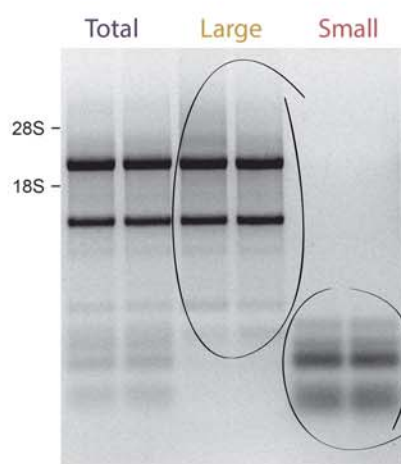


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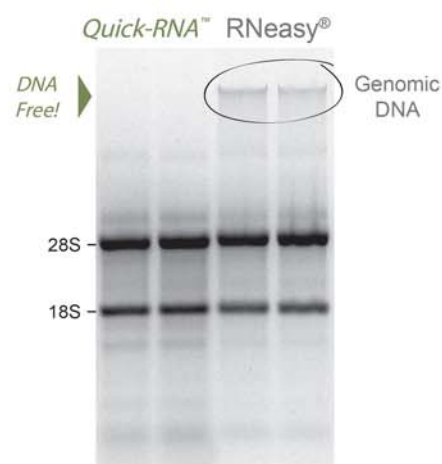
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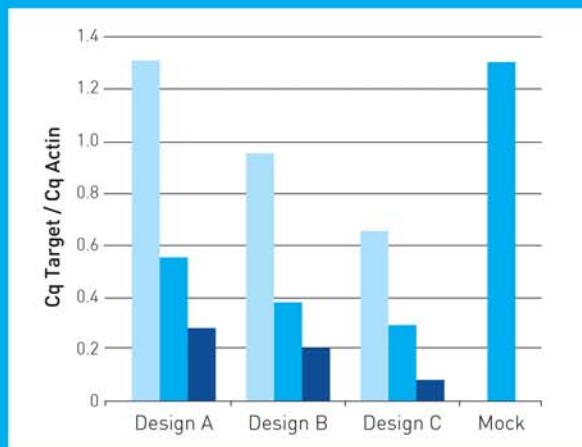
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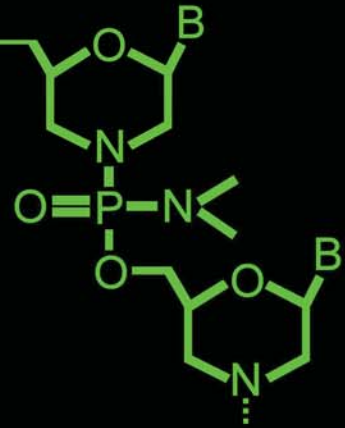
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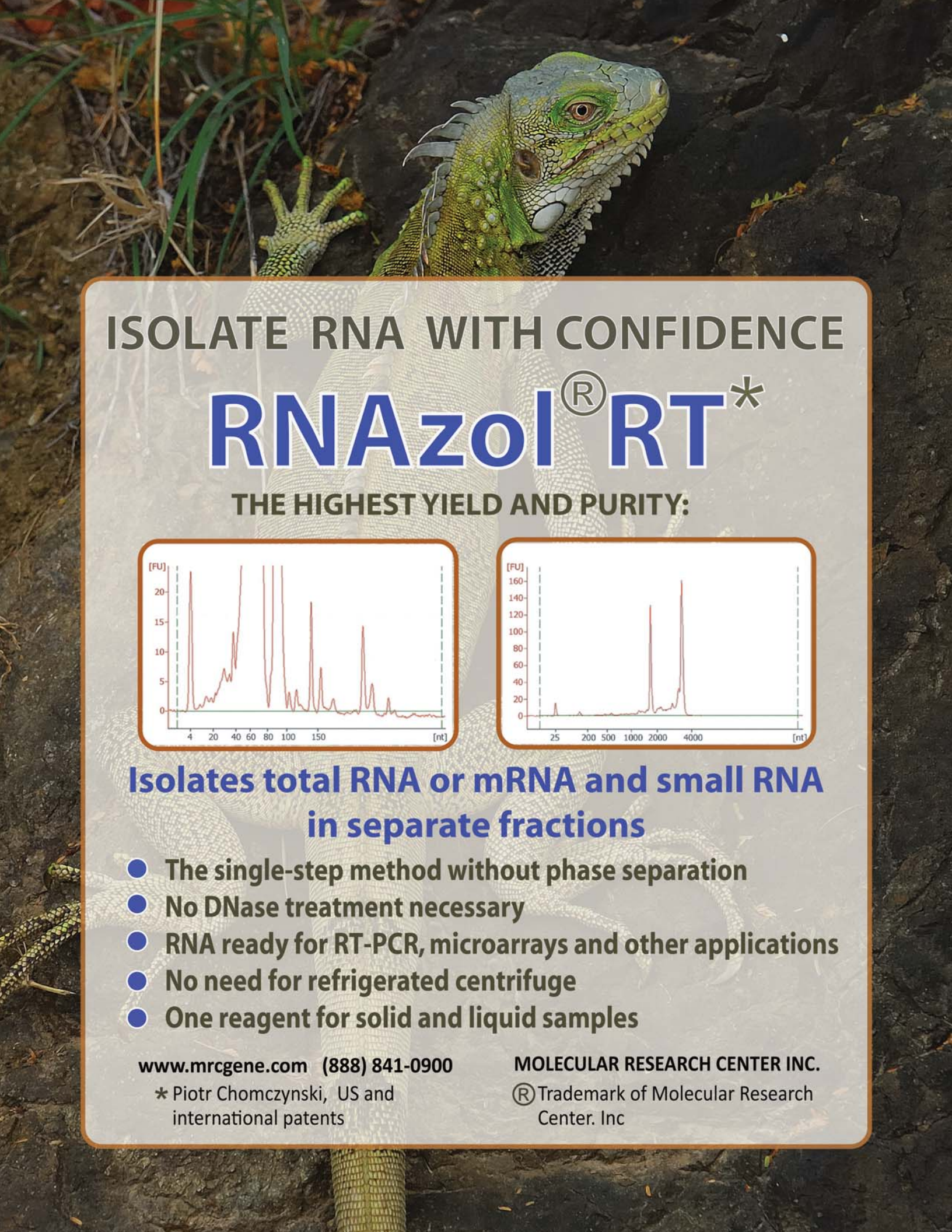


