Because the enzymatic products are resolved on the basis of their m/z ratios, we are exploring the use of MS/MS for the quantification of multiple enzymes in a single dried blood spot by use of a single-platform analytical method in much the same way that MS/MS is currently used to determine the concentrations of multiple metabolites in a single dried blood spot.

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Comprehensive Mu-Opioid-Receptor Genotyping by Pyrosequencing, *Carsten Skarke,* Anja Kirchhof, Gerd Geisslinger, and Jörn Lötsch* (*pharmazentrum frankfurt*, Institute of Clinical Pharmacology, Johann Wolfgang Goethe-University, Theodor Stern Kai 7, 60590 Frankfurt, Germany; * author for correspondence: fax 49-69-6301-7636, e-mail skarke@em.uni-frankfurt.de)

The human μ-opioid receptor, encoded by the *OPRM1* gene *(1, 2)*, is the major site for the analgesic action of opioids. The *OPRM1* gene is therefore a first-line candidate for evaluating the role of mutations on the clinical effects of opioids. The mutant allele of the 118A>G single-nucleotide polymorphism (SNP) in the *OPRM1* gene, which codes for an Asn40Asp μ-opioid receptor, has been associated with decreased opioid activity in carriers of the 118G allele. Morphine 6-glucuronide (M6G) and morphine have lower potencies for pupil constriction in carriers of the mutation, who also vomit less often after treatment with M6G than noncarriers *(3)*. Carriers of the 118G allele need more alfentanil for postoperative analgesia but have less pain relief than noncarriers *(4)*. The 118G allele has also been associated with a greater cortisol response to opioid receptor blockade with naloxone *(5)*.

Regarding opioid addiction, the mutant allele of the 17C>T SNP was found more frequently in drug addicts than in nonaddicts *(6, 7)*. An association between the mutant alleles of the 118A>G (exon 1) and 691C>G (intron 2) SNPs and opioid dependence was reported for Chinese heroin addicts, although this was based on a small study group *(8)*. The frequency of the mutated 118G allele was higher in Indian heroin addicts than in controls *(9)*. Addicted individuals carrying both the mutated 118G allele and the mutated 31A allele in intron 2 consumed higher doses of heroin than individuals who did not carry these mutations *(10)*. The simultaneous presence of the mutated alleles for SNPs −1793T>A, −1699-(−1698)insT, −1320A>G, −111C>T, and 17C>T is associated with substance dependence *(11)*. In European Americans, allele −2044A and haplotypes that include −2044A were found to be associated with susceptibility for substance dependence *(12)*.

To promote further investigation of an association of *OPRM1* mutations with altered opioid effects or substance dependence, we describe a rapid screening method for several mutations in the *OPRM1* gene. SNPs in the *OPRM1* gene that qualify for large-scale screening in patients were selected to become part of the screening method when they met one of the following three criteria: (*a*) in vitro or human studies had revealed a functional consequence; (*b*) the mutation causes an amino acid exchange, encoding an altered opioid receptor protein; or (*c*) the SNP has a high reported allelic frequency, which implies that it could have immediate clinical relevance for the administration of opioids in a large part of the population. On that basis, a total of 23 SNPs in the promoter region; in exons 1, 2, and 3; and in the second intron were chosen for screening (see Table 1 for details). To this we added the SNPs −54G>T in the promoter, 24G>A in exon 1 and 942G>A in exon 3 because their close proximity to SNPs −38C>A, 17C>T and 877G>A, respectively, allowed their detection by use of the already available PCR templates with a small extension of the respective assays (see the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol50/issue3/). Thus, our method includes a total of 26 SNPs.

To detect the 26 selected SNPs in *OPRM1*, we developed a set of 14 assays (Table 1) based on the real-time pyrophosphate detection method Pyrosequencing™ *(13)*.

Table 1. SNPs in the *OPRM1* gene detected by pyrosequencing.

