with acridinium ester. Importantly, our assay runs on standard immunoassay analyzers. After a technical validation of the assay, we evaluated the clinical performance on 4 Alzheimer disease miRNAs.

RESULTS: Our assay has an analytical specificity of 99.4% and is at the same time sensitive (concentrations in the range of 1 pmol/L miRNA can be reliably profiled). Because the novel approach did not require amplification steps, we obtained high reproducibility for up to 40 biological replicates. Importantly, our assay prototype exhibited a time to result of <3 h. With human blood samples, the assay was able to measure 4 miRNAs that can detect Alzheimer disease with a diagnostic accuracy of 82% and showed a Pearson correlation >0.994 with the gold standard qRT-PCR.

CONCLUSIONS: Our miRNA immunoassay allowed the measurement of miRNA signatures with sufficient analytical sensitivity and high specificity on commonly available laboratory equipment.

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A substantial number of molecules, including DNA, RNA, microRNAs (miRNAs),[7] proteins, and methylated sites in the genome or metabolites, are reported as disease markers for various human pathologies, but only a small fraction will be translated to clinical routine use. One challenge is often poor diagnostic specificity or sensitivity, which can be overcome in some instances by combining biomarkers. The second major challenge is the reliable measurement of novel markers on platforms that are commonly used in clinical laboratories. Although current molecular methods used to measure DNA or miRNA biomarkers, such as quantitative RT-PCR (qRT-PCR) and next-generation sequencing (NGS), are available in selected clinical laboratories, they are rather expensive. Moreover, compatibility with clinical high-throughput workflows is challenging. The adaption of miRNA assays to platforms and technologies that would overcome those issues may foster their use.

Small noncoding RNAs such as miRNAs have important functions in nearly all cellular processes owing to their ability to regulate the expression of many protein-coding genes (1). Associations have been described for a large fraction of the >2000 known miRNA diseases, which have been collected in databases such as the Human miRNA and Diseases Database (2). Because of their ability to regulate target gene translation through either silencing or degradation of the target mRNA, miRNAs are involved in pathological processes such as cancer, neurological disorders, and heart disease (3–5). Furthermore, complex miRNA signatures have been increasingly recognized as stable and powerful biomarkers for hu-

man pathologies *(6–14)*, making them ideal bio-marker candidates. For the application of biomarkers in routine clinical settings, body fluids such as serum, urine, and cerebrospinal fluid represent preferable sources for biomarkers. Notably, blood cells contain a rich repertoire of disease-related markers.

Specific miRNA expression signatures for many human cancer and noncancer diseases have been identified *(6, 15–18)*. Following biomarker discovery studies with limited sample cohorts, the suitability of blood-based miRNA expression signatures as early disease detection biomarkers is increasingly being investigated in larger validation studies, either in comparison to or in combination with known serum protein biomarkers *(18)*. In particular, the first tissue-based tests for measurement of specific miRNA expression signatures are already commercially available on qRT-PCR platforms (Rosetta Genomics). However, such tissue-based qRT-PCR tests have important downsides. First, they require substantial hands-on time. Second, qRT-PCR platforms are not used in many clinical laboratories, and tests performed on these platforms are usually less integrated into workflows than immunoassays. The lesser penetration of qRT-PCR and other molecular methods in the clinical laboratory compared with immunoassays is also reflected by the fact that molecular methods other than blood bank tests made up only 5% of all in vitro diagnostic sales in 2011, compared with a 25% market share of immunoassays (excluding blood bank tests) *(19)*. Third, tissue-based miRNA expression signatures require invasive sampling and are therefore more complicated to implement than blood-based tests in routine diagnostic applications. Given these downsides to tissue-based tests, blood-based miRNA diagnostics by use of immunoassay represents an interesting opportunity to introduce miRNA testing into clinical laboratories.

To promote the translation of miRNA tests further into routine use, and to address the challenges mentioned above, we developed a new miRNA measurement principle on the basis of an immunoassay format. Immunoassay platforms are already routinely used in clinical laboratories worldwide, and many immunological tests such as cardiac troponin are carried out on these commercial systems. After successfully setting up the assay format, we evaluated the assay performance on an Alzheimer disease (AD) miRNA panel *(11)*.

