Feasibility of using microbeads with holographic barcodes to track DNA specimens in the clinical molecular laboratory

We demonstrate the feasibility of using glass microbeads with a holographic barcode identifier to track DNA specimens in the molecular pathology laboratory. These beads can be added to peripheral blood specimens and are carried through automated DNA extraction protocols that use magnetic glass particles. We found that an adequate number of microbeads are consistently carried over during genomic DNA extraction to allow specimen identification, that the beads do not interfere with the performance of several different molecular assays, and that the beads and genomic DNA remain stable when stored together under regular storage conditions in the molecular pathology laboratory. The beads function as an internal, easily readable specimen barcode. This approach may be useful for identifying DNA specimens and reducing errors associated with molecular laboratory testing.
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Introduction

Events that negatively impact, or could negatively impact, patient care because of quality concerns with DNA-based clinical genetic testing are rare, occurring in <0.5% of tests performed (Hofgartner & Tait 1999). Despite this low rate of error, further reduction remains a priority of clinical laboratories. Errors can occur in all phases of testing (pre-analytical, analytical, and post-analytical), with the majority of problems occurring in the pre-analytical phase. This emphasizes the importance of quality assurance in all phases of testing. In this brief report, we describe the use of small glass microbeads containing a unique numeric code to barcode DNA eluates from peripheral blood specimens. The beads can be added directly to the peripheral blood specimens and are carried through automated DNA extraction protocols that use magnetic glass particles. A bead reader system can identify the bead number in the specimen, which can function as an internal barcode for specimen identification. This can be used to check the identity of DNA specimens that, for example, give unexpected results. Likewise, this approach could be used to check specimens at a set interval as part of the laboratory quality assessment program. Incorporation of this microbead system directly into blood draw tubes could be used to immediately barcode the specimen very early in the pre-analytical phase of testing. Preliminary data suggest that this approach can be used to track DNA specimens without interfering with DNA storage or downstream molecular testing. With the increased use of nucleic acid testing to guide precision or personalized medicine, this method provides a unique approach to decrease laboratory error.

Materials and Methods

Specimens
The use of peripheral blood specimens in this study was approved by a Stanford University IRB.

**VeraCode glass microbeads**

VeraCode microbeads were provided by Illumina, Inc. (San Diego, CA) in microcentrifuge tubes, each containing approximately 40,000 beads in 70% ethanol. VeraCode microbeads are cylindrical glass beads measuring 240 microns in length by 28 microns in diameter. A digital holographic element containing a numeric code is embedded within the beads serving as a unique identifier. When excited by a laser, each bead emits a unique code image that is detected by Illumina’s BeadXpress Reader System.

**DNA extraction with VeraCode microbeads**

Tubes containing the VeraCode microbeads were centrifuged in a microcentrifuge at greater than or equal to 10,000 rpm. Most of the 70% ethanol was removed from the tubes, leaving ~150 μL of residual 70% ethanol with the 40,000 beads. Subsequently, 200 μL of peripheral blood was added to the 70% ethanol and bead mixture using a 1 mL pipet and mixed thoroughly. The peripheral blood specimens containing the bead mixtures were then extracted on the Qiagen BioRobot EZ1 Workstation using the EZ1 DNA Blood 350 μL Kit (Valencia, CA) following the manufacturer’s standard protocol with a 200 μL elution volume. The eluate containing the VeraCode beads was transferred to the well of a 96-well round bottom microplate (Corning Inc., Corning, NY). A KingFisher 96 pin magnet head with a tip comb (Thermo Fisher Scientific, Waltham, MA) was used to remove residual Qiagen magnetic beads that could potentially interfere with the BeadXpress Reader System.

**Determination of the number of VeraCode microbeads carried through the DNA extraction process**
On seven independent days, VeraCode microbeads were added to three peripheral blood specimens. As is outlined in Table 1, the same sets of peripheral blood specimens were used for two or three days. Following the extraction process described in the above section, the beads were transferred to a 76.2 x 25.4 mm standard glass microscope slide and a 24 x 50 mm cover glass was used. The VeraCode microbeads were counted using a microscope under 100X magnification.