Assay no.	Mutation[a]	Amino acid location	Exon/Intron/ Promoter	Nucleotide sequence to be analyzed,[b] 5′–3′	dNTP[c] dispensing order	Pyrosequencing assay type	Mutated allele frequency, %	
							Reported	Observed
1	−2044C>A		Promoter	TCTT/GCTCTGAAACTA	GTCTGACTC	Reverse simplex	0.8 *(12)*	2.2
2	−1793T>A			A/TTTTAAGTAATGAGAAGAC	GTATACGTATG	Forward simplex	1.4 *(11)*	0.9
3	−1699-(−1698)insT			T/GACTCCAAGGTCAG	CTGTACTCA	Forward simplex	1.4 *(11)*	1.3
4	−1320A>G			CT/CCAGTTGG	GCTCTAGT	Reverse simplex	1.4 *(11)*	0.4
5	−692G>C			AC/GAAACCTGTTGGGAACAA	TACGTACTG	Reverse simplex	4.3 *(11)*	Not found
6	−172G>T			GC/ATGAGCATCTGAC	CGCAGTGCAGTAGAT	Reverse duplex	11.4 *(11)*	3.4
	−111C>T			TGCTA/GTTTCTTACAG			1.4 *(11)*	Not found
7	−54G>T			G/TCCAGGAGCACCGAGAA/ CTTTTCGGGTTCCA	AGTGCAGAGCACGAGACGT	Reverse simplex	Not reported *(27)*	Not found
	−38C>A						1.4–5 *(7, 11)*	Not found
8	12C>G	S4R	Exon 1	C/TGTGGGGG/ACA GCC/GCTGCTGTCCA	ACTCGTGAGCAGCGCGT	Reverse simplex	Not reported *(2)*	Not found
	17C>T	A6V					1.9 *(6)*	0.9
	24G>A	T8T					2 *(6)*	0.4
9	118A>G	N40D		C/TGCCATCTAAGTGGG	GCTAGCATC	Reverse simplex	11.5–15.4 *(6, 16, 25, 26)*	12.1
10	440C>G	S147C	Exon 2	CTC/GCATAGATTACTATA/ GACATGTTC	GCTGCGATAGATACTATGAGC	Forward simplex	<1 *(16)*	Not found
	454A>G	N152D					~1.4 *(11, 27)*	Not found
11	IVS2 31G>A		Intron 2	T/CTCCTTCCCTCAGGC TGGTAACATCACTCACCTTGCC	GCTGCTCTACTG CGAGCAGCTGTGAG	Reverse triplex	4.2–14.3 *(10, 11)*	8.2
	IVS2 106T>C			AAAATTACAG/ATGTGACTAAGAC			1.4 *(11)*	Not found
	IVS2 691C>G (=1031C>G)			ATTG/CATTTTTAGCCTTGACCA			42.9 *(11, 16)*	53.4
12	IVS2 397T>A			GGA/TTTGTTCAATATTCTGAT ATTAGGTGTAGAAAGAT ACATTTGCCATGT	TCTATGTATACA CATAGTCTAGCGAGTAT	Reverse duplex	1.4 *(11)*	Not found
	IVS2 438G>A			T/CGGCTCCAGGTAATGGATGTTTTCACTTC ATTTTTTGATGG			4.3 *(11)*	Not found
13	779G>A	R260H	Exon 3	T/CCTTTTCTTTGGAGCCAGG/ AGAGCATGT/CGGACACTCTTGAGGT/ CGCAAGATCATCAGTCCATAGCACACGGT	GTCGTTCTGAGCAGAGAG TCATGTCTGACACTCTGAGTC	Reverse simplex	<1 *(6)*	Not found
	794G>A	R265H					<1 *(6, 22)*	Not found
	802T>C	S268P					<1 *(22)*	Not found
	820G>A	K273A					<1 *(28)*	Not found
14	877G>A	I877V		AC/TGATGAACACA	GCTCGTACTCGA	Reverse duplex	<1 *(10)*	0.4
	942G>A	T314T		C/TGTAGTTT			<1 *(6)*	Not found

[a] SNPs are listed according to their location in the *OPRM1* gene. The allelic frequencies are indicated according to the literature and as found in our study population.

[b] The sequence to analyze given is long enough to be accepted by the Pyrosequencing software when entering the assay. Note that some nucleotides entered at the end of that sequence will not be analyzed with the dispensed deoxynucleotide triphosphates.

[c] dNTP, deoxynucleotide triphosphate.