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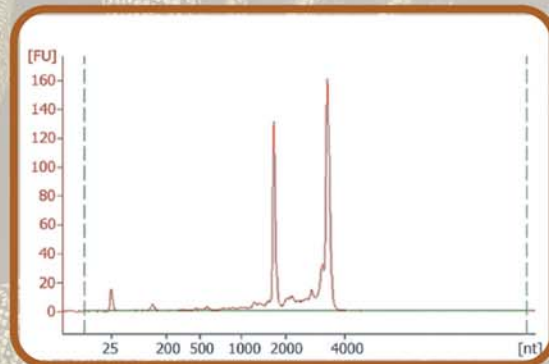
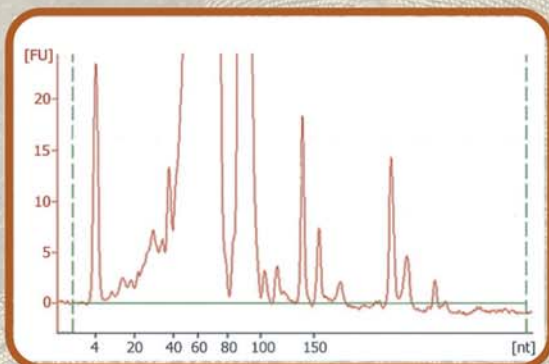
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Profiling microRNA levels in breast cancer using SmartRNAplex™ Assay, a unique microRNA detection platform

Introduction

MicroRNAs (miRNAs) are directly involved in the development of many cancers, including lung, breast, liver, colon, and leukemia. MiRNAs may act as either oncogenes or tumor suppressors by regulating the expression of genes that control cellular proliferation, differentiation, apoptosis or other pathways involved in tumorigenesis¹.

