TECAN.

Detection of a SNP in the Adenosine A2a receptor gene (ADORA2A) using fluorescence polarization of template-directed dye-terminator incorporation (FP-TDI)

Introduction

Except for identical twins or clones, no two organisms are genetically identical. There is a large range of genetic variation between two members of a species. In humans, two unrelated genomes are estimated to vary between 1 in 500 - 1000 base pairs. Given an estimated size of the human genome of 3 x 10^9, then there are approximately 3 x 10^6 DNA sequences that are non-identical between two unrelated individuals. Most of the variations occur as **S**ingle **N**ucleotide **P**olymorphisms (**SNPs**). These variants are the substitution of a single base in the DNA sequence for another, such as a C being replaced with a T (or U). SNPs are hypothesized to account for much of the genetic variation between individuals and are thought to affect processing of pharmacological agents and certain SNPs may confer higher risk of developing complex genetic diseases, such as, cancer, hypertension, diabetes etc. Although SNPs are numerous, genotyping of this form of genetic polymorphism has been difficult in many cases. One recently described method of genotyping SNPs, FP-TDI (**F**luorescence **P**olarization **T**emplate directed **D**ye **I**ncorporation) is simple, non-radioactive, relatively inexpensive, is done in a single tube/microplate well (homogeneous assay), has a very short development time for each assay, works for most SNPs and can be used for high-throughput genotyping.

Assay Theory

In practice, a PCR amplification product is generated using oligonucleotide primers that encompass the SNP (size 50 - 150 base pairs). After the PCR reaction, the unused PCR primers and free dNTPs are degraded simultaneously with *E. coli* exonuclease I (ExoI) and Shrimp Alkaline Phosphatase (SAP), respectively, in the same well. A third oligonucleotide (sequencing primer, or “probe”) that is complementary to the 20 - 25 bases just 5’ to the SNP base is then annealed and a “single-base extension” (SBE) reaction using fluorescently labeled dideoxynucleotides (ddNTPs) and no dNTPs is performed. Given the structure of the ddNTP, only one base can be added to the sequencing primer in this reaction and it will be complementary to the SNP base.

FP detection is based upon the rotational properties of the fluorescently labeled ddNTPs (di-deoxynucleoside triphosphate): free labeled ddNTPs rotate faster in solution than ddNTPs that have been added to the relatively larger oligonucleotide used as a sequencing primer. When the smaller ddNTP molecule is excited with plane-polarized light, the emitted light is largely depolarized because the molecule rotates rapidly in solution during the fluorescence lifetime (= time between excitation and emission), resulting in a low polarization value. If the ddNTP is added to the larger oligo-nucleotide, thereby increasing the effective molecular volume, the rotation is slowed to emit light in the same plane in which it was excited, resulting in an increase of the polarization value (G.J.Parker et al, 2000).