Methods

SAMPLE COLLECTION

We carried out miRNA measurements with PAXgene Blood RNA tubes (Preanalytix, Becton Dickinson). These tubes can be used to collect 2.5 mL blood from donors, according to the manufacturer's recommendations. We collected blood samples from 40 healthy volunteers. The Institutional Ethics Committee of the Uni-

versity Erlangen-Nuremberg approved the study. All donors met the relevant guidelines *(20, 21)* and tested negative for human immunodeficiency virus, hepatitis B virus, and hepatitis C virus.

miRNA EXTRACTION

The pellets from 2.5 mL blood collected in PAXGene tubes were obtained by 10-min centrifugation at $4500g$ according to the manufacturer's instructions, and the supernatant was removed immediately. The pellets were then resuspended in 4 mL RNase-free water by vortex-mixing and collected by 10-min centrifugation at $4500g$. We then isolated total RNA including miRNA from the pellets with the miRNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. Isolated RNA was pooled, divided into aliquots, and stored at $-80\ ^\circ C$ until use.

miRNA qRT-PCR MEASUREMENT

We analyzed the miRNAs using stem-loop primers for qRT-PCR with TaqMan® probes on a Stratagene MX-3005P real-time cycler, essentially as previously described *(22)*. The master mix for real-time PCR, M-MuLV H Plus Reverse Transcriptase, dNTPs, and RNase inhibitor were obtained from Peqlab and stored at $-20\ ^\circ C$. The synthetic miRNAs were obtained from Biomers.net. The sequences of primers are described in Supplemental Table 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol61/issue4. We dissolved the synthetic miRNAs in Rnase-free water with 30 mU/μL RNase inhibitor to a concentration of 100 μmol/L and divided the miRNA solution to 5 μL/tube to be stored at $-80\ ^\circ C$ until use. The calibration curve was determined by qRT-PCR with the synthetic miRNA from 0.1 pmol/L to 1 nmol/L. The primer sets for qRT-PCR were obtained from Biomer.net. The sequences of primers for measuring the synthetic miRNAs are described in online Supplemental Table 2.

miRNA IMMUNOASSAY

The miRNA immunoassay presented in this study is a 2-step nucleic acid capture immunoassay adapted to the Advia Centaur® Immunoassay System (Siemens Healthcare Diagnostics). This immunoassay analyzer platform can be used to measure protein and small molecule analytes by respective assays with acridinium ester technology *(23)*. The components of our assay prototype consisted of the solid phase (containing streptavidin-linked microparticles), a biotinylated catcher oligonucleotide complementary to a single miRNA species (the biotinylated catchers are described in online Supplemental Table 3), and finally a monoclonal antibody to DNA/RNA heterohybrids *(24)* labeled with acridinium ester. The antibody, which was developed in the 1980s, specifically

