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inants and eluting bound total RNA or on (ii) cell lysis with guanidinium thiocyanate and phenol:chloroform:iso-propanol extraction, and we tried (iii) to produce first-strand cDNA directly from cell lysates, thereby eliminating the RNA isolation step.

In our experiments with as few as about 3000 microdissected cells, we were not able to perform RT-PCR directly from cell lysates without initial RNA isolation. We could not confirm the successful results obtained with the widely used silica-gel binding RNA isolation procedures (5,8,9). In our experiments with only 3000 cells, these methods based on matrix binding did not give satisfying total RNA recovery rates in the elution step, and DNA contamination was found in control RT-PCRs without adding reverse transcriptase, possibly because of an insufficient on-column DNase digestion. In the original protocols based on phenol:chloroform:iso-propanol extraction, it was impossible to precipitate sufficient yields of pure undegraded RNA from the aqueous layer because of the limited initial amount of cells. Therefore, we developed an optimized protocol that combines a guanidinium thiocyanate-based denaturation with a modified RNA precipitation and purification protocol (1), as shown in Table 2. The quality of the isolated DNA-free total RNA was examined by RT-PCR (Figure 1).

Generating target samples from small microdissected sections suitable for hybridization of microarrays requires linear RNA in vitro amplification. We used a modified T7 RNA polymerase linear amplification protocol (2,6) combined with a template-switching mechanism (9), as described in detail in Table 3, which also indicates changes to the original protocols.

To prove the quality and efficiency of our labeled target, ArrayLink™ control slides (GeneScan Europe, Freiburg, Germany) were hybridized according to the manufacturer’s protocol. Briefly, arrays were prehybridized with GeneScan hybridization solution containing 1 µg salmon sperm DNA and washed in 2× SSC buffer. Hybridization was performed overnight at 37°C with 5 µL labeled cDNA in 20 µL hybridization solution, followed by three washing steps with SSC/0.1% SDS buffer. Cy5-fluorescence signals can be seen for all housekeeping gene transcripts (Figure 2).

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SNP Analysis by Allele-Specific Extension in a Micromachined Filter Chamber

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Single-nucleotide polymorphisms (SNPs) are distributed across the genome with an estimated prevalence of 1 SNP per 1300 bases (4,12) and hence are useful as genetic markers in linkage studies, pharmacogenomics, forensics, and the analysis of loss of heterozygosity. Consequently, the high prevalence of SNPs throughout the genome requires efficient methods to analyze these sequence variants. Several high-throughput techniques are now available for this purpose. Many of the methods use allele-specific hybridization to discriminate between allelic variants. These methods include allele-specific hybridization in microarray formats (13), molecular beacons (11), dynamic allele-specific hybridization (3), and TaqMan® PCR amplification (5). Some other widely used genotyping technologies are minisequencing (8) and ligation assays (7). Allele-specific amplification (6) and extension (9) have also been used for the analysis of genetic variations.

A recently developed technique for SNP genotyping is pyrosequencing (1). The technique relies on the incorporation of nucleotides by DNA polymerase and the release of pyrophosphate (PPi), which will be converted to ATP and then to detectable light by sulfurylase and luciferase, respectively (10). In the standard procedure of pyrosequencing, iterative addition of dNTPs is performed, and as the process continues, the complementary DNA strand is built up and the sequence is determined. In an alternative approach for SNP analysis, allele-specific extension with alternating 3’ end primers and the addition of all four nucleotides may be utilized. The direct consequence of this approach will be that the DNA polymerase fully extends a complete matched primer-template, and an equivalent amount of PPi will be released. If the primer-template does not match at the 3’ end of the primer, then
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the DNA polymerase should not be able to extend the primer. In the case of matched primer-template, a very high level of light intensities is detected, which consequently allows the miniaturization of allele-specific pyrosequencing reactions. Currently, pyrosequencing is performed in a microplate format with a reaction volume of 50 µL. To reduce the reaction volume in pyrosequencing, the method was integrated with a flow-through micromachined device. The device is designed for biochemical reactions on beads. Both magnetic and nonmagnetic beads can be collected in the filter chamber (reaction chamber) without the use of external magnets (2). The device is not sensitive to gas bubbles, and clogging of the filter is rare and reversible. The smallest reaction chamber used in the assays had a volume of 12.5 nL. Thus, the principle of allele-specific extension by pyrosequencing chemistry in a flow-through filter chamber was used to analyze two SNP sites. The SNPs were codon 72 of the p53 gene located on chromosome 17p and wiaf 1764 located on chromosome 9q. As described previously (1), a multiplex outer PCR was performed to amplify the SNP sites. The outer PCR was followed by region-specific inner PCRs in which one of the primers was biotinylated at one of the primers was biotinylated at the 5′ end to facilitate immobilization and strand separation. Five microliters of total human DNA (5 ng/PCR), isolated from blood samples, were used as starting material for outer PCR amplification. The inner PCR products were immobilized onto nonmagnetic streptavidin-coated 5.5-µm beads (2 × 10⁶ beads/PCR) (Bangs Laboratories, Fishers, IN, USA), and ssDNA was obtained by alkali treatment. The immobilized strand was resuspended in water, and 10× annealing buffer (100 mM Tris-acetic acid, pH 7.75, 20 mM Mg-acetate) was added to the single-stranded templates. The solution was then divided into two wells in a microplate, and allele-specific primers (1 µM) were added in each well. The primers differed in their 3′ ends, allowing allele-specific analysis of each sample. The p53 primers with alternating 3′ ends (shown in bold) had the sequence 5′-GCTGCTGGTGCAGGGGCCACG-3′ and 5′-GCTGCTGGTGCAGGGGCCACG-3′. The wiaf 1764 primers differing in their 3′ ends had the sequence 5′-ACTCCCTTCAGATCA-3′ and 5′-ACTCCCTTCAGATCC-3′. Hybridization was performed by incubation at 94°C for 40 s and then cooling to room temperature. The ssDNA with annealed primer was captured in the filter chamber (12.5 nL), and a pyrosequencing mixture was added (1). However, the pyrosequencing mixture contained 4 µM of each nucleotide (final concentration), and the nucleotide-degrading enzyme apyrase was excluded. The extension procedure was carried out in a dark box. An external CCD camera detected the output of light resulting from nucleotide incorporations versus time.