**Utilization of VeraCode microbeads for specimen identification**

Six peripheral blood specimens submitted for cystic fibrosis gene mutation testing underwent DNA extraction as described above with and without VeraCode microbeads added to the peripheral blood; beads containing a unique bead identification code were added to each peripheral blood specimen. DNA eluates with and without the beads were tested with the Cystic Fibrosis Genotyping Assay (Abbott Laboratories, Abbott Park, IL). In brief, this assay detects 32 cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations via multiplex PCR amplification and oligonucleotide ligation assays followed by detection via capillary electrophoresis on an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies Corporation, Carlsbad, CA). The resulting electropherograms were interpreted and evaluated for equivalency by two molecular pathologists (JDM and IS). A plate containing the residual DNA specimens with and without the beads was covered, frozen, and shipped to Illumina, Inc. to use the BeadXpress Reader System in order to determine the bead number associated with each specimen. We required that \( \geq 20 \) beads with the same barcode be detected by the instrument for definitive specimen identification for all experiments. In internal experiments, Illumina has determined that the bead mis-identification rate is under 0.5%, suggesting that a requirement for 20 beads for sample identification is more than sufficient.
We selected six independent peripheral blood specimens from individuals without a known cancer diagnosis, and for three of the specimens, we spiked in a cell line containing the \textit{IGH-BCL2} translocation at a 1:10,000 dilution to generate positive sensitivity controls near the lower limit of detection of the \textit{IGH-BCL2} translocation assay. DNA extraction was performed as described above with and without VeraCode microbeads added to each of the six peripheral blood specimens. Beads containing a unique bead identification code were added to each peripheral blood specimen. DNA eluates with and without the beads were tested with our assay used to detect \textit{IGH-BCL2} translocations involving the major breakpoint region, minor cluster region and intermediate cluster region of \textit{BCL2}. This assay uses multiplex, nested PCR followed by detection of amplification products via agarose gel electrophoresis. The resulting gel photographs were interpreted and evaluated for equivalency by two molecular pathologists (JDM and IS). A plate containing the residual DNA specimens with and without the beads was covered, frozen, shipped to Illumina, Inc., and scanned on the BeadXpress Reader System in order to determine the unique bead identification code associated with each specimen. The group using the BeadXpress Reader System was blinded to which specimens had beads added and to which bead identification code was used in each sample.

\textbf{Examination of VeraCode bead and DNA stability}

Three peripheral blood specimens were used for this portion of the study – each was submitted to our molecular pathology laboratory for testing with either the Cystic Fibrosis Genotyping Assay described above, the quantitative \textit{JAK2 V617F MutaQuant} assay (Ipsogen, Stamford, CT), or our laboratory-developed Fragile X syndrome assay. The \textit{JAK2 V617F MutaQuant} assay is an allele-specific, real-time PCR assay that quantifies both \textit{JAK2 V617F} and corresponding wild-type alleles. Our laboratory-developed Fragile X syndrome assay uses PCR optimized for
GC-rich amplicons followed by capillary electrophoresis on an Applied Biosystems 3130xl

Genetic Analyzer to detect (CGG)\textsubscript{n} trinucleotide repeat expansions in the 5’ untranslated region of \textit{FMR1}. Each specimen was assigned a unique bead identification code. Beads with this identification code were added to four aliquots of each peripheral blood specimen, and the specimens were extracted as described above. Eluates containing the beads were incubated at 25°C. Two aliquots of each specimen were removed after 50 days and 90 days. Each specimen was re-tested using the same assay for which the specimen was originally submitted, and the results were interpreted and evaluated for equivalency by two molecular pathologists (JDM and IS). A plate containing the DNA specimens with beads was covered, frozen, and shipped to Illumina, Inc. to use the BeadXpress Reader System in order to determine the bead identification code associated with each specimen. The group using the BeadXpress Reader System was blinded to which bead identification code was added to each specimen.

Accelerated stability calculations used the Q\textsubscript{10} model, a simplification of the Arrhenius equation approach (Hemmerich 1998). In this experiment, the method predicts the stability of a well-characterized reagent at \(\leq4°C\) based on stability at the elevated temperature of 25°C. The Q\textsubscript{10} value equals 2. Under the Q\textsubscript{10} model, the time relationship between the accelerated temperature (T\textsubscript{accel}) and the storage temperature (T\textsubscript{storage}) is given by the equation Q Factor = \(2^{(T_{accel} - T_{storage})/10}\); therefore, for this experiment the Q Factor = \(2^{(25-4)/10}\) = 4.3. One day at 25°C is equivalent to \(~4\) days at 4°C.