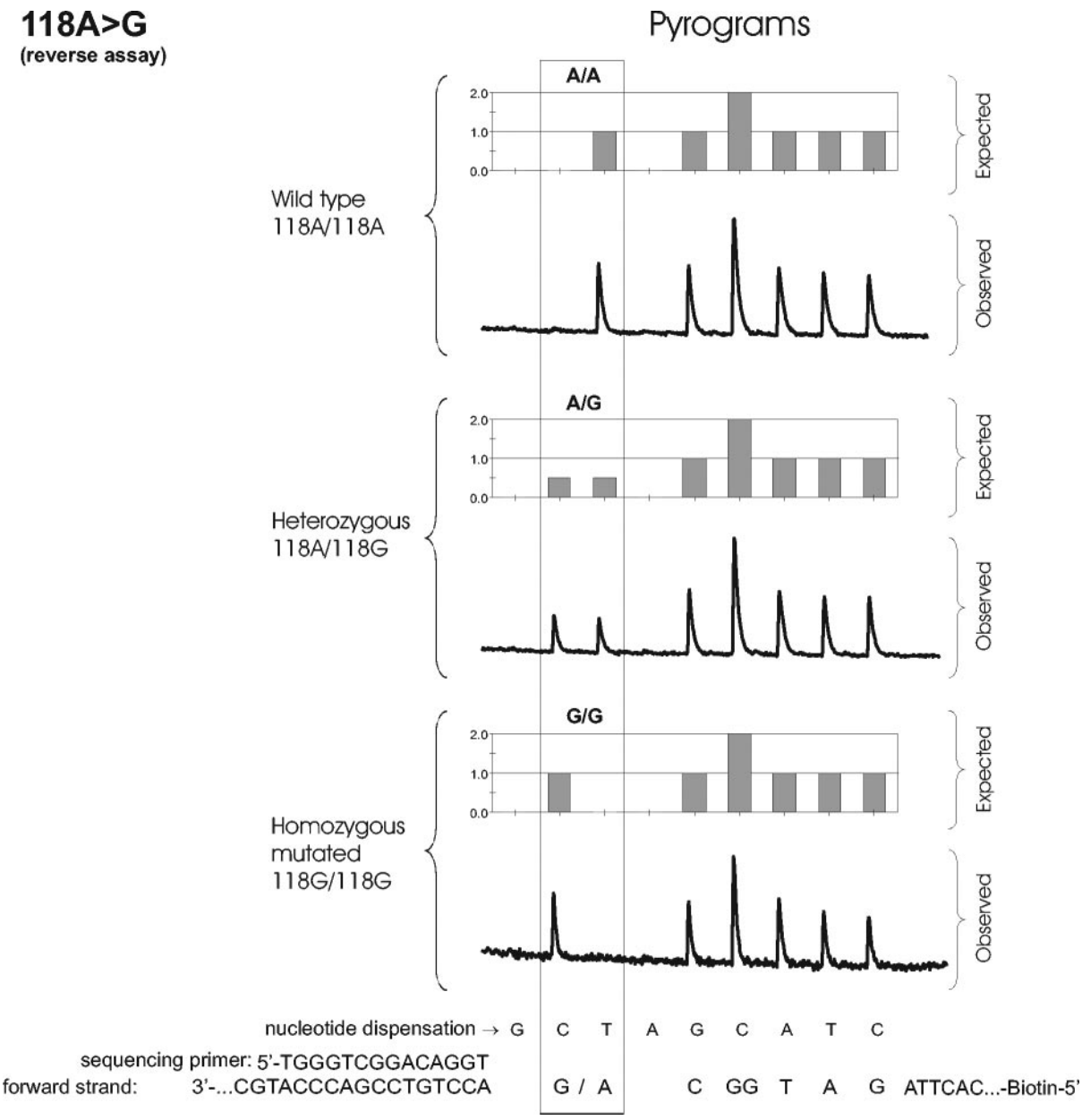

Fig. 1. Assay design with expected and observed pyrograms, i.e., signals produced by the Pyrosequencing method, for the detection of SNP 118G>A in the *OPRM1* gene.

The SNP position is *boxed*. The sequence to be analyzed based on the cDNA position with attached sequencing primer determines the nucleotide dispensation order. The biotinylated forward DNA strand is used in the reverse assay. Note that in the pyrograms the segments corresponding to the dispensation of enzyme and substrate have been omitted.

