Comparative Analysis of three different reagent systems offered for TaqMan®-based 5´exonuclease assays with regards to their compatibility

EPIDAUROS Biotechnologie AG offers to pharmaceutical companies a comprehensive package of custom pharmacogenetic services in a GLP-compliant environment and has already developed more than 70 pharmacogenetic profiling assays. Using these assays, patients/probands can be selected based on their genetic profiles of drug transporters, metabolizing enzymes and drug targets. The correlation between these polymorphisms and certain drug side effects makes it possible to identify patients/probands with a genetic predisposition.

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Introduction

EPIDAUROS Biotechnologie AG provides assay services for the identification of polymorphisms in drug transporters and drug metabolizing enzymes, including several members of the cytochrome P450 superfamily. Cytochrome *CYP2D6* is involved in the metabolism of a range of therapeutically important drugs including some tricyclic antidepressants, antiarrhythmics and morphine derivatives[1,2]. It is polymorphically expressed in the general population and therefore it is of major interest to the pharmaceutical industry to include *CYP2D6* in analytical pharmacogenetic approaches accompanying clinical Phase I or II trials. As a consequence of the polymorphic expression of *CYP2D6*, individuals greatly differ in the occurrence of side effects, therapeutic efficiency and drug interactions. In addition to some sequencing assays, spanning all exons of *CYP2D6*, EPIDAUROS Biotechnologie AG currently offers 13 TaqMan® technology-based genotyping assays for this gene, which also covers all frequent PM alleles (null alleles).

Different methods for the analysis of polymorphisms like SNPs, deletions, insertions or duplications can be applied[3]. TaqMan®-based 5´exonuclease assays are a rapid and economic method used in our laboratory for high and low throughput assays. Since the TaqMan® probes can discriminate between alleles during amplification it takes only 2 to 3 hours until data can be analyzed, enabling genetic testing starting with whole blood in less than a day.

There are many manufacturers offering reagent systems for TaqMan®-based assays used in genetic analysis. Service providers like EPIDAUROS Biotechnologie AG who work in a GLP-compliant environment should not depend on only one supplier for their so-called *critical materials* (which include the chemical compounds for TaqMan®-based assays). Therefore we selected two assays, one investigating the presence of the variant 1846G>A determining the **4* allele in *CYP2D6*[4], and a second very sensitive testing system exploring the copy number of that gene. These assays were used to test three reagent systems for TaqMan®-based assays from three different manufacturers for their mutual compatibility.

Materials and Methods

The TaqMan® technology involves amplification of a PCR fragment with simultaneous detection of the degradation of a labeled probe. Probes are labeled at both ends with an allele-specific fluorescent dye and a quencher. During the amplification reaction the specifically hybridized probe is displaced by the 5' exonuclease activity of the DNA polymerase and is degraded. Upon degradation quencher and dye are separated and the fluorescence signal is increased. An increase in signal is indicative of the presence of the respective allele. In assays for allelic discrimination two probes are present, one for each allele, and fluorescence of both dyes is analyzed and compared to measure the genotype.

A batch of either 32 (1846G>A) or 28 (copy number assay) genomic DNA samples of already known genotype was reanalyzed in the testing approach. The following reagent systems for TaqMan®-based assays were tested in a comparative approach with regards to their power of resolution in allelic discrimination: (1) ABgene® 2x ABsolute™ QPCR ROX Mix (at 500nm ROX), (2) 2x ready-to-use mix from manufacturer A and (3) a mix system consisting of a kit comprising single chemical compounds from manufacturer E.

In the first test assay investigating the presence of the variant 1846G>A, the ABgene® reagent system was compared to that offered by manufacturer A. Reactions containing genomic DNA samples were run in volumes of 10µl, and samples employing oligonucleotide-based artificial references for the homozygous wild-type and the variant in volumes of 5µl. A comparative assay was set up with the mixes and simultaneously amplified in a PE9700 thermal cycler running in the 9600 emulation mode. Fluorescent signals were subsequently recorded in a fluorescence

reader. The fluorescent data of the reporter dyes (VIC reporting for the presence of the wild-type and FAM for the variant) were normalized against ROX and analyzed in a scatter plot.