However, with nearly 5,000 human miRNAs identified, and with over 50% of human miRNA genes located in cancer-linked loci^{2,3}, it can be challenging to determine which miRNAs are important to the phenotype of a particular cancer cell or are predictive of tumor progression. To efficiently identify miRNA biomarkers, therefore, it is important to measure the relative levels of multiple miRNAs in both normal and tumor tissues through miRNA profiling⁴.

Current methods for miRNA profiling include quantitative RT-PCR (qRT-PCR), microarray analysis and RNA sequencing, and each method has its particular advantages and challenges. Here, we use a novel miRNA profiling platform to simultaneously quantify multiple miRNAs and correlate their expression levels with that of an established tumor marker.

The SmartRNAplex™ miRNA Profiling Assay consists of bio-inert, hydrogel particles that can be customized to hybridize to any miRNA from any species as identified in miRbase⁵. By using a standard flow cytometer for data acquisition, up to 68 unique miRNA targets can be simultaneously evaluated in each well of a 96 well plate, providing a very comprehensive and quantitative miRNA profile without requiring any specialized expertise. The details of the technology are outlined in Figure 1.

This study describes how the SmartRNAplex™ Assay was used for multiplexed quantitation of miRNA expression in HER-2-positive tumor breast tissues and adjacent normal (HER-2-negative) breast tissue samples derived from the same patient. Additionally, multiplexed miRNA assessment was conducted on well-known mammary gland-derived cell lines, MCF-10A (immortalized but not transformed) and BT-474 (invasive breast carcinoma). We cross-validated these data using qRT-PCR to confirm that the SmartRNAplex™ Assay could be used as a complementary tool for miRNA assessment.

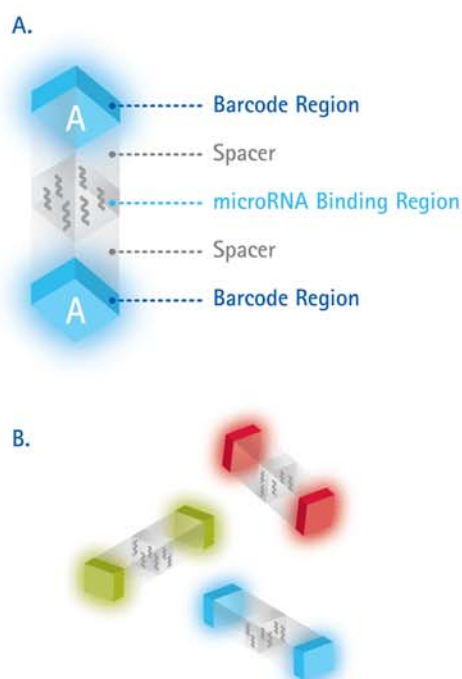


Figure 1. SmartRNAplex™ Assay: Firefly™ hydrogel particle anatomy. The Firefly™ hydrogel particle can be broken down into five separate sections as shown in (A). The center contains the target-specific miRNA binding region, where the target miRNA is recognized by a complementary sequence. The regions on the ends (labeled A in blue) contain the barcode that identifies which particle specifically recognizes which miRNA target. Spacers separate each region, so that when the particle flows through a flow cytometer, three separate events are recorded. Adding a pooled collection of various encoded hydrogel particles to the sample being analyzed enables multiplexed miRNA quantitation (B).

For this study, the SmartRNAplex™ Assay was performed using a customized pool of hydrogel particles recognizing specific miRNAs associated with breast cancer. A variety of miRNA targets were evaluated for this study (human miR-21, miR-22, miR-210, miR-10b, and miR-125b). Each particle was functionalized to hybridize to the targets described and pooled together to enable multiplexed miRNA profiling of each sample.

Results

We obtained formalin-fixed, paraffin-embedded breast cancer and adjacent normal breast tissue samples from the same patient. Immunohistochemistry indicated that the breast tumor tissue sample, but not the adjacent normal tissue, was HER2-positive (data not shown), indicating a strong likelihood that the tumor sample represented an aggressive, metastatic form of breast cancer.