Figure 1 shows the principle of allele-specific extension by pyrosequencing chemistry. This principle was used in a flow-through micromachined device for biochemical reactions on beads. Figure 2A shows a schematic view of the complete flow-through microfluidic device. Figure 2B shows a scanning electron microscope photograph of the filter chamber device. The beads with the immobilized strand (hybridized to allele-specific primer) are applied at the inlet and collected in the filter chamber. As shown in Figure 2, the waste chamber is surrounding the filter chamber and is connected to the outlet. Thus, the principle of allele-specific extension in a flow-through filter chamber device was used to analyze two SNPs at codon 72 of the p53 gene and wiaf 1764. The coding SNP (cSNP) on the p53 gene involves either a C or G residue, corresponding to the amino acids proline (CCC) and arginine (CGC), while wiaf 1764 contains G or T residues. To analyze the SNP positions, immobilized ssDNA with annealed allele-specific primer was captured in the filter chamber (reaction chamber). Pyrosequencing reagents (excluding apyrase) together with the four dNTPs were applied on the inlet channel of the device (1 µL). The mixture was then pulled into the reaction chamber through manual sucking from the outlet. The reaction mixture was then sucked out to the outlet. This procedure leads to 5–10 s of contact between pyrosequencing mixture and the immobilized DNA target. Immediately thereafter, the flow-through device was...
placed on an external CCD camera in a dark box, and the produced light was collected. By applying backpressure, the beads and the immobilized DNA were removed from the filter chamber and washed, and the next sample with the alternative allele-specific primer was captured in the device. All three possible genotypes of the two SNPs could be scored using the same flow-through chip (12.5-nL filter chamber).

As the reaction mixture flows into the microfluidic chip and comes in contact with the immobilized DNA in the filter-chamber, elongation of primer-template takes place and PP1 will be released. However, the reaction mixture and PP1 continue to flow through and end up in the outlet of the chip; thus, most of the produced light is detected at the outlet. Figure 3 shows the results of allele-specific extensions in the microfluidic device for codon 72 of the p53 gene (left) and wiaf 1764 (right). In homozygous samples, one of the allele-specific primers is a complete match to the target DNA template and is shown as a continual line, and one primer will have a 3′-end mismatched base to the template, and the light signal is shown as dotted line. In heterozygous samples, both primers form matched configurations to the target DNA and thus are shown as continual lines. Because the signal for each extension slightly varies by time, an average signal value was calculated. The ratios were determined by dividing the average signal for matched primer-template configuration to the average signal for mismatched primer-template configuration. However, in the case of heterozygous samples, both allele-specific primers form matched configurations, and, thus, the higher average signal is divided with the lower signal. The ratios for the three variants of codon 72 of the p53 gene were 7.8 (homozygous C), 1.2 (heterozygous C/G), and 2.4 (homozygous G). The ratios for the three variants of wiaf 1764 were 5.4 (homozygous G), 1.2 (heterozygous G/T), and 3.1 (homozygous T). However, in the present setup, the procedure depends on manual handling of the liquids, but a micro-machined flow-through device with 100 filter-chambers has been designed and fabricated that allows parallel analysis of SNPs. The device will be in-
tegrated with a valve-less diffuser micropump to control the pressure, which opens up the possibility for base-by-base sequencing in the filter chamber.

In summary, this work demonstrates the possibility of typing SNPs by allele-specific extensions in a microfluidic flow-through filter chamber using pyrosequencing chemistry. This miniaturization of pyrosequencing can lead to a 4000-fold reduction of the reagent consumption compared to the present standard volume.

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