127 \textbf{Results}

128 We initially observed that VeraCode microbeads containing a holographic identification code could be added to whole blood specimens and subsequently carried through the genomic DNA
extraction process on instruments that use magnetic glass particles. We observed equivalent
results using the BioRobot EZ1 Workstation (Qiagen) and MagNA Pure LC (Roche Diagnostics
Corporation, Indianapolis, IN), and used the BioRobot EZ1 Workstation for subsequent work
described in this manuscript. Given potential applications for specimen identification and
quality assurance in the molecular pathology laboratory, we performed a set of experiments
designed to evaluate the potential feasibility of using the VeraCode microbeads to track
specimens in a clinical molecular laboratory. We found that a sufficient number of microbeads
are consistently carried over during genomic DNA extraction to allow specimen identification,
that the beads do not interfere with several different molecular assays, and that the beads and
genomic DNA are stable when stored together over extended periods of time.

We first evaluated if a sufficient number of microbeads would be carried through the
DNA extraction procedure to be read by the BeadXpress Reader System (Illumina), thereby
allowing identification of the specimen being tested. Although in practice even a single bead can
be read and detected by the system, we conservatively established that at least 20 microbeads
with the same identification code should be present to allow reliable detection with this system.
As is shown in Table 1, well in excess of 20 microbeads were carried through the extraction
procedure using multiple different specimens over multiple days. In total, 21 individual
extractions were evaluated, yielding a median of 242 microbeads per extraction (range 83 –
1096). Given that 40,000 microbeads are added to the initial whole blood aliquot, the efficiency
of carryover to the DNA eluate is low, but a sufficient number of beads are consistently
transferred to allow detection by the system. Of note, the measured DNA yield of extractions
that included the beads was generally lower than that of the same specimen extracted without
microbeads – average of 57 ng/μL without beads and 34 ng/μL with beads (Table 2). PCR can
be reproducibly performed on nanogram quantities of genomic DNA, and our laboratory
generally uses between 1-1,000 ng of genomic DNA per PCR reaction. Therefore, the DNA
ccentration and total yield were for sufficient for molecular testing, and we usually had
micrograms of residual DNA. It is unclear why the presence of the microbeads during the
extraction process reduced the amount of DNA in the eluate. It is possible that some of the DNA
was bound to the glass microbeads and therefore not available to be measured in solution.
Collectively, these data indicate that a sufficient number of microbeads are carried through the
DNA extraction procedure to be read by the BeadXpress Reader System, and that inclusion of
the microbeads does not appreciably impact the DNA extraction process.

We subsequently examined whether inclusion of the VeraCode microbeads affected the
performance of two clinical assays commonly performed in our molecular pathology laboratory
and whether the beads could be used to track DNA specimens within our laboratory. We
hypothesized that the beads would not interfere with molecular assays because they are
commonly used in related genotyping assays (Lin et al. 2009). Six peripheral blood specimens
submitted for CFTR mutation testing underwent DNA extraction with and without VeraCode
microbeads added to the peripheral blood. A unique bead identification code was added to each
peripheral blood specimen, essentially adding a readily readable molecular barcode to the
specimen. DNA eluates with and without the beads were tested with our laboratory’s cystic
fibrosis carrier screening assay. The resulting data were interpreted by two molecular
pathologists, and the results of the cystic fibrosis assays were the same in the paired peripheral
blood specimens with and without the beads (Table 3). In addition, peak positions and heights
were equivalent in the specimens with and without beads (Figure 1 illustrates a representative
example), indicating that the presence of the microbeads does not significantly interfere with the
performance of this assay. Subsequently, members of our group blinded to which bead was added to each specimen successfully used the BeadXpress Reader System to identify the correct bead identification code originally added to each specimen (Table 3).

We also performed a similar set of experiments to that described above with the cystic fibrosis carrier assay, using our laboratory assay for the qualitative detection of \( IGH-BCL2 \) translocations by PCR. As is seen in Table 3 and Figure 2, the results were the same for all six specimens tested and assay performance was equivalent. Likewise, we were able to re-identify in a blinded manner which bead was added to which specimen.

The cystic fibrosis and \( IGH-BCL2 \) experiments also allowed examination of time required for operation of the BeadXpress Reader System. Since the plates containing the specimens with microbeads can be directly loaded into a drawer in the BeadXpress Reader System after thawing, minimal hands-on time is required for this step. Once the instrument is initialized, the time to get results for 1, 12, or 96 specimens is approximately 5, 10, and 60 minutes, respectively.