To determine which miRNAs might be functionally related to the tumor's malignant phenotype, we designed a SmartRNAplex™ Assay panel to quantify several miRNA targets previously reported to play either an oncogenic or tumor-suppressing role. The experimental protocol for quantifying miRNAs using the SmartRNAplex™ Assay is outlined in Figure 2.

SmartRNAplex™ analysis of FFPE breast tissue samples

Total RNA isolated from tissue samples was incubated with the pool of Firefly™ particles from the SmartRNAplex™ Assay. Following hybridization and labeling steps (Figure 2), fluorescent-labeled particles were detected using the guava easyCyte™ 8HT flow cytometer. The Firefly™ Analysis Workbench software provided statistical data based on the fluorescence signal detected for each miRNA target within each sample. Fluorescence signal intensity positively correlates to levels of miRNA target. The fluorescence signal detected from tumor breast samples are divided by the signal obtained from normal breast tissues to calculate fold change.

Compared to fluorescent signals from normal tissue samples, we observed increased signals from tumor samples corresponding to key oncogenic miRs 21, 22, 10b, and 210 (Figure 3). MiR-21, known as a true "oncomiR," has been shown to be an indicator of an aggressive phenotype in



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breast cancer, with expression levels higher in cancerous tissue than in normal tissue⁵. The high level of miR-21 expression (showing over 250-fold upregulation in tumor vs. normal when analyzed using the SmartRNAplex™ Assay) has been associated with large tumor size, advanced stage, high grade, ER negativity and HER2 positivity⁵. Similarly, miR-22 is part of the miR-17-92 cluster of targets, which regulates many processes during tumor formation⁶. In the SmartRNAplex™ Assay, tumor samples contained approximately 25-fold more miR-22 than did normal samples.

Other miRNAs for which we observed upregulation have also been previously reported to have tumorigenic roles⁷⁻⁸, confirming that our multiplexed assay yielded physiologically relevant data. Our study also identified miR-125b as a tumor suppressor (Figure 3), consistent with previous work showing that the miR-125b gene coded for miRNAs that acted as tumor suppressor genes in HER-2-positive breast cancer⁹.

Fold changes as determined using the SmartRNAplex™ assay were compared to qRT-PCR results (Figure 3B and data not shown). Where significant fold changes were determined, there was very good correlation between the two assay methods.

Conclusions

Given the inpatient and intratumor heterogeneity displayed by most cancers, it is increasingly crucial to identify multiple biomarkers to increase the sensitivity and specificity with which tumor progression is quantified. For example, HER-2-positive breast cancers are increasingly being seen as a heterogeneous class of tumors, with the source of heterogeneity possibly being differential expression of hormone receptors¹⁰. Multiplexed biomarker analysis can not only help stratify heterogeneous populations, but it also helps conserve patient samples, which can be difficult to obtain and small in volume.

We have demonstrated a novel method for miRNA detection that provides a way to obtain relative quantification of multiple miRNA targets all within a single sample. Up to 68 unique miRNAs can be detected simultaneously, enabling efficient identification of miRNAs for further investigation as potential cancer biomarkers. We used this technology to simultaneously assess the levels of five miRNAs in HER-2-positive breast cancer samples along with adjacent normal (HER-2-negative) breast tissue. Qualifying additional biomarkers of breast cancers with poor prognosis may help guide research and, ultimately, therapeutic strategy.

