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Pancreatic Cancer

Methods and Protocols

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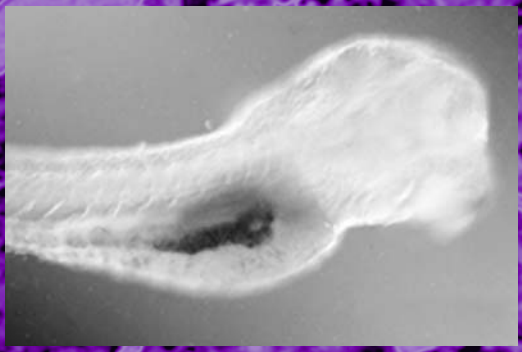

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Digital Single-Nucleotide Polymorphism Analysis for Allelic Imbalance

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Summary

Digital single-nucleotide polymorphism (SNP) analysis is developed to amplify a single template from a pool of DNA samples, thereby generating the amplicons that are homogeneous in sequence. Different fluorophores are then applied as probes to detect and discriminate different alleles (paternal vs maternal alleles or wild-type vs mutant), which can be readily counted. In this way, digital SNP analysis transforms the exponential and analog signals from conventional polymerase chain reaction (PCR) to linear and digital ones. Digital SNP analysis has the following advantages. First, statistical analysis of the PCR products becomes available as the alleles can be directly counted. Second, this technology is designed to generate PCR products of the same size; therefore, DNA degradation would not be a problem as it commonly occurs when microsatellite markers are used to assess allelic status in clinical samples. Last, digital SNP analysis is designed to amplify a relatively small amount of DNA samples, which is available in some clinical samples. Digital SNP analysis has been applied in quantification of mutant alleles and detection of allelic imbalance in clinical specimens and it represents another example of the power of **PCR** and provides unprecedented opportunities for molecular genetic analysis.

Key Words: Digital; molecular genetics; mutation; allelic imbalance; polymerase chain reaction; single-nucleotide polymorphism.

1. Introduction

Genetic instability is a defining molecular signature of most human cancers ***(1,2)***, and at the molecular level it is characterized by allelic imbalance (AI), representing losses or gains of defined chromosomal regions. Analysis of AI is useful in elucidating the molecular basis of cancer and also provides a molecular basis for cancer detection. There are, however, at least two major problems associated with the current methods for assessing AI using microsatellite markers.

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First, DNA purified from microdissected tissues or body fluids is a mixture of neoplastic and non-neoplastic DNA and the latter, released from non-neoplastic cells, can mask AI because it is difficult to quantify the allelic ratio using microsatellite markers. Second, such DNA is often degraded to a variable extent, producing artifactual enrichment of smaller alleles when microsatellite markers are used for analysis ***(3)***. To overcome these obstacles associated with the molecular genetic analysis of AI, we employed a recently developed polymerase chain reaction (PCR)-based approach called digital single-nucleotide polymorphism (SNP) analysis in which the paternal or maternal alleles within a plasma DNA sample are individually counted, thus allowing a quantitative measure of such imbalance in the presence of normal DNA. Digital SNP analysis is based on the concept of digital PCR ***(4)***. Therefore, digital SNP analysis is a powerful tool to assess allele status of tumor cells when the presence of contamination from normal DNA is inevitable or only a minimal amount of DNA is available for assay.

2. Materials

1. Molecular beacons (MB) and oligonucleotide primers (*see* **Subheading 3.1.**).
2. Regular PCR reagents (10X PCR buffer provided by the company [DMSO], 50 m*M* $MgCl_2$, and 25 m*M* dNTP) and *Taq* enzyme.
3. TE Buffer: 10 m*M* Tris and 1 m*M* EDTA.
4. Biomek2000 Working Station (Beckman) or equivalent.
5. 96- and 384-well plates.
6. Salad spinner or centrifuge (spin the 384-well plate).
7. PCR machine with block for 384 wells.
8. Fluorometer with appropriate adaptor to read 384-well plates.

3. Methods

3.1. Molecular Beacon and Primer Design

1. Several SNP websites are available to identify appropriate SNPs, such as http://www.genome.wi.mit.edu/SNP/human/index.html or http://www.ncbi.nlm.nih.gov/SNP.
2. If the NCI website is used, http://lpg.nci.nih.gov/html-snp/imagemaps.html → select one's chromosomal regions by directly clicking the SNP map under the chromosome → click the left column (distribution of SNP) of the SNPs one prefers based on gene name, adjacent microsatellite marker, genetic, physical maps or banding segment → SNP viewer → self-explanatory and you will see the flanking sequence data around the SNPs.
3. Choose the SNPs with allelic frequency close to each other [e.g., 12 A and 12 G from 24 different expressed sequence tags (ESTs)]. Go to Tool → Design Primer → copy the sequence to new file.

4. Design the primers that amplify approx 100 bp products containing the SNPs using the same guidelines as usual (approx 18–20 bp length, T_m: approx 58–62°C, end with 5'-CG-3' if possible). Design the molecular beacon with the following features: length: 18–21 bp with the SNP around the middle of the sequence, then add the stem loop structure as underlined (5'-CACG-nnnnnnnnnnnnnn-CGTG) so the total length of the molecular beacon is 26–29 bp. Check the T_m of the molecular beacon around 51.5 ± 2°C (without the stem sequence) or 72 ± 3.4°C (the whole molecular beacon). Try to avoid secondary structure within the beacon other than the stem loop on both ends (only 4 bp). Then design the molecular beacon for the other allele with the same sequence except for the SNP. A website is available to assist the design (*see* **Note 1**).
5. Repeat the above procedures to find more SNPs.
6. Order molecular beacons. Beacons should be 5' labeled with either fluorescein or HEX (or other fluorophores) and the 3' should be labeled with Dabcyl (the quencher). A 200 μ*M* scale should be sufficient for at least 60 assays. All beacons should be gel purified.