All three reagent systems (ABgene®, manufacturers A and E) were tested in a second assay to determine the copy number of *CYP2D6*. This assay was developed at EPIDAUROS Biotechnologie AG and analyzes the *CYP2D6*-specific probe reaction (FAM-labeled) in correlation to that of a second probe (VIC-labeled) being specific to a reference gene of a constant copy number. The assay reactions of the three testing compounds were simultaneously amplified and recorded in the real-time mode. The same sample line of genomic DNA was used as for the first assay dealing with the **4* allele of *CYP2D6*.

Results

A scatter graph (Figure 1) was generated for allelic calling of the *CYP2D6*4* assay (1846G>A) using normalized values of the two fluorescent TaqMan® probes. The assay showed an excellent resolution in the formation of distinct clusters for each of the following sample categories: (i) NTCs (No Template Controls = negative controls), (ii) homozygous wild-types, (iii) homozygous variants and (iv) heterozygotes. The quality of resolution was the same for both assays, either run with the ABgene® ABsolute™ QPCR ROX Mix or with the product of manufacturer A. The samples of both assays agreed in their genotyping values. With both mixes the signals generated by the artificial references for the wild-type formed separate clusters exhibiting stronger signal intensities than the genomic samples of the same genotype. These references are artificially created template DNAs based on the usage of synthetic oligonucleotides comprising the annealing sites for the amplification primers and the corresponding allelic hybridization sites of one of the two probes. Thus artificial references derive from a very homogenous source and therefore generate a stronger fluorescent signal than that of genomic DNA samples (all TaqMan®-based assays developed and run at EPIDAUROS Biotechnologie AG use artificial references generated in-house). The results clearly demonstrate that the ABgene® ABsolute™ QPCR ROX Mix can be substituted for the product of manufacturer A.