Fig. 1. The 4 fully automated main steps carried out on the immunoassay analyzer. AE, acridinium ester.
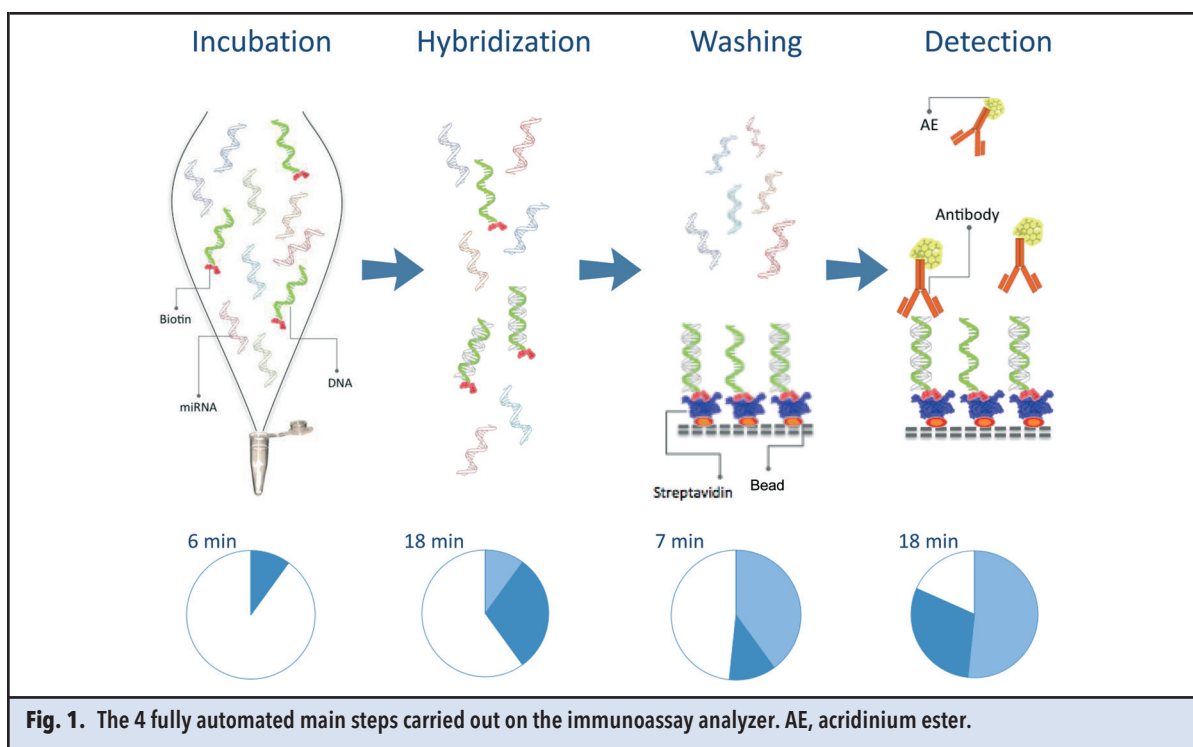
binds to DNA/RNA hybrids without any obvious bias toward a specific sequence *(24, 25)*.

In the assay, the purified miRNA from a blood sample is first hybridized to the biotinylated catcher oligonucleotide, generating perfectly matched DNA/RNA heterohybrids. In a second step, these biotinylated DNA/RNA heterohybrids are then incubated with and bound to the streptavidin-labeled solid phase. In the next step, the acridinium ester–labeled antibody to DNA/RNA heterohybrids is added. This antibody can bind only to perfectly matched heterohybrids and does not bind to mismatched heterohybrids. The amount of antibody bound will therefore be proportional to the amount of perfectly matched heterohybrids present in the reaction, which again is proportional to the amount of that specific miRNA species present in the blood sample. Chemiluminescence is then triggered by addition of acid and base reagent *(26)*.

The following 9 automated steps were carried out with the Advia Centaur system. (*a*) Pipetting 75 μL samples in a cuvette. (*b*) Pipetting 75 μL reagent (20 mmol/L sodium phosphate, pH 7.2, 300 mmol/L NaCl, 0.1% Triton X-100, 0.5% bovine serum albumin, 0.02% sodium azide) containing biotinylated oligonucleotides (10 nmol/L) and incubating for 6 min at 37 °C. (*c*) Pipetting 150 μL solid phase and incubating for 18 min at 37 °C. (*d*) Separating solid phase from the mixture and removing the liquid phase. (*e*) Washing the cuvette with washing solution 1 and incubating for 6.75 min at 37 °C. (*f*)