DNA from 116 healthy Caucasians who had given written informed consent was used to generate *OPRM1* DNA fragments. Genotyping was approved by the University of Frankfurt Medical Faculty Ethics Review Board. Primers were designed with "Oligo" primer analysis software (Molecular Biology Insight, Inc.) with use of the *OPRM1* nucleotide sequences provided with Ensembl Gene ID ENSG00000112038 (Table 2 in the online Data Supplement). A standard thermal cycling protocol (see Table 2 in the online Data Supplement for the number of cycles) was used. Cycling was for 30 s at 95 °C, 60 s at primer-specific temperatures (Table 2 in the online Data Supplement), and 30 s at 72 °C. Pyrosequencing primers (Table 2 in the online Data Supplement) were designed with use of SNP Primer Design software (Pyrosequencing AB; http://www.pyrosequencing.com). A series of simplex, duplex, and triplex assays were generated on a PSQ 96MA (Pyrosequencing AB; Table 1, Fig. 1, and online Data Supple-

ment). In most cases, reverse assays were identified to be best suited for detection. A forward assay was applied in the case of the SNPs −1793T>A and −1698-(−1699)insT, and the duplex assay covering 440C>G and 454A>G (see the online Data Supplement). For each pyrosequencing assay, PCR template for each *OPRM1* SNP was incubated in a shaker (10 min) with streptavidin-coated Sepharose beads (Amersham Pharmacia Biotech) and prepared with 672 mL/L 96% ethanol and denaturation buffer in a Vacuum Prep Workstation (Pyrosequencing AB) for transfer of the biotinylated templates into 55 μL of the corresponding 0.35 μmol/L sequencing primer (Table 2 in the online Data Supplement). Sequencing took place in a PSQ 96MA after incubation for 2 min at 80 °C. For confirmation of the pyrosequencing results, a total of 48 samples [4 randomly chosen samples for each of the 12 DNA templates (Table 1), including 2 mutated DNAs where available] were sequenced by conventional methods on an ABI PRISM 310 Genetic Analyzer (PE/Applied Biosystems). For haplotype analysis, all pyrosequencing results were submitted to HAPLOTYPER computer software for Linux (Harvard University) *(14)*. As an alternative to haplotyping by software, molecular haplotyping would be possible with pyrosequencing using allele-specific PCR templates *(15)* in an otherwise identical assay.

The observed pyrograms corresponded to the predicted theoretical assay outcomes, allowing clear identification of the genotypes in all SNPs. Pyrosequencing results agreed with the results obtained with conventional sequencing of random samples. A pyrogram exemplifying the assay for the 118A>G SNP is shown in Fig. 1; more examples are available in the online Data Supplement. The observed allelic frequencies of the mutations are given in Table 1. The most frequent SNP was 691C>G in intron 2 (frequency of the mutated allele, 53.4%). The 118A>G SNP in exon 1 was found at a frequency of 12.1% (mutated allele). The observed allelic frequencies corresponded to those published previously (Table 1) except for the −692C and −38A alleles, which had previously been reported to have frequencies of 4.3% and 1.4–5% *(11)*, respectively, but which we did not find in any of the 116 participants in our study. We found fewer carriers than reported previously for the mutated allele −172T [3.4% vs 11.4% *(11)*], whereas we found the mutated 691G allele at a higher frequency in our study population [53.4% vs 42.9% *(11, 16)*]. The most frequent haplotype (39%) contained an IVS2 691G>C mutation and no mutation at any of the other 25 analyzed loci. The second most frequent haplotype had no mutations in any of the 26 positions (frequency, 33%). A haplotype with a mutation at only 118A>G was found at a frequency of 10%, and a combination of IVS2 mutations 31G>A and 691G>C was found at a frequency of 7%. The 118G allele was found together with the IVS2 691G>C allele, without any other mutations, at a frequency of 1.3%.

Receptors encoded by the mutated allele of the 118A>G SNP were reported to have a threefold higher binding affinity for β-endorphin compared with wild-type receptors in AV-12 cells transfected with the 118G cDNA *(6)*. However, the binding in HEK293 cells transfected with mutated 118G cDNA was not affected *(17)*. Clinical data have indicated decreased opioid activity in heterozygous carriers of the 118A>G SNP *(3, 4, 18)*. In a previous report, a patient not responding to morphine therapy was heterozygous for the 118G allele, whereas a patient who responded was wild type at this position *(19)*. In another report, a 118G carrier with renal failure tolerated high plasma M6G, whereas a patient who did not have the mutation was sedated as a consequence of M6G accumulation *(20)*. The mutated allele of SNP 802T>C SNP has been shown to produce altered receptor desensitization and receptor signaling with decreased G-protein coupling *(21)*. The affinity of μ-opioid-receptor agonists such as morphine; diprenorphine; D-Ala(2),*N*-MePhe(4),Gly(5)-olenkephalin (DAMGO); β-endorphin; metenkephalin; and dynorphine was not changed, but the potency and efficacy of DAMGO, β-endorphin, and morphine were decreased *(22)*. Mutated alleles containing SNPs 779A>G and 877G>A are possibly involved in altered desensitization of the μ-opioid receptor *(23)*. Finally, mutated alleles carrying the −T1793A SNP and an inserted thymine at position −1699 were found to potentially influence transcriptional regulation *(24)*.

