concentrations between the trisomy 18 and control cases. This implies that a relatively low sensitivity and specificity would result if maternal serum βhCG mRNA measurement were used as the sole predictor for pregnancies with trisomy 18. In ROC curve analysis (using MedCalc 5.0 software), the mean (SE) area under the ROC curve was 0.734 (0.067) with a 95% confidence interval of 0.651–0.806. On the other hand, a larger scale study may be necessary to explore whether maternal serum βhCG mRNA is a useful marker in trisomy 21 screening.

In summary, our findings provide the first evidence for the value of circulating placental mRNA measurement in the noninvasive detection of a fetal chromosomal aneuploidy. The current study is designed primarily as a proof-of-concept investigation. The main technical advantage of the mRNA technology is the relative ease with which new mRNA markers can be developed, including genes coding for proteins for which no immunoassays are currently available. We believe that the availability of microarray technology could lead to development of panels of placenta-specific mRNA markers for future fetal aneuploidy screening.

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References


Universal Sensing Strategy for the Detection of Nucleic Acid Targets by Optical Biosensor Based on Surface Plasmon Resonance, Bi-feng Yuan, Yu-hua Hao, and Zheng Tan’ (Laboratory of Biochemistry and Biophysics, College of Life Sciences, Wuhan University, Wuhan 430072, People’s Republic of China; * author for correspondence: fax 86-27-8788-2661, e-mail tanclswu@public.wh.hb.cn)

PCR is an important technique for identifying specific nucleic acid targets; for example, sequences associated with diseases and pathogens in clinical, environmental, and food samples. Many techniques currently used for sequence-specific detection of PCR products either require manual processing or are limited in the speed or scale of analysis. The development of biosensors in recent years has provided promising techniques for efficient sequence-specific DNA analysis (1). Biosensors have been used to detect target sequences in PCR products. Most studies have been conducted with the commercially available biosensor BiAcore, which is based on surface plasmon resonance (SPR) technology. In those investigations, either sequence-specific oligonucleotide probe (2–8) or amplified products themselves (8–11) were immobilized on the sensor chip, limiting their application to one sequence or to one particular sample. Such a strategy suffers several drawbacks: (a) it compromises the automation and high-throughput capability of such instruments when different targets are analyzed; (b) chip-to-chip variation makes it difficult to compare different measurements; and (c) probe immobilization and chip consumption make running costs substantial.

To overcome these drawbacks, we describe here a “one-chip-for-all” strategy that is capable of, in principle, detecting different target sequences by use of the same sensor chip. Target sequences are amplified by asymmetric PCR using a primer pair in which the low-concentration primer carries a common tag sequence that is identical to that of the oligonucleotide capture probe immobilized on the sensor chip. The PCR product is then injected and captured on the sensor chip, and its sequence identity can be further verified by use of a target-specific probe (see Fig. 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue6/). We exemplify this application by analyzing three different genes: the cystic fibrosis transmembrane conductance regulator gene (CFTR), the mutation of which causes cystic fibrosis (12); the hTERT gene, which encodes the catalytic component of the human telomerase complex (13); and the human gene for the tumor suppressor p53 (Table 1).

Asymmetric PCRs were conducted with 100 ng of human genomic DNA from HeLa cells in a final volume of 50 μL, containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1 mM MgCl2, 0.2 mM deoxynucleotide triphosphates, 1 μM high-concentration primer; 0.1 μM low-concentration primer, and 3 U reaction of Taq polymerase (MBI). Thermal cycling was conducted on a Biometro thermal cycler with initial denaturation at 94 °C for 3 min, followed by 55 cycles of 94 °C for 30 s, 58 °C for 30 s, and
Table 1. Primers and probes used for CFTR, hTERT, and p53 amplification and detection.