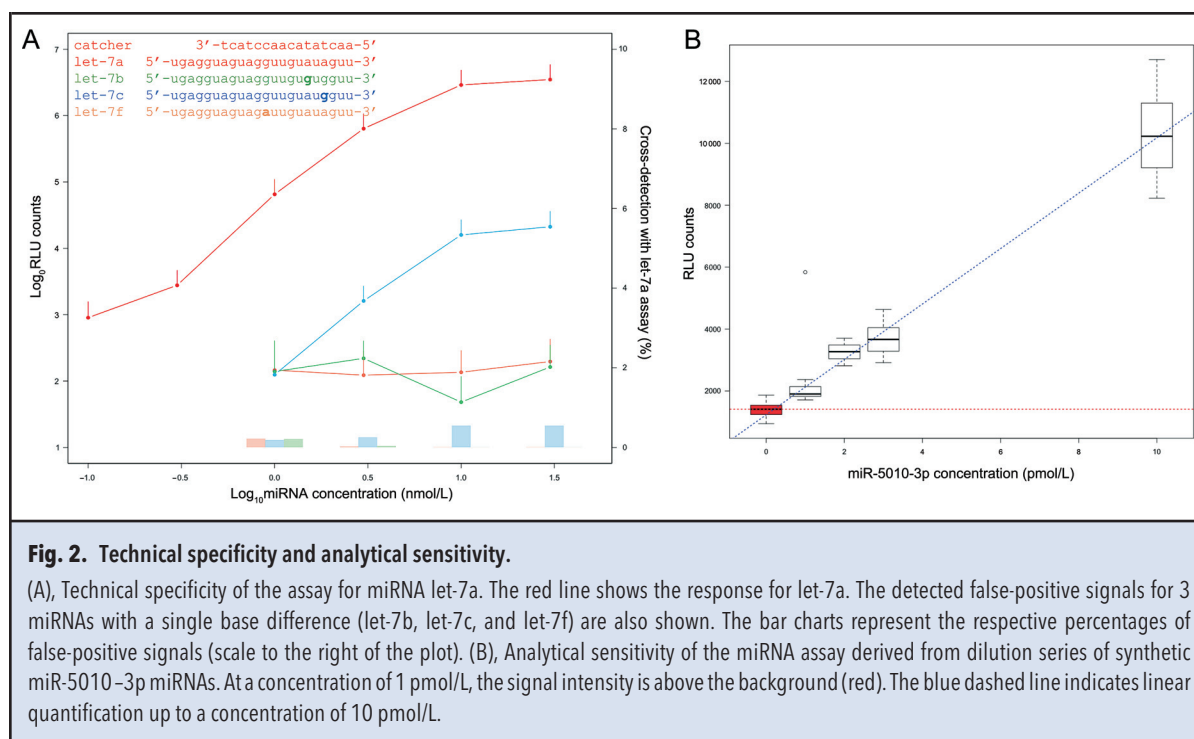Pipetting 95 μL antibody reagent and incubating for 18 min at 37 °C. (*g*) Separating solid phase from the mixture and removing the liquid phase. (*h*) Washing the cuvette with wash solution 1. (*i*) Pipetting 300 μL reagent A (acid) and 300 μL reagent B (base) to generate a chemiluminescence signal. The workflow is presented schematically in Fig. 1. The concepts and information presented in this article represent research and are not commercially available.

CALIBRATION CURVES AND CALCULATION OF CONCENTRATIONS

We measured the calibration curve with synthetic miRNAs from a concentration of 1 pmol/L to 1 nmol/L on an the Advia Centaur system, carrying out a second-degree polynomial analysis to determine the equation of the relationship between relative light unit (RLU) counts and miRNA concentration. We then measured the biological samples on the same Advia Centaur system. The concentration of a certain miRNA of biological samples was calculated from the RLU counts on the basis of the equation of the calibration curve.

STATISTICS

We carried out all statistical calculations with the freely available R programming language (version 3.0.2). Hypothesis tests were carried out, if not mentioned explicitly, as 2-tailed unpaired tests. In cases where

Fig. 2. Technical specificity and analytical sensitivity.

(A), Technical specificity of the assay for miRNA let-7a. The red line shows the response for let-7a. The detected false-positive signals for 3 miRNAs with a single base difference (let-7b, let-7c, and let-7f) are also shown. The bar charts represent the respective percentages of false-positive signals (scale to the right of the plot). (B), Analytical sensitivity of the miRNA assay derived from dilution series of synthetic miR-5010–3p miRNAs. At a concentration of 1 pmol/L, the signal intensity is above the background (red). The blue dashed line indicates linear quantification up to a concentration of 10 pmol/L.

the parametric *t*-test was applied (evaluating the null hypothesis that the means of 2 normally distributed populations are equal), approximate normal distribution was verified by Shapiro–Wilk test (evaluating the null hypothesis that measurements come from a normally distributed population).

