Over the past 10 to 15 years, genomic research has identified unique patterns of changes in gene expression that are characteristic of specific diseases, disease prognosis and therapeutic response. These patterns are called gene expression "signatures" and they are an important and necessary component of personalized medicine. A large number of gene signature tests are now in clinical development,¹ and a few examples have already been cleared by the FDA for in vitro diagnostic (IVD) use.²

Despite the significant potential for healthcare improvement and cost savings, the broad scale use of gene expression signatures in the clinic has been hampered by several factors including (1) the high technical complexity of the tests (2) the high cost of materials and labor to perform them and (3) the limited availability of validated tests. Novel technology developed at DxTerity will speed the development of new tests as well as lower the cost and broaden the availability of existing tests through the introduction of the DxDirect™ assay method and DxCollect™ sample collection systems.

DxDirect enables the rapid, simple and inexpensive analysis of gene signatures and is compatible with a wide variety of existing genomic analysis systems, including the Luminex xMap, Capillary Electrophoresis (CE) systems such as the Caliper LabChip Gx/Dx and ABI 3500, as well as planar microarray systems. In addition to making it faster, easier and more cost-effective to perform genomic testing in the clinic and at the point of care (POC), DxDirect simplifies the development and validation of new gene signatures by substantially reducing the development cost and improving the reproducibility of genomic analysis.

DxCollect provides a simple and reliable method for ensuring the integrity of blood samples during transport, and eliminates the need for sample preparation when used in conjunction with DxDirect. DxCollect consists of standard 1.5 mL vacuum draw tubes designed to interface seamlessly with standard phlebotomy methods, as well as a capillary collection kit that takes advantage of the excellent sensitivity of the DxDirect system, which only requires 25 µL of whole blood.

DxDirect™ Assay Principle

Sample collection, preservation and preparation methodologies are major barriers in the routine use of RNA-based genomic signatures in the clinic. Unlike DNA, which is very stable, RNA degrades quickly and care must be taken to stabilize RNA for transport and storage. RNA is usually stabilized by freezing the sample at -70°C or by addition of a harsh chemical denaturant that inactivates the RNases. Unfortunately, freezing requires complicated shipping conditions, and the chemical denaturants are potent inhibitors of the downstream amplification and assay methods. Furthermore, the enzymes that are routinely used for target amplification in molecular diagnostic testing are unable to directly amplify RNA, and the Reverse Transcriptase enzyme that is used to convert RNA to cDNA usually requires an input of purified RNA. The processing steps associated with isolating and purifying RNA are very costly and time consuming.

DxDirect overcomes these limitations by changing the chemistry associated with genomic testing. DxDirect makes a seemingly minor change to the testing process by replacing the traditional enzymatic ligation step of the MLPA³-⁴ (Multiplex Ligation Dependent Probe Amplification) and related LDA⁵ (Ligation Dependent Amplification) methods with a non-enzymatic chemical ligation reaction (Figure 1). This change simplifies the testing process in profound ways. Unlike its enzymatic equivalent, the chemical ligation reaction is able to directly target the RNA, eliminating the need for RNA isolation and cDNA production. Furthermore, the chemical ligation reaction is extremely tolerant of sample impurities and works just as well in the presence of RNA stabilization reagents as with highly purified RNA. As a result, it can be used to directly analyze stabilized RNA in whole blood and tissue, hence the name DxDirect.

The general design of the DxDirect probe sets is outlined in Figure 2. For every gene in the assay, a unique probe set is designed consisting of two DNA oligonucleotides (S-probe and L-probe) that bind to adjacent sites of the genomic target. Each S- and L-probe pair contains a target-specific hybridization sequence (red) and a unique identifier sequence (blue) that enables separation and analysis based on length (for measurement by CE) or capture probe binding sequence (for measurement by planar microarray or bead array). All S- and L-probes contain universal upstream and downstream primer sequences (black) to allow for multiplex PCR amplification of all ligation products with a single PCR primer. DxTerity has

* For Research Use Only. Not for use in diagnostic procedures.
developed a proprietary software package that automates the design process.

In the DxDirect method, ligation products are generated directly in the denatured sample, captured on magnetic beads to remove un-reacted probes, and then amplified by PCR directly from the beads. The assay is quantitative because the amount of each ligation product made during the DxDirect reaction is proportional to the concentration of its RNA target sequence, and the relative ratios of the ligation products are maintained during PCR amplification since amplification uses only a single PCR primer. Quantification is relative to one or more housekeeping genes in the assay and readout is by capillary electrophoresis (CE), planar microarrays or bead arrays.