Experimental Setup

Test Procedure

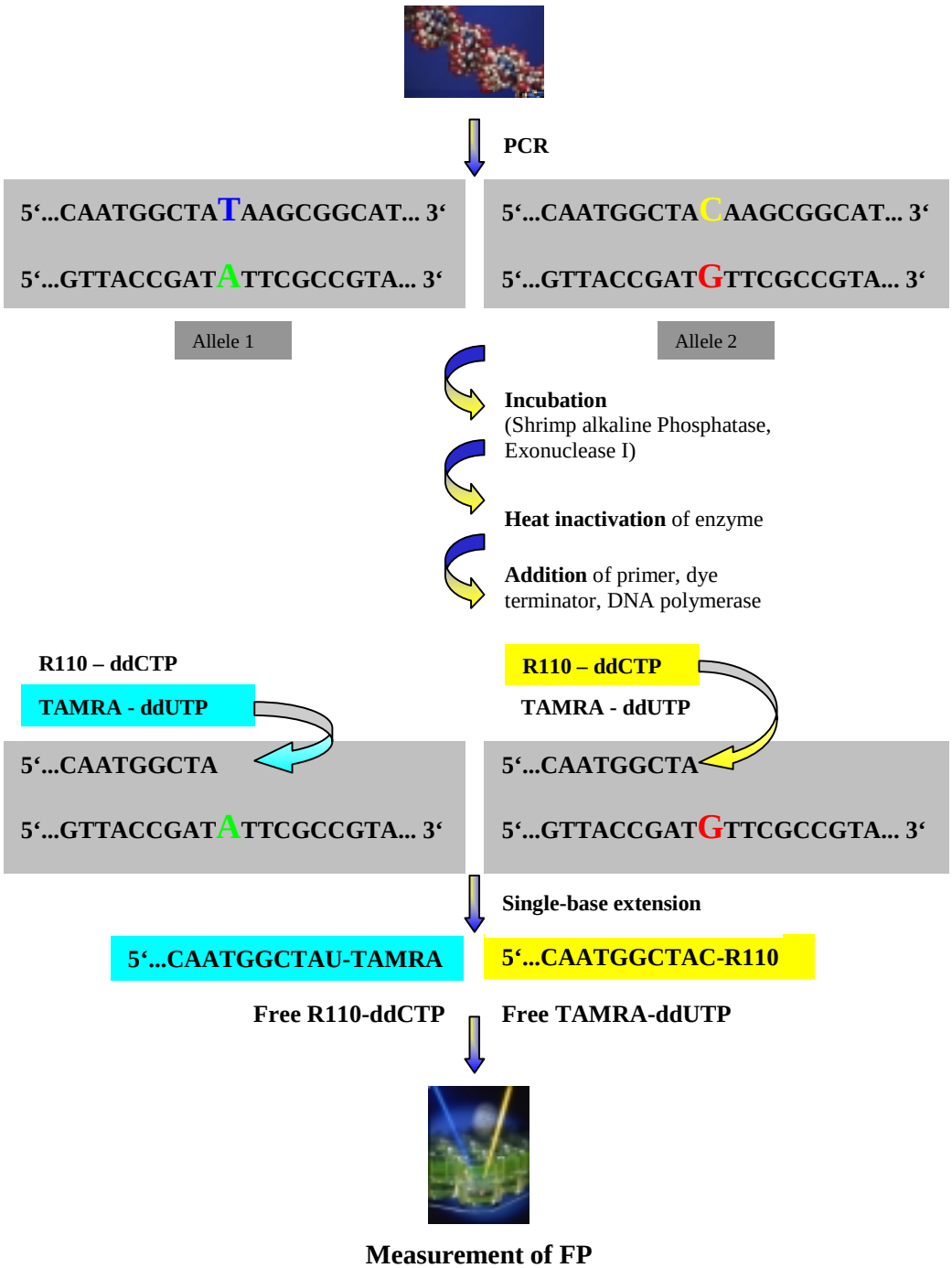

Figure 1 Outline of Assay Principle

Assay Procedure

A SNP in ADORA2A was assayed using Fluorescence Polarization detection of template-directed dye-terminator incorporation (FP-TDI), essentially as described by Chen et al. (1999). Briefly, 20 ng of genomic DNA was first amplified in 5 µl, using 1.5 mM $MgCl_2$, 1M anhydrous betaine, 20 mM Tris (pH 8.4), 50 mM KCl, 50 µM dNTP, 200 nM primer (A2A-F, 5'-AGGCAGCAAGAACCT TTCAA-3'; A2A-R, 5'-CTAAGGAGCTCCACGTCTGG-3'), and 0.25 unit of Platinum *Taq* DNA polymerase (GibcoBRL).

The reaction was cycled by using a touchdown protocol starting at 94°C for 3 min, followed by 10 cycles of 94°C for 30 sec, 65-55°C for 30 sec (decreased by 1°C intervals per cycle), and 72°C for 30 sec, followed by 30 cycles of 90°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec, with a final 10 min at 72°C. The reactions were performed on an MJ Research PTC-200 Thermal Cycler in 384-well plates (Marsh).

The remaining primers and dNTPs were degraded by adding to the PCR product a 5 µl aliquot containing 1 units of shrimp alkaline phosphatase (SAP) (Roche) and 0.5 units of *E. coli* exonuclease 1 (Exo1) (Amersham Pharmacia) in SAP buffer (50 mM Tris-HCl [pH 8.5] and 5 mM $MgCl_2$). This reaction was incubated for 90 min at 37°C, followed by inactivation at 95°C for 15 min.

To this reaction 5 µl of the FP-TDI mix of 50 mM Tris-HCl (pH 9.0), 50 mM KCl, 5 mM NaCl, 5 mM $MgCl_2$, 8% glycerol, 0.1% Triton X-100, 125 nM each of unlabeled ddATP and ddGTP, 109.2 nM each of unlabelled ddCTP and ddUTP (Amersham Pharmacia Biotech), 15.6 nM each of R110-ddCTP and TAMRA-ddUTP (NEN Life Science), 1.0 µM primer (A2A-Probe, 5'-AGTGCTCCCCACCCTG-AGCGGAGGCCCAATGGCTA-3'), and 0.4 units of Thermosequenase DNA polymerase (Amersham Pharmacia Biotech) were added. This mixture was incubated at 95°C for two min, followed by cycling 35 times at 94°C for 15 sec and 55°C for 30 sec, and then held at 4°C until the next step.