Thus, evidence from in vitro experiments and clinical studies points to a functional importance of several *OPRM1* SNPs. Research on SNPs in the *OPRM1* gene is, therefore, of immediate interest for assessing the clinical effects of opioid analgesics and for studying the epidemiology of substance addiction. We provide a screening method suitable for large-scale genetic diagnosis of 26 SNPs in *OPRM1*. The method includes SNPs with reported high allelic frequencies as well as rare SNPs with demonstrated or potential functional relevance. The method presented could facilitate identification of *OPRM1* mutations with clinical relevance and thus enable individualized opioid pharmacotherapy in the near future.

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Comparison of Methods for Polycythemia Rubra Vera-1 mRNA Quantification in Whole-Blood Leukocytes and Purified Granulocytes, *Lars Palmqvist,[1†] Philipp Goerttler,[4] Carina Wasslavik,[1] Peter Johansson,[2,3] Björn Andreasson,[2,3] Soodabeh Safai-Kutti,[2] Jack Kutti,[2] Heike L. Pahl,[4] and Anne Ricksten[1*]* ([1] Department of Clinical Chemistry and Transfusion Medicine, Institute of Laboratory Medicine and [2] Hematology and Coagulation Section, Department of Medicine, Sahlgrenska University Hospital, Göteborg, Sweden; [3] Department of Medicine, Uddevalla Hospital, Uddevalla, Sweden; [4] Department of Experimental Anaesthesiology, University Hospital, Freiburg, Germany; † current address: BC Cancer Research Centre, Terry Fox Laboratory, Vancouver, BC, Canada; * address correspondence to this author at: Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden; fax 46-31-828458, e-mail anne.ricksten@clinchem.gu.se)

In the absence of pathognomonic markers, the diagnosis of the two chronic myeloproliferative disorders polycythemia vera (PV) and essential thrombocythemia (ET) has relied on a set of clinical and laboratory criteria *(1–5)*. The cloning of the cell surface receptor polycythemia rubra vera-1 (PRV-1) has recently been described *(6)*, and the consistent overexpression of PRV-1 mRNA observed in PV patients indicates that this might constitute a new diagnostic marker for the disease. In the initial cohort examined by Northern blot analysis, PRV-1 expression was increased in all PV patients examined as well as in some ET patients, but not in healthy controls *(6)*. These results have also been verified and extended using a quantitative reverse transcription-PCR (RT-PCR) assay. All PV as well as 50% of ET patients displayed increased PRV-1 expression *(7, 8)*. Patients with secondary erythrocytosis and healthy controls tested showed PRV-1 concentrations within the reference interval. Interestingly, the observed increase in PRV-1 mRNA expression does not lead to a corresponding increase in protein expression on the cell surface *(9)*. Erythropoietin-independent colony growth and PRV-1 overexpression seem to go hand in hand in both PV and ET patients *(7)*, raising the hope that RT-PCR for PRV-1 could replace the need for the technically demanding erythropoietin-independent colony assay, although a recent report suggests that the erythropoietin-independent colony growth assay is a more reliable method *(10)*. The aim of the present work was to develop a quantitative RT-PCR method to measure PRV-1 transcripts in whole-blood leukocytes and to determine the potential usefulness of the method in the differential diagnosis of polycythemias and thrombocytosis. The assay was compared with a method using isolated granulocytes *(7, 9)* to assess whether granulocyte purification is necessary before RNA extraction. Granulocyte fractionation is cumbersome to standardize, and an assay that uses whole-blood leukocytes would simplify the analysis and perhaps make it more suitable for a routine laboratory setting.

Blood samples were collected from 78 patients with