**DxCollect and RNA Stabilization**

DxCollect and DxDirect are designed to work together to streamline the genomic testing process. Similar to the industry-leading PAXgene product, DxCollect preserves mRNA integrity by lysing the cells and inactivating RNases. Importantly, the PAXgene mechanism of action includes forming a protective shell of hydrophobic cations around the mRNA, which leads to a precipitation of the mRNA from solution. In comparison, DxCollect was designed to inactivate the RNases and to keep the mRNA in solution so that it can be used in downstream processing without requiring a centrifugation step. Furthermore, while RNA can be isolated and purified from the DxCollect buffer if needed, the buffer composition has been optimized to enhance the performance of the DxDirect chemical ligation reaction and enable testing directly from the primary sample, without RNA extraction and purification.

DxCollect stabilizes RNA for more than 1 week at room temperature (Figure 3), up to a month at 4°C and up to 6 months at −70°C. DxTerity is working with major suppliers of blood collection systems to develop easy-to-use devices that allow for collection and ambient transport of blood by both venipuncture and finger stick. Finally, the DxCollect buffer can be used to directly test RNA in FFPE tissue, and from fresh-frozen tissue samples such as needle biopsies using the same sample processing methodology as blood samples.

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**Figure 1:** Schematic Illustration showing how replacing the enzymatic ligation reaction in MLPA/LDA with a chemical ligation reaction dramatically simplifies the testing process and reduces upfront processing time from 2 days to 1 hour.
The correlation of RNA isolated and purified from blood to the DxCollect stabilized blood was measured for an 11-plex gene expression signature. The blood was collected from a donor and immediately processed using the Norgen Blood Total RNA kit. After isolation, the purified RNA had an RNA Integrity Number (RIN) of 8.0. This RNA sample, as well as the original stabilized blood sample without any processing were tested using an 11-plex gene expression panel of genes commonly expressed in blood. Comparison of the isolated RNA to the blood gave a correlation coefficient ($r^2$) of 0.93 (Figure 4).

**Figure 2:** DxDirect assay method for mRNA gene expression. S and L-probes containing target specific regions (red), universal primers (black) and a target specific, unique identifier sequence (blue) are designed for each RNA target. There is a 1:1 conversion of labile RNA to a stable, engineered, synthetic DNA ligation product. Following purification, this product is amplified and then detected by the readout platform of choice.

**Figure 3:** DxDirect Signal Stability. Whole blood is mixed with an equal volume of DxCollect buffer, and then stored at room temperature for 2 weeks. At each time point, a sample was removed and assayed using the DxDirect CE method on the ABI 3500 Genetic Analyzer.

**Figure 4:** Comparison of 11-plex gene signature in DxCollect stabilized whole blood and purified blood RNA. Analysis is by CE.
**Adaptability of DxDirect™ to Multiple Detection Platforms**

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*Table 1: DxDirect Assay Readout*

DxDirect assay reaction products can be analyzed using instruments that are routinely found in research and clinical laboratories. Not all instruments are created equally and it is important to consider the performance characteristics of each one, including the multiplex capability, dynamic range, sensitivity, and ease of assay development before deciding on an instrument system (Table 1).

- **Real-Time PCR** provides the greatest dynamic range and fastest time-to-answer; however the single tube multiplexing capability is limited by the color resolution of the instrument (typically 5-color maximum).

- **Planar microarrays** can be used to multiplex an almost unlimited number of targets, but they require significant downstream processing steps, additional expense related with purchasing the microarrays, and the reproducibility is inferior to CE and PCR. DxTerity has used custom planar microarrays (Applied Microarrays, Tempe, AZ) to simultaneously analyze up to 450 independent probe pairs, and observed a linear dynamic range of 3 to 3.5 logs (data not shown). However, a probe-based technology such as DxDirect is better suited for lower multiplex (under 100), due to the time and expense of developing large numbers of unique, gene-specific probe sets.

- The Luminex xMAP system offers a simple and easy way to access the multiplexing power of arrays; however the usable linear range is limited to about 1.5-2.0 logs of target concentration. The Luminex LX200 bead array platform can multiplex up to 100 targets, while the less common (and more expensive) Luminex FlexMap allows multiplexing of up to 500 targets. Main advantages of the Luminex LX200 are the rapidity and ease of developing new bead assays, the large number of installed systems, and the potential for using the same system for protein and gene expression assays. For the DxDirect application, however, the analytical performance is inferior to CE and RT-PCR, and it is not well suited for assays that require measurement of low gene expression fold changes.

- **CE** is DxTerity's preferred readout platform. CE offers a unique combination of attributes, including low cost (less than $3 per read), multiplexing capability (up to 40 genes), reproducibility (run-to-run CVs less than 5%), and ease-of-use (direct sampling of amplified material). Most CE systems show at least 2 logs of linear performance, and there is a very large existing installed base in clinical and research laboratories today.