Fluorescence polarization was measured directly after transfer of 10 ul to a black polystyrene 384-well plate with the TECAN ULTRA.
To perform the assay the following steps were performed to set-up the ULTRA:

1. Optimum z-position: An arbitrary well containing the fluorophores is selected and the ULTRA automatically performs a scan in z-direction. Once found, the optimum z-position can be kept as a constant for following plates if the same volume and type of plate is used.

2. Determination of the G-Factors: The G-factor corrects for variances within optical elements used for the measurements with the polarizers in parallel and perpendicular position. In order to determine the G-factors for both filter combinations used for R110 and TAMRA the following layout was used:

- Wells A1 to A3: BGG Buffer, 10 µl
- Wells B1 to B3: R110/TAMRA with C/U nucleotide in BGG buffer, 10 µl

For having a common reference both were referenced to zero. The G-factors are automatically determined by the instrument and stored for further readings.

3. For both **R110 and TAMRA dichroic mirrors** were used to enhance sensitivity. The following filters are used:

- R110: excitation 485 nm, emission 535nm/ dichroic 505 nm
- TAMRA: excitation 535 nm, emission 590 nm/ dichroic 560 nm

4. The **FP values** were derived as described (Chen et al., 1999), and expressed in the dimensionless unit mP.

Results

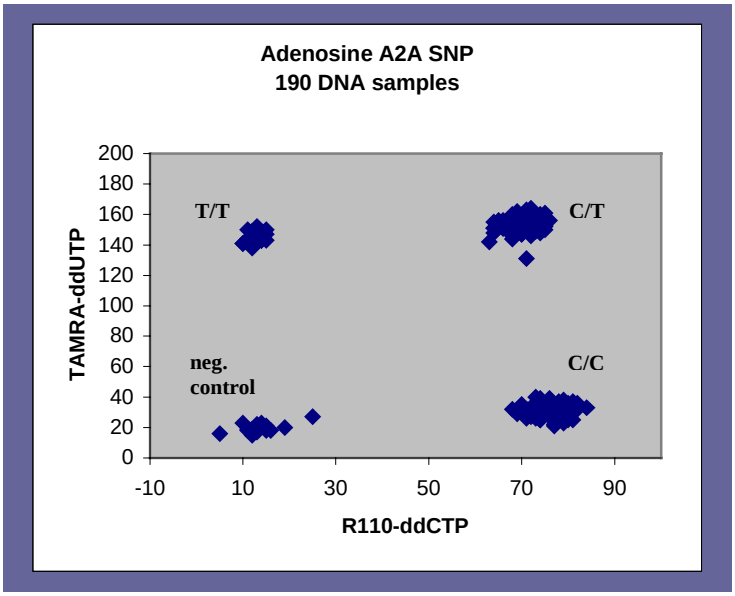

Figure 2 DNA from 190 individuals was genotyped as described above. Four clusters of readings were observed. In the lower left-hand cluster are values from 2 no DNA control wells and wells in which the initial PCR amplification failed. In the upper left-hand cluster, there has been incorporation of ddUTP, but not ddCTP, indicating that these individuals are homozygous T/T (U and T are interchangeable). The opposite occurs in the lower right-hand cluster, there is incorporation of ddCTP but not ddUTP, hence this cluster is formed of individuals of genotype C/C. The remaining cluster, in the upper right-hand corner, is composed of individuals who incorporate both dyes and are therefore heterozygotes (C/T) for this polymorphism.

Conclusion

As seen in Figure 2, four clusters of mP values are observed from the DNA samples. These represent the genotypes C/C, C/T, T/T and null as described in the figure legend. These DNA samples are from families and all the observed genotypes are transmitted in a Mendelian manner. Genotypes for a SNP in hundreds of individuals can be generated within 6 hours after receipt of the appropriate oligonucleotide primers. This is substantially faster than many of the competing high-throughput methods, such as those that use gels or microarrays, and therefore adds considerable flexibility in usage. In summary, genotyping of Single Nucleotide Polymorphisms (SNPs) by detection of incorporation of fluorescently labeled ddNTPs into an oligonucleotide primer using a TECAN ULTRA is fast, simple and accurate.

Literature References

CHEN X., LEVINE L., and P.Y. KWOK Fluorescence polarization in homogeneous nucleic acid analysis. Genome Res. *9*, 492-498, 1999.

PARKER G.J., T.L. LAW, F.J. LENOCH and R.E. BOLGER, Development of High Throughput Screening Assays using Fluorescence Polarization: Nuclear Receptor-Ligand Binding and Kinase/Phosphatase assays. J.Biomolec. Screening Vol. 5 (2), pp 77 – 88, 2000

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