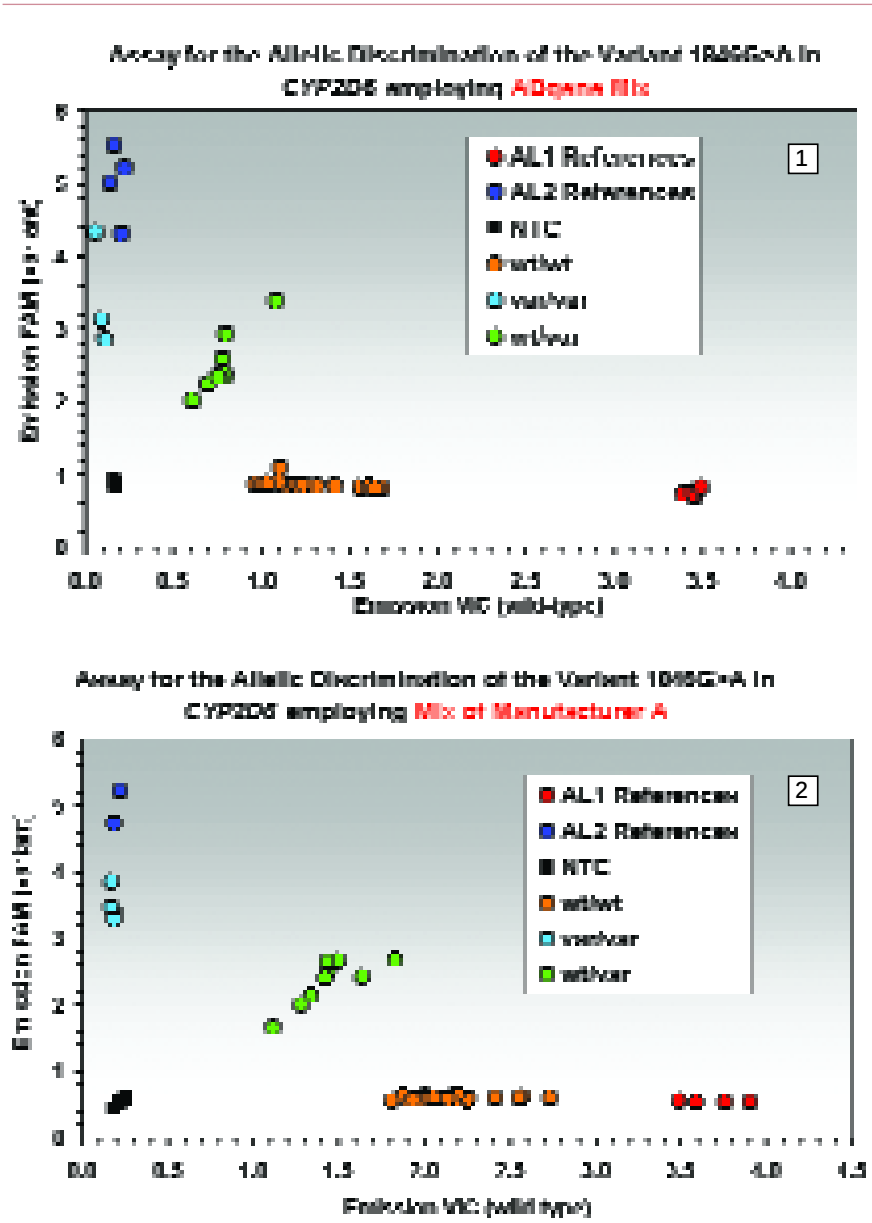

Figure 1: Scatter graph of the fluorescent data of the assay investigating the presence of the variant 1846G>A in the *CYP2D6* gene run with the ABgene® ABsolute™ QPCR ROX Mix (1) and the product of manufacturer A (2). AL1: wild-type allele, AL2: variant allele, NTC: No Template Control.

Figure 2 shows the result of the assay investigating the copy number of *CYP2D6*. In this assay, the copy number of the gene is determined by the ratio of the fluorescent signals raised by the degradation of two gene-specific but differentially labeled probes during the amplification reaction. One probe is specific for the *CYP2D6* gene, the other for a reference gene of a constant copy number of two. This type of assay was developed at EPIDAUROS Biotechnologie AG and is routinely analyzed in a semi-automated manner with an Excel-based software tool also developed in-house. The frequency distribution of the ratios of the two dyes of the sample line amplified with (1) ABgene® ABsolute™ QPCR ROX Mix, (2) the product of manufacturer A and (3) with the product of manufacturer E is shown in the graphs. The inclusion of reference samples of known copy numbers of *CYP2D6* allows the assignment of a specific gene dose value to the different clusters formed by the signal ratios of the samples. The best resolution is indicated by a polymodal distribution of sharp and distinct sample clusters referring to the copy number as a function of the signal ratios. The ABgene® ABsolute™ QPCR ROX Mix gives the best resolution followed by those generated with the product of manufacturer A. In contrast, the product of manufacturer E gave a lower resolution, resulting in the generation of ambiguous results for two samples, the signals of which were located in between the clusters formed by the signal ratios of samples of the copy number two and three.

The reaction kinetics generated with the three different reagent systems in the assay investigating the copy number of *CYP2D6*

were compared (data not shown). With all three reagent systems the kinetics of both probes showed no significant differences in pattern, with a strong increase in signal intensity between PCR cycles 21 and 24 for the VIC reaction and with an earlier on-set (cycle 20) for the FAM reaction.

Conclusions

The assay investigating the copy number of *CYP2D6* is one of the great successes and key tools in our genetic studies accompanying clinical trials. However, the assay proved to be very sensitive to inhibition from contaminants, requiring pure genomic DNA with regards to the $A^{260}/_{280}$ ratio. Prior to this comparative analysis, we obtained only reliable and reproducible results employing the reaction mixture of manufacturer A. In this testing approach, the ABgene® ABsolute™ QPCR ROX Mix performed comparably with the corresponding product of manufacturer A. Since the chemical compounds used for the TaqMan®-based allelic discrimination (AD) assays in our GLP-compliant genotyping studies are critical materials requiring the availability of alternative suppliers, it is of major benefit to us that the ABgene® mix is fully interchangeable with the product of manufacturer A. Testing of the ABgene® mix with several other TaqMan® AD assays developed in-house showed equivalent performance with products of other manufacturers (data not shown), which confirmed the results presented here.