To show the distribution of miRNA measurements, we generated box-whisker plots, and to provide a per-measurement representation, we provided bee swarm plots as included in the beeswarm R package.

Results

miRNA IMMUNOASSAY

As shown in Fig. 1, our assay works as follows. Total RNA isolated from PAXgene blood is used for the hybridization assay. The total RNA is hybridized with a biotinylated DNA catcher and forms a DNA-miRNA duplex. Streptavidin-coupled magnetic beads are added to the solution, and the DNA catcher binds to the beads through biotin–streptavidin interaction. Unbound miRNAs and other RNAs are washed away so that just the DNA-miRNA duplex remains. A monoclonal antibody specific to DNA-miRNA hybrids labeled with acridinium ester is added to the solution, binding to the DNA-miRNA hybrids. A light signal proportional to the number of DNA-miRNA hybrids is monitored and reported. Altogether, the entire experimental setup, including RNA purification and miRNA profiling, requires <3 h.

SPECIFICITY OF THE IMMUNOASSAY

To evaluate the analytical specificity of the immunoassay, we distinguished members of the let-7 family that differed by just a single base. The miRNA to be quantified was selected to be hsa-let-7a. Synthetic molecules of this miRNA were added in 6 concentrations from 0.1 to 30 nmol/L to the respective catcher, leading to background-corrected results between 1201 counts (0.1 nmol/L) and 4.6 million counts (30 nmol/L). Next, we carried out the same measurement with the 3 miRNAs hsa-let-7b, hsa-let-7c, and hsa-let-7f. For the lowest concentrations, signals were beyond the detection limit; for the higher concentrations, we measured up to 21 222 counts (hsa-let-7c, 30 nmol/L). The results are shown in Fig. 2A. In this figure, the lines represent log10 values of raw counts and the bars correspond to the percentage of crosstalk (false-positive light signals) with hsa-let-7a. Whereas the signals for hsa-let-7b and hsa-let-7f remained in the range of the background even for the highest concentrations, for hsa-let-7c, low signals at very high concentrations could be measured. The crosstalk never exceeded 0.6%, demonstrating a specificity of 99.4% for the miRNA immunoassay.

SENSITIVITY AND LOWER LIMIT OF DETECTION OF THE IMMUNOASSAY

Next, we systematically evaluated the limit of detection of the immunoassay. We selected 1 of the miRNAs included in our AD panel *(11)*, namely hsa-miR-5010–3p.

With a catcher probe, we performed 20 replicates for different concentrations between 1 and 10 pmol/L. Additionally, we performed 20 replicates of blank controls representing the background signal. As shown in Fig. 2B, we were able to measure signals substantially exceeding the background noise even for miRNAs at concentrations of 1 pmol/L. Whereas the blank controls (shown in red) had a median intensity of 1411 RLU counts (SD 211) (horizontal red dashed line), 1 pmol/L hsa-miR-5010−3p resulted in 1904 RLU counts (SD 863) ($P =$ 0.001, 2-tailed unpaired t-test). For 2 pmol/L hsa-miR-5010−3p, 3270 RLU counts (SD 314) were reported; for 3 pmol/L 3666 RLU (SD 493), and for 10 pmol/L 10 226 RLU (SD 1208). Altogether, the concentration of hsa-miR-5010−3p correlated significantly with the counts measured by our assay (Pearson correlation 0.998, $P = 0.0001$). In all measurements carried out with our immunoassay, we recorded just a single outlier (Fig. 2B, concentration of 1 pmol/L, Grubbs test $P < 0.001$).