Finally, to examine the long-term stability of the VeraCode microbeads we used accelerated stability experiments, and we also evaluated the integrity of the DNA under these conditions. We selected three peripheral blood specimens submitted to our molecular pathology laboratory for testing with either the Cystic Fibrosis Genotyping Assay, a quantitative \( JAK2 \) V617F MutaQuant assay, or our laboratory-developed Fragile X syndrome assay. Beads with a number specific to each specimen were added to multiple aliquots of each peripheral blood specimen, and DNA was extracted from the specimens. Eluates containing the beads were incubated at 25°C, which was our elevated storage temperature. Using the Q\(_{10}\) model, one day at 25°C is equivalent to just over four days at or below 4°C. Two aliquots of each specimen were
removed from incubation at 25°C after 50 days and 90 days, which is projected to represent 4ºC incubation for 215 days and 387 days. Each specimen was re-tested using the same assay for which the specimen was originally submitted, and the results were equivalent in both replicates at both time points. In addition, the correct bead number associated with each replicate was identified in a blinded manner. We note that the accelerated stability calculations using the $Q_{10}$ model is a conservative approach in the assignment of $Q_{10} = 2$. In addition, if this method is used to calculate the stability reagents stored at -15ºC or below, additional stability may be conferred due to the phase transition. Collectively, these data indicate that the beads and the DNA in the eluate are stable for at least one year at 4ºC and possibly longer when stored frozen.

Discussion

In this report, we evaluated the feasibility of using the VeraCode microbeads to track DNA specimens in the clinical molecular laboratory. We found that a sufficient number of microbeads are consistently carried over during genomic DNA extraction to allow specimen identification, that the beads do not appear to interfere with several different molecular assays, and that the beads and genomic DNA are stable when stored together over extended periods of time. The beads function as an internal, easily readable specimen barcode. This method may provide an additional approach to identifying DNA specimens and minimizing errors associated with molecular laboratory testing. Presently, this approach could be used to recheck the identity of DNA specimens that give unexpected results or to check specimens at a set interval as part of the laboratory quality assurance program. This would be a novel way to monitor for possible specimen mix-ups. Although the hands-on time and scanning time are reasonable, our
experience suggests that it would not be practical to examine every specimen tested by a busy molecular pathology laboratory with our method.

We suggest that this approach represents a novel mechanism to track DNA specimens in the molecular pathology laboratory, and the data presented provide a proof of concept that such an approach is possible. However, a couple of technical issues related to the microbead size currently limit the potential utility of this approach. The present microbeads are too large to co-elute with DNA using column-based extraction methods, and consequently this microbead tracking system cannot be used with many DNA and RNA extraction methods. Likewise, the relatively large size of the microbeads makes it difficult to consistently transfer a sufficient number of beads from the DNA eluate to downstream assay steps due to the microliter volumes typically used during molecular testing. As an example, we found that addition of DNA from the tube containing the DNA eluate with microbeads to a standard PCR reaction did not reliably result in the transfer of a sufficient number of beads to be detected by the barcode reader. However, further manipulation of the beads or extraction process may allow for these limitations to be overcome and for this application to become more broadly applied.

Other factors must be addressed and studies performed before the method can be incorporated into routine clinical practice. First, the BeadXpress Reader System and associated VeraCode microbeads were not designed for this application. Consequently, the final retail list price of the reader, $98,000, was likely higher than an instrument designed solely for applications described in this manuscript. Furthermore, the configuration of VeraCode microbeads used in this study was custom prepared and is not commercially available at this time. Second, we evaluated this method to track DNA specimens extracted from peripheral blood. For this method to be applied to all areas of the molecular pathology laboratory, further
examination of RNA extracted specimens would need to be performed. Likewise, specimens other than peripheral blood would need to be evaluated, and in the case of tissue specimens, the protocol would likely need to be modified to avoid, among other issues, destruction of the glass microbeads during the homogenization process. Third, we examined four assays in this study, and a thorough validation would require examination of a larger number of qualitative and quantitative assays with associated statistical assessment of interference.

In summary, we have demonstrated a proof of concept that glass microbeads with a holographic numeric code can be used to barcode DNA eluates from peripheral blood specimens. This represents a novel way to track DNA specimens in the molecular pathology laboratory, and we suggest that with appropriate modifications and further evaluation such an approach could minimize the potential for errors associated with molecular laboratory testing.
References

Hemmerich KJ. 1998. General Aging Theory and Simplified Protocol for Accelerated Aging of Medical Devices. *Medical Plastics and Biomaterials* 5:16-23.