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligonucleotide</th>
<th>Sequence*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFTR</td>
<td>Immobilized capture probe</td>
<td>5’-Biotin-C12-TGCCTGAGCCAGCGAGAGAAA-3’</td>
</tr>
<tr>
<td></td>
<td>High-concentration primer</td>
<td>5’-GCTAAGTCCTTTTGTCTCACTTG-3’</td>
</tr>
<tr>
<td></td>
<td>Low-concentration primer</td>
<td>5’-GGAGCCAGCGAGAGATGAAACATGAAAGGAGAAATCCAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, full match</td>
<td>5’-ATCGATTTGTTGTCCTGGGATCTCAAACGCTGAGAAATCCAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, 1 mismatch</td>
<td>5’-ATCGATTTGTTGTCCTGGGATCTAAACGCTGAGAAATCCAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, 2 mismatches</td>
<td>5’-ATCGATTTGTTGTCCTGGGATCTGAAACGCTGAGAAATCCAGG-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, 3 mismatches</td>
<td>5’-ATCGATTTGTTGTCCTGGGATCTTAAACGCTGAGAAATCCAGG-3’</td>
</tr>
<tr>
<td>hTERT</td>
<td>High-concentration primer</td>
<td>5’-CATCCTCTCTAGTTCTACGCA-3’</td>
</tr>
<tr>
<td></td>
<td>Low-concentration primer</td>
<td>5’-GGAGCCAGCGAGAGATGAAATGACGCGAGAAATG-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, full match</td>
<td>5’-TGGGGTTCCTCAAAACTTGTGATGAAATGGAGCTGC-3’</td>
</tr>
<tr>
<td>p53</td>
<td>High-concentration primer</td>
<td>5’-CCGTCATCTCCCTTGCCCTCC-3’</td>
</tr>
<tr>
<td></td>
<td>Low-concentration primer</td>
<td>5’-GGAGCCAGCGAGAGATGAACTTGGCTGTCCTCGAGAATGC-3’</td>
</tr>
<tr>
<td></td>
<td>Probe, full match</td>
<td>5’-AAGAAGCCGCAAGCGAAAAACCGTACCTGCGCTGGTGTT-3’</td>
</tr>
</tbody>
</table>

* The underlined uppercase bases indicate the tag sequences. The underlined lowercase bases indicate mismatches.

72 °C for 30 s for CFTR and of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s for hTERT and p53. Symmetric PCRs were conducted under the same conditions except that 0.1 μM each of both primers were used. PCR products were run in 12% polyacrylamide gels at 20 V/cm for 1 h in Tris-borate-EDTA buffer [90 mmol/L Tris-borate (pH 8.0), 2 mmol/L EDTA] and visualized by ethidium bromide staining.

Detection of PCR products by SPR was conducted at 30 °C on a BIACore X biosensor with a streptavidin-coated SA5 sensor chip using HEPES-buffered saline containing 300 mmol/L NaCl, and 0.05 mL of the surfactant P20 as running buffer. The biotinylated probe was immobilized on the sensor chip surface by injection of 30 μL of probe at 6 ng/μL into one flow cell, giving a capture of ~300 response units (RU) of probe. The PCR sample was mixed with an equal volume of HEPES-buffered saline containing 1.6 mol/L NaCl, and a 20-μL aliquot of this mixture was injected at 2 μL/min followed by a flow of running buffer. In some experiments, 10 μL of 10 ng/μL sequence-specific or irrelevant oligonucleotide was subsequently injected at 2 μL/min, followed by a flow of running buffer. The sensorgram of a blank cell was simultaneously recorded as reference and subtracted from that of the sensing cell. The sensor chip was regenerated by a pulse of 5 μL of NaOH (20 mmol/L) in NaCl (1 mol/L).

Taking CFTR as an example, gel electrophoresis (Fig. 1A) showed that the tag sequence in the low-concentration primer was introduced into the symmetric and asymmetric PCR products based on their molecular sizes. The asymmetric PCR produced two weak bands with slower migration in addition to the double-stranded product. Evidence that these two bands were single-stranded DNA was that they were sensitive to mung-bean nuclease, which specifically digests single-stranded DNA. When the sample was incubated with the 40mer probe complementary to the CFTR gene before electrophoresis, these bands disappeared and a new band appeared, indicating that they were both the single-stranded form of the expected products. Similar results were obtained for hTERT and p53.