ABILITY TO MEASURE MODERATE CHANGES IN miRNA ABUNDANCE

The variation in blood-based miRNA concentrations in diseases is frequently limited. We have found that variations in circulating miRNA patterns are usually moderate (2-fold expression changes). We thus explored the potential of the miRNA immunoassay to measure changes in concentrations typical for miRNAs found in previous studies. Specifically, we carried out 2 experiments on different concentration scales. First, we started at a concentration of 3 pmol/L and increased the concentration by 0.3 pmol/L in each step until we reached an absolute concentration of 4.8 pmol/L after 7 dilution steps. The R^2 between the concentration and RLU counts reached 0.91 (see online Supplemental Fig. 1). For all measured data points, we found deviation between the expected measurement given the linear regression line and the actual measurement to be <5%. For the 3.6 pmol/L data point, a slightly higher difference was observed (expected according to regression line, 3760 RLU; actually measured, 3995 RLU). Nevertheless, our assay was able to measure even 10% changes reliably in the lower concentration range. To demonstrate that this could also be achieved for the higher-abundance miRNAs, we performed similar experiments, increasing abundance by an order of magnitude. Specifically, we started at 30 pmol/L concentration and increased it by 3 pmol/L up to 60 pmol/L in the 11th step. The R^2 value was even higher and reached 0.98 (Fig. 3). These results demonstrate the linearity of measurements for concentrations of >3 orders of magnitude and also provide evidence that even small changes in miRNA abundance can be quantified by our prototype assay.
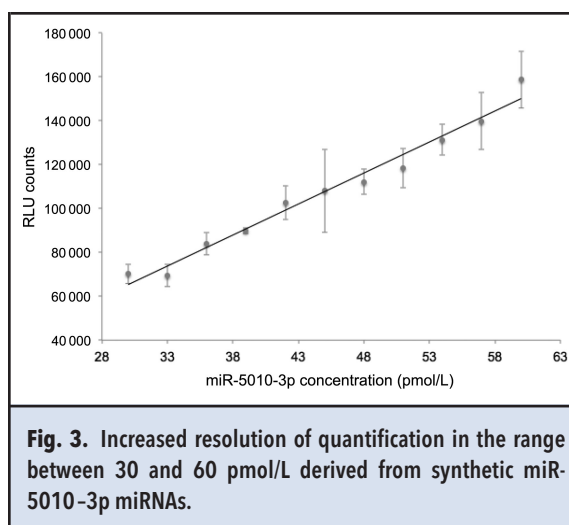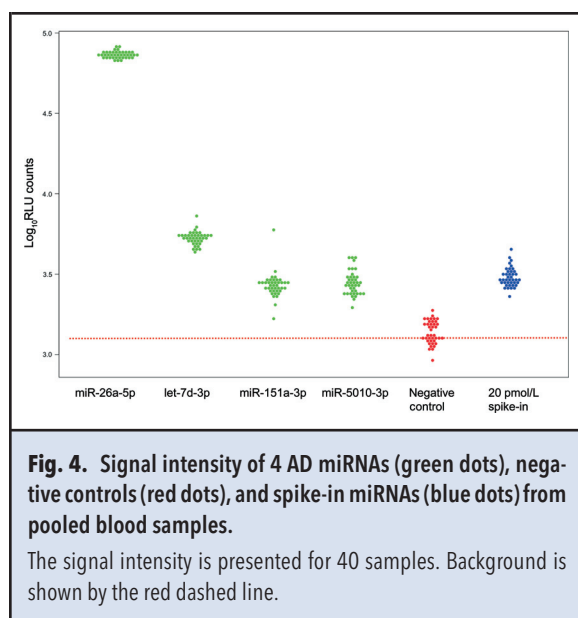

Fig. 3. Increased resolution of quantification in the range between 30 and 60 pmol/L derived from synthetic miR-5010−3p miRNAs.

MULTIPLEX IMMUNOASSAY

Originally, the assay format was designed as single-plex assay. Although this setup does not prevent routine application, an automated measurement of several miRNAs from the same sample would be beneficial. Therefore, we explored the potential of serial multiplexing. We mixed 8 synthetic miRNAs (miR-5010−3p, miR-151a-3p, let-7d-3p, miR-107, miR-26b-5p, miR-103a, miR-26a-5p, and let-7f-5p) in increasing concentrations. Starting from the miRNA with lowest concentration, we performed measurement of the single-plex assay. The supernatant, however, was not discharged but reentered the measurement cycle with the next miRNA. The same experiments were also done for aliquots of the single-plex assay. The results of single-plex vs multiplex are shown in online Supplemental Fig. 2. Generally, we observed a good correlation; however, those miRNAs with just a single mismatch, such as miR-26a and miR-26b, showed slight variations. Additionally, the experiments revealed a lower performance for let-7f-5p. These preliminary results demonstrate that 8-plex measurements are possible but that increasing the degree of multiplexing decreases the analytical specificity and sensitivity of the assay.