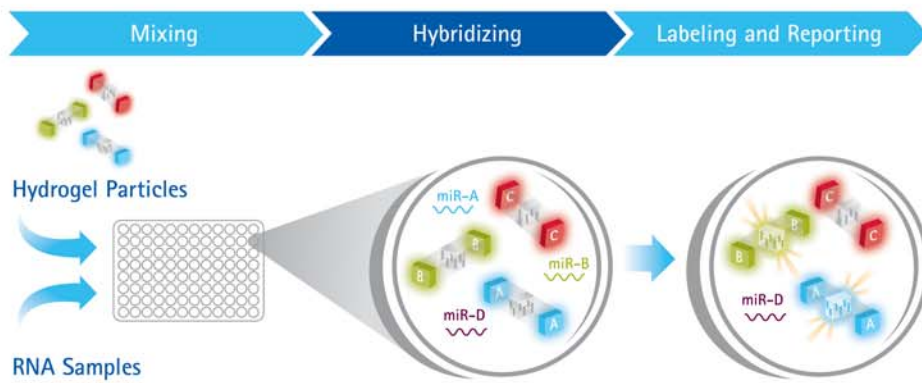
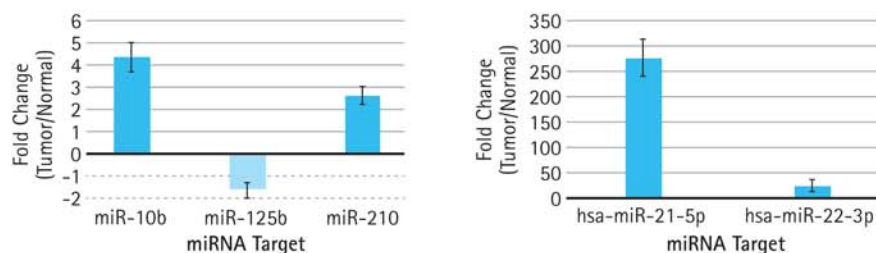


Figure 2. Workflow for SmartRNAplex™ detection of miRNAs in RNA samples. Uniquely encoded Firefly™ hydrogel particles (containing specific miRNA recognition sequences and barcodes for identification) are mixed with samples in the included MultiScreen® 96-well filter plate and incubated to allow hybridization. Each successfully hybridized particle is then ligated to a fluorescent label, resulting in fluorescence as detected by a flow cytometer. Non-complementary strands or inadequate hybridization will not allow labeling, resulting in no fluorescence.

A.



B.

	miR-10b	miR-125b	miR-210	miR-21	miR-22
Direction of regulation in tumor vs. normal (by SmartRNAplex™ assay)	UP	DOWN	UP	UP	UP
Direction of regulation in tumor vs. normal (by qRT-PCR)	UP	DOWN	UP	UP	UP

Figure 3. Multiplexed miRNA profiling in breast tissue samples using SmartRNAplex™ assays. Breast tissue samples (tumor vs. adjacent normal) were assessed by SmartRNAplex™ profiling and the resulting fluorescence signal (corresponding to expression level) was determined for each miRNA. Fold change between tumor and normal samples were calculated and plotted (A). Results were correlated with qRT-PCR results (B).