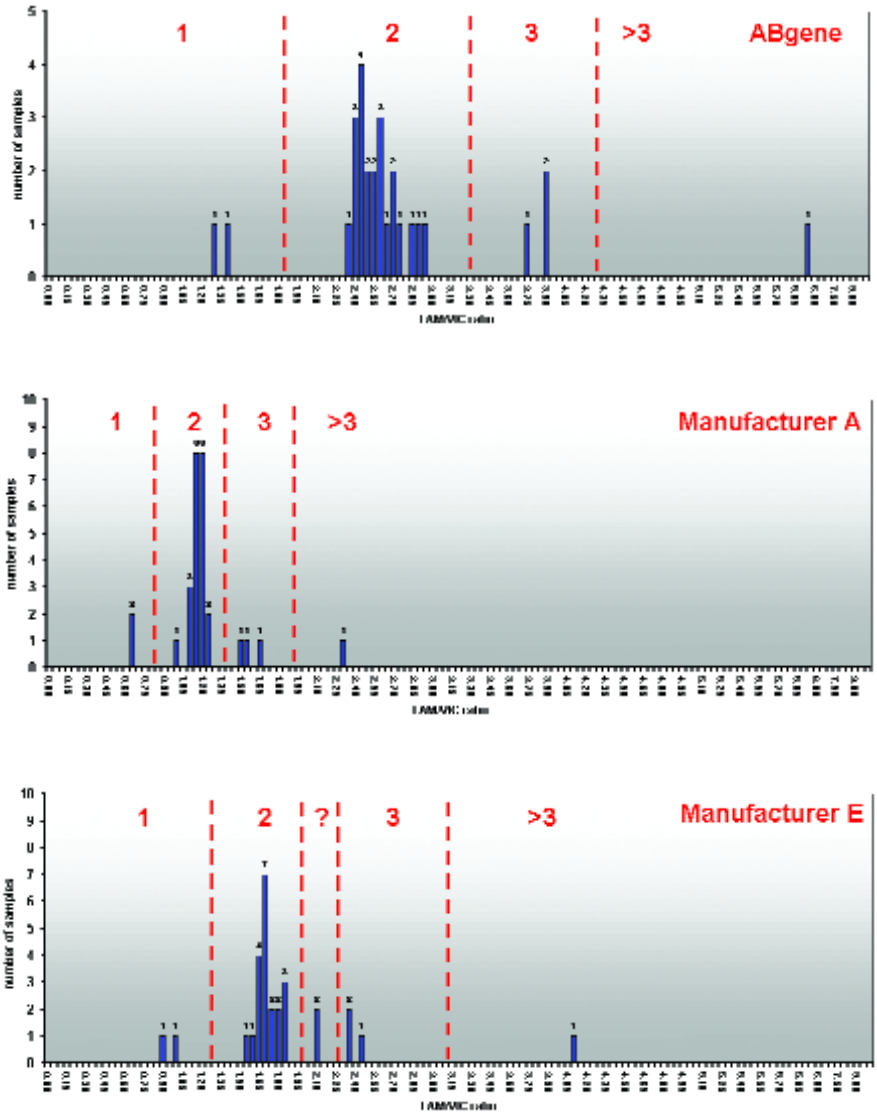

Figure 2: Comparison of the frequency distribution of the ratios of the *CYP2D6* -specific signal and the signal of the reference gene in a TaqMan® assay investigating the copy number of *CYP2D6* . The assays used ABgene® ABsolute™ QPCR ROX Mix (top), the product of manufacturer A (middle) and of manufacturer E (bottom). The copy numbers were assigned to the signals following the formation of clusters in the polymodal pattern of the frequency distribution.

References

1. Cytochrome P450 drug interaction table. Indianapolis, Indiana: Indiana University, Department of Medicine; 29-April-2002. http://medicine.iupui.edu/flockhart/.

2. CYP2D OMIM entry. Baltimore, Maryland: John Hopkins University;28-May-2002. http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?id=124030.

3. Shi MM. (2001) Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. *Clin Chem* 47(2):164–172.

4. *CYP2D6* allele nomenclature. Stockholm, Sweden: Human Cytochrome P450 (CYP) Allele Nomenclature Committee. http://www.imm.ki.se/CYPalleles/cyp2d6.htm.