### TRANSFER TO BIOLOGICAL MEASUREMENTS

After exploring the limit of detection, analytical sensitivity, and specificity of our miRNA immunoassay with synthetic miRNAs, we tested 4 miRNAs of our AD miRNA panel, hsa-miR-5010−3p, hsa-miR-26a-5p, hsa-miR-151a-3p, and hsa-let-7d-3p, with 40 replicates of biological samples to evaluate their potential for clinical application beyond the measurement of the synthetic miRNAs presented above. The miRNAs were selected so that most informative markers of the signature were combined while ensuring that lower-abundance markers were also included. Thus, we purposely selected the three -3p

Fig. 4. Signal intensity of 4 AD miRNAs (green dots), negative controls (red dots), and spike-in miRNAs (blue dots) from pooled blood samples.

The signal intensity is presented for 40 samples. Background is shown by the red dashed line.

Fig. 5. Correlation with qRT-PCR for 4 Alzheimer miRNAs in blood samples.

mature and the higher-abundance -5p mature form of miR-26a. We generated calibration curves for all 4 miRNAs, as described in Methods, to enable quantification with our novel assay.

These previously published miRNAs allow for detecting patients with AD with diagnostic accuracy, specificity, and sensitivity of 82%, 85%, and 80%, respectively (area under the curve 0.91) *(11)*. On the immunoassay analyzer system, we measured 40 replicates for the 4 miRNAs and controlled the process with 20 pmol/L spike-in controls (Fig. 4). The measurements were carried out with aliquots of the pooled samples by use of the single-plex assay. Again, even for the lowest-abundance miRNAs hsa-miR-5010–3p and hsa-miR-151a-3p, stable signals above the background were observed. For the background, we calculated 1391 RLU (SD 222). For miR-5010–3p, RLU counts were already 2835 (SD 516) (2-tailed unpaired *t*-test between background and miR-5010–3p, $P < 10^{-20}$). For miR-151a-3p, 2738 RLU (SD 604) was found, with 2-tailed unpaired *t*-test significance of $<10^{-20}$, indicating that the difference between this miRNA and the background was highly significant.

In all 240 measurements, 2 outliers (0.8%) were observed. For miR-26a-5p, the mean concentration was 561.3 pmol/L (SD 19.9), let-7d-3p had a mean concentration of 38.3 pmol/L (SD 9), miR-151a-3p had a mean concentration of 5 pmol/L (SD 0.8), and miR-5010–3p had a mean concentration of 3.5 pmol/L (SD 0.5). Given these mean values and SDs, we calculated CV values of 0.04 (miR-26a-5p), 0.24 (let-7d-3p), 0.16 (miR-151a-3p), and 0.13 (miR-5010–3p). Although the CV values were generally low (miR-26a-5p showed a CV of 0.04),
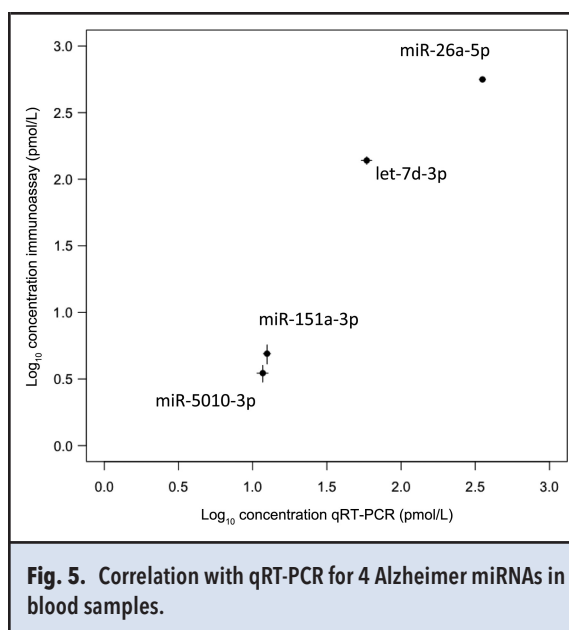
let-7d-3p showed an increased CV. The CV values of the blood samples were in the same range as the technical evaluation CV values.