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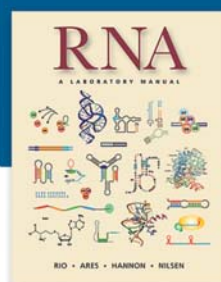
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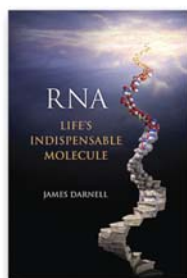
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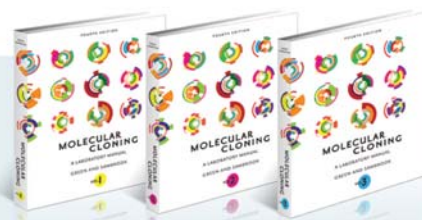
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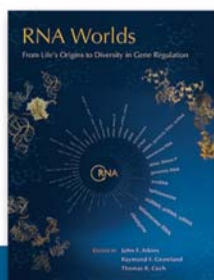
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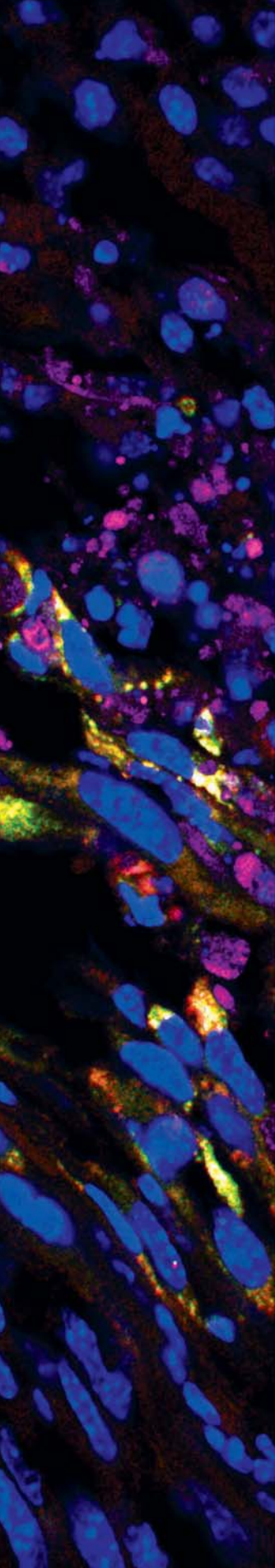
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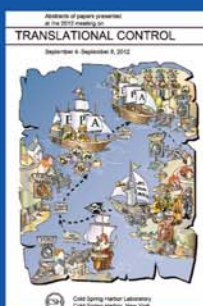
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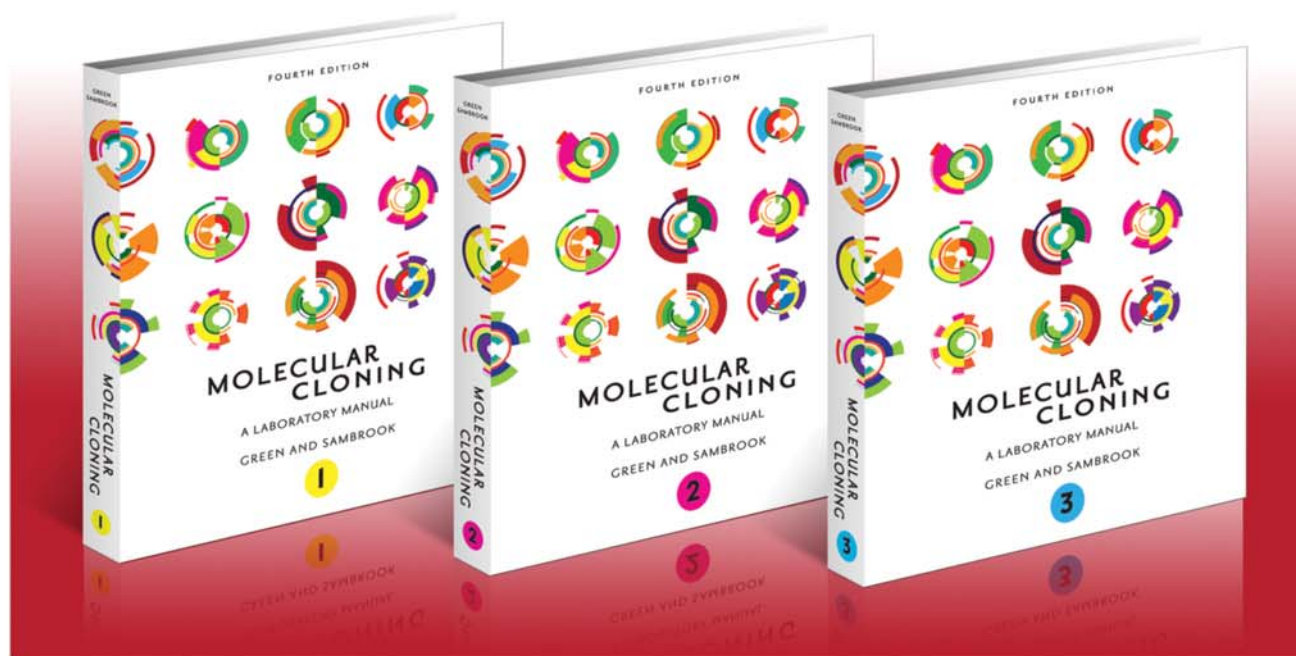
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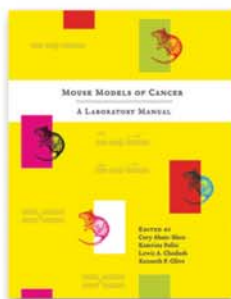
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