The BIACore system monitors changes in mass of analyte at sensor chip surfaces by changes in refractive index (14). The tag sequence introduced to the single-stranded PCR product is complementary to and can hybridize with the capture probe immobilized on the sensor chip. When PCR sample is injected, the hybridization between the PCR product and the probe is reflected by an increase in RU. The response is proportional to the quantity of PCR product captured (15). As shown in Fig. 1B, the amplified product hybridized to the capture probe, as demonstrated by the increase in RU. Little hybridization was detected when the tag sequence was not present in the low-concentration primer or the gene was omitted or amplified by symmetric PCR (data not shown).

The sequence identity of the captured PCR product was further verified by an injection of target-specific probe complementary to the CFTR sequence, which led to an additional increase of 143 RU, indicating that the captured product was indeed what was expected. Because the target-specific probe had 40 and the expected PCR product had 127 bases, an increment of 143 RU corresponded to 95% of the captured DNA was the expected PCR product. The captured PCR product had a response of 500 RU before injection of the target-specific probe. This means that >90% of the captured DNA was the expected PCR product of CFTR. Note that the captured PCR product underwent a slow dissociation from the immobilized probe. Taking this into account, we estimated that >95% of the captured DNA was the expected PCR product. When noncomplementary probes were injected, no hybridization was detected. These results indicate that the detection is specific. Similar results were obtained when we used this same sensor chip to detect the other two targets, i.e., the hTERT and p53 genes (Fig. 2 in the online Data Supplement).

Because of mutations or single-nucleotide polymorphisms (16), target genes may not fully match the sequence-specific probes. Probes with one to three mismatches provide specificity that allows for detection of one target, and this method is not limited to the other two targets, i.e., the hTERT and p53 genes (Fig. 2 in the online Data Supplement).
matches were injected after PCR product was captured on chip surface. The results shown in Fig. 1C demonstrate that for a 40mer probe, mismatches of up to 3 bases had little effect on the hybridization. This indicates that target can be verified even when a few mismatches are present. On the other hand, this method should also be applicable to the detection of point mutations if a shorter probe (≈10 bases) is used for the second hybridization (T1).

Optimization was conducted with respect to salt concentrations and temperatures (Fig. 3 in the online Data Supplement). Salt greatly enhanced the hybridization. This can be explained by the fact that both DNA and the carboxymethylated dextran coating at the sensor surface are negatively charged and salt will reduce the repulsion between them. Increasing the temperature also enhanced hybridization, although occasional increased noise signals might be produced at 35°C. We therefore conducted our formal measurements at 30°C in a high concentration (0.8 mol/L) of NaCl to obtain proper sensitivity and stability. For the BIAcore, background noise was within 1 RU (instrument specification). We obtained stable signals at ≈600 RU for ≥50 ng and >500 RU for 10 ng of input genomic DNA (data not shown).

Flow-type biosensors such as the BIAcore biosensor are expected to be a valuable platform in diagnostics when automation and high-throughput capability are desired. Because there is no need to switch the chip for different targets or samples, the one-chip-for-all strategy described here should be especially suitable for automated, high-throughput analysis of large numbers of samples. The SPR technique avoids post-PCR processing, and the detection is highly specific, efficient, sensitive, and reproducible. The chip can be reused for hundreds of measurements in this particular case. After more than 260 measurements, the binding capacity of the chip we used decreased by <6%. Using one chip for different samples and targets should simplify operation, improve efficiency for routine analysis, and reduce the running costs associated with chip consumption and probe immobilization.