Hofgartner WT, and Tait JF. 1999. Frequency of problems during clinical molecular-genetic testing. *Am J Clin Pathol* 112:14-21.

Lin CH, Yeakley JM, McDaniel TK, and Shen R. 2009. Medium- to high-throughput SNP genotyping using VeraCode microbeads. *Methods Mol Biol* 496:129-142.
Table 1 (on next page)

Number of Veracode microbeads carried through the DNA extraction process.
| Specimen 1 | Specimen 2 | Specimen 3 | Specimen 4 | Specimen 5 | Specimen 6 | Specimen 7 | Specimen 8 | Specimen 9 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| 534       | 464       | 743       | 108       | 203       | 135       | 231       | 255       | 308       |
| 1096      | 778       | 362       | 168       | 242       | 83        | 140       | 174       | 101       |
| 105       | 279       | 257       |           |           |           |           |           |           |

Days 1, 2 and 3

Days 4 and 5

Days 6 and 7
Effect of including Veracode microbeads during DNA extraction on resulting DNA concentration.
| Specimen | DNA concentration when extracted without beads | DNA concentration when extracted with beads |
|----------|-----------------------------------------------|---------------------------------------------|
| 1        | 43 ng/µL                                      | 22 ng/µL                                   |
| 2        | 51 ng/µL                                      | 33 ng/µL                                   |
| 3        | 44 ng/µL                                      | 24 ng/µL                                   |
| 4        | 73 ng/µL                                      | 44 ng/µL                                   |
| 5        | 49 ng/µL                                      | 25 ng/µL                                   |
| 6        | 79 ng/µL                                      | 53 ng/µL                                   |
Table 3 (on next page)

Using Veracode microbeads to identify DNA specimens and examining their effect on two molecular pathology assays.
| Specimen number | Bead number added | Bead number detected | Assay         | Assay result with microbeads added | Assay result without beads added | Equivalent amplification |
|-----------------|-------------------|----------------------|---------------|------------------------------------|----------------------------------|-------------------------|
| 10              | 4096              | 4096                 | CF32          | homozygous delF508                 | homozygous delF508               | Yes                     |
| 11              | 272               | 272                  | CF32          | homozygous delF508                 | homozygous delF508               | Yes                     |
| 12              | 40                | 40                   | CF32          | homozygous delF508                 | homozygous delF508               | Yes                     |
| 13              | 136               | 136                  | CF32          | no mutations detected              | no mutations detected            | Yes                     |
| 14              | 2560              | 2560                 | CF32          | no mutations detected              | no mutations detected            | Yes                     |
| 15              | 1040              | 1040                 | CF32          | no mutations detected              | no mutations detected            | Yes                     |
| 16              | 1040              | 1040                 | BCL2          | IGH-BCL2 detected                 | IGH-BCL2 detected               | Yes                     |
| 17              | 2560              | 2560                 | BCL2          | IGH-BCL2 not detected             | IGH-BCL2 not detected           | Yes                     |
| 18              | 136               | 136                  | BCL2          | IGH-BCL2 detected                 | IGH-BCL2 detected               | Yes                     |
| 19              | 40                | 40                   | BCL2          | IGH-BCL2 not detected             | IGH-BCL2 not detected           | Yes                     |
| 20              | 272               | 272                  | BCL2          | IGH-BCL2 detected                 | IGH-BCL2 detected               | Yes                     |
| 21              | 4096              | 4096                 | BCL2          | IGH-BCL2 not detected             | IGH-BCL2 not detected           | Yes                     |

**CF32** – Abbott Laboratories Cystic Fibrosis Genotyping Assay

**BCL2** – Laboratory-developed assay for detection of *IGH-BCL2* translocations
**Figure 1**

Representative capillary electropherograms from a multiplex PCR amplification and oligonucleotide ligation assay to detect 32 different mutations in the **CFTR** gene.

The presence of beads during the extraction process and downstream steps (panel A) does not appear to affect either peak height or assay results when compared to analysis of the same specimen without beads (panel B). No **CFTR** mutations were detected in the specimen with or without the beads and each of the peaks is present at the same position.
Figure 2

Agarose gel electrophoresis demonstrating control amplification reactions for a PCR-based IGH-BCL2 translocation assay.

The presence of beads during the extraction process and downstream steps does not significantly affect the control amplification for this assay. This control amplicon is a 270 bp product derived from the F5 gene.