In developing a new test, it is important to benchmark it against the gold standard, in this case qRT-PCR. We quantified the same samples by qRT-PCR as described in Methods. We found a high correlation between qRT-PCR and the Advia Centaur system (Pearson correlation >0.994, $P = 0.006$) (Fig. 5). For hsa-miR-5010–3p, hsa-miR-151a-3p, let-7d, and hsa-miR-26a-5p, the concentrations on the immunoanalyzer system were 3.5, 5, 38.3, and 561.3 pmol/L, respectively, and on qRT-PCR the concentrations were 11.7, 12.5, 58.7, and 335.6 pmol/L. Although these results indicated differences between the technologies, the results showed a general concordance.

Discussion

Our novel method involves hybridization of miRNA from a patient sample to complementary biotinylated DNA oligonucleotides, followed by detection of the DNA-miRNA hybrids by a monoclonal antibody that specifically binds to DNA-miRNA hybrids. Using this setup, we were able to obtain a prototype assay that can measure miRNAs from biological samples without any preamplification step. Our assay has an analytical specificity of 99.4%, a limit of detection in the range of 1 pmol/L, and a time to result of <3 h, including RNA purification and miRNA profiling. We obtained stable results over a dynamic range of 4 orders of magnitude. Additionally, the amplification-free detection allows for less biased miRNA measurements. This advantage, how-

ever, results in a current lower limit of detection of 1 pmol/L. Although many blood-based miRNAs can be profiled with the proposed assay, the sensitivity has to be further improved to measure other samples with lower miRNA concentrations, such as serum. Another drawback of our assay is the currently limited multiplexing capability. We demonstrate first results on a multiplexing concept here, but more work is required to obtain the same specificity as for the single-plex assay. Another point that has to be taken into account is that the used antibody can react with different DNA-RNA hybrids with different affinity *(24)*, influencing the sensitivity of the assay for this miRNA and requiring additional calibration.

In a test on clinical samples, we found an outstanding correlation with qRT-PCR data (Pearson correlation >0.994), which as of now represents the gold standard for miRNA expression analysis. Our assay is currently a research assay that aims to lay the basis for further development, with the challenging goal to promote the usage of miRNAs as clinical IVD tests.

Besides its application to measure miRNAs, our assay design bears the potential to be extended to other nucleic acid test formats, in particular to those that still require preamplification of the target nucleic acid. For example, the method described by Yehle et al. *(25)*, which allows bacterial typing by hybridization of 16s rRNA to strain-specific oligonucleotides, could be adapted to our automated assay format. Moreover, high-abundance mRNAs or rRNAs could be quantified by hybridization to complementary DNA oligonucleotides in the assay format described in this article.

Our miRNA immunoassay has a low time-to-result, comparable to that of qRT PCR, and is still faster than NGS, for which typically at least 1 day (and frequently several days) is required. At the same time, our assay is inexpensive, with costs in the same range as established and marketed immunoassays, which are below those of qRT-PCR or even NGS, and microarrays, which are still in the range of several hundred dollars. In turn, NGS has a much higher multiplexing capability and allows for integrative screening of all miRNAs, even those that are not annotated in databases. NGS is thus a perfect biomarker discovery tool, whereas our assay is tailored for much higher throughput in terms of samples at a decreased degree of multiplexing. Among the most important points with respect to our miRNA immunoassay is that the required hardware is installed in many central laboratories of hospitals worldwide.

In summary, we developed a method that has the potential to change the current practice to measure miRNAs, by providing a means to analyze miRNAs on commonly used immunoassay analyzers, thus providing substantial advantages over existing methodologies.

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