3.2. Molecular Beacon Testing

1. Use the panel of control DNA from several healthy individuals.
2. For each molecular beacon set, sequence the PCR products amplified from control DNA (usually four or five samples are enough to obtain homozygous alleles).
3. Test the molecular beacon on seven control DNA samples without sequencing them.

3.3. Set Up the Reactions in a 96-Well Plate

1. Make up the PCR premix for one 384-well plate (1253 μL of water, 145 μL of 10X PCR buffer, 88 μL of DMSO, 45 μL of 50 m*M* $MgCl_2$, 12 μL of 25 m*M* dNTP) without *Taq*, primers, beacons, and template. The detailed protocol has been described ***(5–9)***. They are good at –20°C for at least 3 mo.
2. Before the experiment, for one 384-well plate one need to add 14 μL of *Taq*, 3 μL of primers-F (1 μg/μL), 12 μL of primers-R (1 μg/μL), and 20 μL of MB mixture (10 μ*M*) into the PCR premix and aliquot to a 96-well plate.
3. Add DNA templates (*see* **Note 2**), two allele-specific, homozygous control DNA samples and negative control (TE buffer).

3.4. Transfer from a 96-Well Plate to a 384-Well Plate

1. Apply 5 μL of mineral oil in each well of 384-well plates using automatic pipetting system. Open the BioWorks software for Biomek2000 Working Station → set up the plate configuration to make 5 μL per well → and run the program.
2. Apply the PCR mixture from 16 wells or 32 wells in a 96-well plate to a 384-well plate by Biomek2000 → set program to make 3 μL per well for the purpose of DNA dilution with 0.5 genomic equivalent per well in 384-well plate → make sure the pipet tip used is correct (20 μL) → run → accept all.

3.5. PCR Protocol

1. Place the adhesive plastic plate cover on the plates. Rub the cover evenly and gently with the plastic device. This rubbing is critical to keep samples at edge from evaporation. Salad spinning is highly recommended before PCR.
2. PCR was performed in a single step with the following protocol: 94ºC (1 min); four cycles of 94°C (15 s), 64°C (15 s), 70°C (15 s); four cycles of 94°C (15 s), 61°C (15 s), 70°C (15 s); four cycles of 94°C (15 s), 58°C (15 s), 70°C (15 s); 60 cycles of 94°C for (15 s), 55°C (15 s), 70°C (15 s); 94°C (1 min), and 60°C (5 min). Use hot lid, simulated plate, and volume set to 8 μL.

3.6. Reading Using Cytofluor Galaxy (BMG)

1. Open the BMG cytofluor in the program → control → plate out → insert the plate → plate in → measure. If the reading is near 65,000 in raw data, decrease the gain of the specific fluorophore and repeat the above until the optimal intensity range is achieved (the highest reading approx 45,000–55,000) (*see* **Note 3**).

3.7. Analysis of Digital SNP

1. In the analysis format, go to Summary in Excel sheet → adjust the ratio of one control allele to be 1 and the other control above 1.
2. To obtain digitalized results, the number of positive wells (green, red, or yellow) should not exceed 250 (optimal: 150–220 positive wells per plate).
3. SPRT analysis. To determine whether there is statistical significance for AI, we employed the sequential probability ratio test (SPRT) ***(10)***. This method allows two probabilistic hypotheses to be compared as data accumulate, and facilitates decisions about the presence or absence of allelic imbalance after study of a minimum number of samples. The details and application of the SPRT in allelic counting have been previously reported ***(6–12)***. If the ratio is above lower curve, then interpretation is allelic imbalance; if below higher curve, then interpretation is allelic balance; if between higher and lower curve, then interpretation is not informative and more wells are required to conclude anything. Alternatively, a receiver-operating characteristic (ROC) curve can be constructed to determine the sensitivity and specificity using a series of allelic ratio cutoffs ***(12)***.

4. Notes

1. Alternatively, a software is available for design of molecular beacon and primer. Please refer to "Beacon Designer 2" for Molecular beacon, TaqMan® probe and primer pair design software for Windows. http://www.premierbiosoft.com/
2. The DNA samples are purified form Qiagen PCR kit and the final volume in elution buffer is approx 150 μL. To determine the amount of DNA for digital SNP analysis, the DNA concentration was measured using the PicoGreen® dsDNA quantitation kit (Molecular Probes, Inc.) following the manufacturer's instruction. The fluorescence intensity was measured by a FLUOstar Galaxy fluorescence microplate reader with an excitation at 480 nm and an emission at 520 nm.

3. One can adjust the gain by selecting one positive control well → select one fluorophore → gain adjustment (well) → repeat for the other fluorophore. Alternatively, one can select the whole plate for gain adjustment by selecting all the wells. The instrument will then determine the highest reading of any well and adjust the gain so that this reading is no higher than 90% of 65,000.

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