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References
Molecular Beacons for Multiplex Detection of Four Bacterial Bioterrorism Agents, Mandira Varma-Basil, Hiyam El-Hajj, Salvatore A.E. Marras, Manzour Hernando Házbon, Jessica M. Mann, Nancy D. Connell, Fred Russell Kramer,* and David Alland** (1 Department of Medicine, Division of Infectious Disease, New Jersey Medical School, The University of Medicine and Dentistry of New Jersey, Newark, NJ; 2 Department of Molecular Genetics, The Public Health Research Institute, Newark, NJ; 3 current affiliation: Department of Microbiology, Vallabhbhavi Patil Chest Institute, University of Delhi, Delhi, India; * address correspondence to this author at: Division of Infectious Disease, New Jersey Medical School, 185 South Orange Ave., MSB A920C, Newark, NJ 07103; fax 973-972-0713, e-mail allandda@umdnj.edu)

The advent of bioterrorism has highlighted the need for rapid, simple, and robust diagnostic assays to detect select agents. Mortality from select agents may be greatly reduced by prompt treatment (1); however, treatment may be delayed if diagnostic assays are outsourced to reference laboratories. Most bacterial species that would likely be used as bioterrorism agents infect the blood stream during the course of life-threatening disease. Furthermore, even “nonseptic” syndromes may produce hema-

togenous bacterial DNA that could be detected by a sensitive assay (2). This means that a rapid “molecular” version of a blood culture would fulfill many of the rapid diagnostic needs for biodefense.

Bacteria can be detected in blood and other sterile body sites by the identification of species-specific DNA sequences in their 16S rRNA genes. These species-specific sequences are flanked by conserved sequences, permitting most rRNA targets to be amplified by PCR using a limited set of “universal” primers (3). Real-time PCR is well suited for sensitive and specific pathogen detection because it is performed in hermetically sealed wells, which greatly reduces the risk of cross-contamination, and it does not require post-PCR analysis (4). Real-time PCR assays have been developed for some select agents, most of which use fluorogenic 5′-nuclease (TaqMan) probes (5–7). However, TaqMan probes are difficult to use in multiplex PCR assays (8, 9). In contrast, molecular beacons are real-time PCR probes that are particularly amenable to multiplexing (10). They can be labeled with differently colored fluorophores (11), use a common nonfluorescent quenching moiety (9), and have thermodynamic properties that favor highly specific detection of nucleic acid sequences (12).

Here we describe a real-time PCR assay that simultaneously detects four bacterial agents that could be used in bioterrorism. This assay is specifically designed to test sterile body fluids, where a rapid and simple assay would be beneficial. We developed a flexible assay format that can easily be adapted to the wide range of spectrofluorometric thermal cyclers that are in common use, including thermal cyclers that have only one- or two-color capabilities, and others that can detect four or more colors simultaneously.

Phenol–chloroform extraction of DNA from standard strains of Bacillus anthracis Vollum and Sterne, Yersinia pestis CO92, and Burkholderia mallei (ATCC 23344) was performed as described previously (13) in a biosafety level III laboratory certified to work with select agents (registration number C20011016-798; entity number C20031123-0125). A 180-bp amplicon for use as template inFrancisella tularensis assays was also constructed in vitro from two overlapping oligonucleotides (Invitrogen). DNA was also extracted from clinical isolates of Staphy-

lococcus aureus, S. epidermidis, Streptococcus pneumoniae, Klebsiella pneumoniae, Escherichia coli, Enterobacter cloacae, and Serratia marcescens to serve as controls.

The conservation of 16S rRNA gene sequences among bacteria enabled us to design primers FUHP (5′-GTG-GAC-CTTAGATACCCCTGGTATCCAC-3′; underlined sequence indicates additional nucleotides added to create a hairpin structure) and RUP (5′-GGCTTGCATCGAATTTAAA-3′) to amplify short segments of the 16S rRNA genes of Y. pestis, F. tularensis, and B. mallei by PCR. The benefits of hairpin-shaped primers have been noted previously (14–16). A second primer pair, FBA (5′-TGACGACAAC-GACTTAGATACCCTGGTAGTCCAC-3′) and RBA (5′-ATGTTGTATTCA-TGAAACCAATCC-3′), was designed to amplify a segment of the 16S rRNA gene of B. anthracis.

We designed molecular beacons (Table 1) that bound to amplicons generated from F. tularensis, B. mallei, and B. anthracis and that could not bind to amplicons generated by other significant human pathogens. However, the Y. pestis molecular beacon also bound to amplicons gener-