**DxDirect Assay Workflow**

The DxDirect assay procedure is simple, rapid and designed for high-throughput analysis. There is variation in workflow depending upon the readout platform (CE, Luminex, RT-PCR or planar microarray); however, the front-end steps associated with the chemical ligation and amplification steps are identical.

For a DxDirect assay designed for readout on a CE instrument, a 96-well plate of samples can be processed from sample input to placement on the CE reader in about 3 hours using common laboratory equipment. The total time from sample-to-answer will depend on the CE reader and the number of capillaries (1, 4, 8, 16, 24 or 96) on the instrument. The ABI 3730XL can read 96 samples in about 35 minutes.

The typical workflow for a blood based assay designed for CE readout is:

1. The stabilized blood sample (50 µL) is mixed with 25 µL of S-probe solution and heated to 80°C for 5 min in a sealed microtube on a standard PCR thermal cycler.
2. The solution is cooled to 55°C and L-Probe solution (25 µL) is added to each reaction, mixed and incubated at 55°C for 1 hour.
3. 5 µL of a magnetic bead solution is added, mixed and incubated for 5 minutes longer.
4. The magnetic particles are quickly collected using a 96-well magnetic collector plate and the supernatant/blood mixture is disposed as biohazardous waste.
5. The beads are washed 3 times with buffer, and the final supernatant removed.

6. PCR master mix is added directly to the wells containing the magnetic beads, and thermal cycling performed (28 cycles).

7. The PCR products are mixed with formamide and a CE size standard and analyzed by CE. Detection and quantification is by a fluorescent label incorporated into the universal PCR primer pair.

**DxDirect Dynamic Range and Sensitivity**

The dynamic range and sensitivity of the DxDirect-CE assay system was evaluated by spiking known concentrations of run-off mRNA transcripts for the human genes TNFAIP6 and ANXA3 into 50 μL of stabilized whole blood. The linear range of CE is about 2 logs, and differences of less than 1 fM of RNA can be distinguished. This corresponds to a sensitivity of less than 1 copy of mRNA per cell for a 50 μL blood sample (Figure 5).

**FFPE Tissue Analysis**

FFPE tissue samples are the standard for clinical oncology tissue sample archiving worldwide. It is estimated that there are approximately 1 billion FFPE tissue cancer samples archived in hospitals and tissue banks around the world, and many of these samples have full clinical annotation, including long term follow-up data. However, it is difficult to use FFPE samples for biomarker discovery because the RNA and DNA within the samples are heavily degraded. Current methods of analyzing gene expression in these samples involve laborious, inefficient and expensive sample preparation protocols, long turnaround times (2 days or longer) and large amounts of FFPE sample input. Furthermore, these methods have shown limited correlation of gene expression between FFPE and matching freshly-frozen (FF) samples.

The DxDirect method is able to directly test FFPE tissue slices with minimal sample processing. Matching prostate and breast tissue samples (FFPE and FF) were purchased, RNA was isolated from the fresh frozen tissue slice using standard methods, and the FFPE tissue was processed into 5 micron slices mounted on glass slides by Asuragen or Asterand. At DxTerity, a single 3 mm x 3 mm x 5 micron section of FFPE tissue mounted on a pathology glass slide was scraped off the slide using a scalpel and placed directly into 50 μL of DxCollect buffer without any processing. This buffer/FFPE sample was sonicated in a 55°C water bath for 5 min, and then processed using the standard workflow methodology. The fold change between breast and prostate for a panel of 15 genes in these tissues was determined for both FF and FFPE samples; the observed correlation was better than 0.93 (Figure 6).
Discussion and Conclusions

The DxDirect technology provides a rapid, simple and cost-effective method for measuring gene expression levels, with state-of-the-art analytical performance. By eliminating the need for RNA purification, RNA characterization and cDNA synthesis, this assay method dramatically shortens result turn-around time and eliminates major sources of assay imprecision. In addition, the DxCollect Stabilization Buffer allows shipment of samples at ambient temperature and direct analysis of blood RNA and FFPE tissue sections without RNA purification or isolation. The DxDirect method provides a practical pathway for converting complex gene signature assays into clinical assays. DxTerity is Enabling Genomic Testing in Routine Medical Care.

References:


2. To date, multiplex gene expression tests have been cleared by the FDA for determining the risk of breast cancer metastasis (Agendia MammaPrint®), determining the origin of cancer cells (PathWork Diagnostics Tissue of Origin test) and assessing the risk of heart transplant rejection (XDx AlloMap®).


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