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BRIEF COMMUNICATION

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A simple genotyping method for *CD247* 3′-untranslated region polymorphism rs1052231 and characterization of a reference cell panel

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CD247 (or CD3-ζ chain) is an essential adaptor and signal-transducing molecule of the T-cell antigen receptor (TCR) complex, and it also couples to NK-cell activating receptors such as NKp46, NKp30 and CD16A (FcγRIII). Noncoding sequence polymorphisms and variations in *CD247* expression, a tightly regulated process, have been related with an altered immune response in multiple health conditions. A single nucleotide polymorphism (T > A) at nucleotide 844 of the *CD247* 3′-untranslated region, rs1052231, has been related with lower *CD247* gene expression and it has been investigated as a potential biomarker of autoimmune disease. We present here a simple, accurate, reliable, time-efficient, and cost-effective method for *CD247*-rs1052231 genotyping. Using this method, based on polymerase chain reaction with confronting two-pair primers (PCR-CTPP), we have also characterized the *CD247*-rs1052231 genotypes in a panel of worldwide available cell lines, which should facilitate study of the role of this polymorphism in immunity and human health.

KEYWORDS
alleles, CD247, CD3-ζ, genetic polymorphism, genotyping, human genetics, T-cell receptor, untranslated region

T-lymphocyte recognition and response to antigens are achieved through a functional complex formed by a variable clonotypic T-cell receptor (TCR), which recognizes antigens, generally peptides presented by Major Histocompatibility Complex (MHC) molecules; and a signaling complex, formed by the invariant chains CD3-δ, -ε, -γ, and CD247 (formerly designated CD3-ζ or TCR-ζ). Assembly and cell-surface expression of the TCR/CD3/CD247-complex are highly regulated processes, which condition T-cell development and function. In general, only fully assembled complexes reach the cell surface, thereby variations in the expression of the invariant chains modulate TCR surface levels and the ensuing T-cell activation in response to antigen.[1] However, CD247 downregulation with conserved TCR/CD3 surface expression has been observed in chronic inflammation, cancer, infection, and autoimmune disease.[2]

CD247 differs from the rest of TCR subunits in its structure, genetic organization, and chromosomal

location.[3] Its long intracellular domain contains three immunoreceptor tyrosine-based activation motifs (ITAMs), unlike CD3-γ, -δ, and -ε chains bearing a single ITAM; and it assembles in disulfide-bond homodimers within the TCR/CD3-complex. It is noteworthy that CD247 is present, not only in T-lymphocytes, but also in NK cells, where it forms complexes with activating NKp46, NKp30, and CD16 (FcγRIII) receptors, enabling signal transduction and the ensuing effector functions.[4,5] In NK cells, loss of the adaptor FcεR1γ-chain (a late maturation event) favors subsequent CD16 engaging to CD247, hence enhancing antibody-dependent cellular cytotoxicity.[6,7] Conversely, replacement of CD247 by the FcεR1γ-chain in T-cell subsets has been observed in pathological conditions.[8,9] Thus, changes in CD247 expression may modulate both innate and adaptive immunity and be potentially pathogenic.

The *CD247* (*CD3Z*) gene is located on chromosome 1 and spans ~88 kbp.[3] Whereas its coding sequence is virtually invariant, multiple noncoding single nucleotide polymorphisms (SNPs), alternative splicing, and post-transcriptional regulation can affect gene expression.[10–13] *CD247* genetic and functional variability has been related to the clinical course or susceptibility to autoimmune disease in humans and rodents, including systemic lupus erythematosus (SLE),[10,14–16] systemic sclerosis,[17,18] rheumatoid arthritis,[19–21] and diabetes.[22,23] The minor allele of rs1052231 T > A, affecting nucleotide 844 of the *CD247* 3′-untranslated region (UTR), has been associated with lower gene and protein expression,[10] which could also be conferred by other *CD247* polymorphisms. Because a genetically determined decrease in *CD247* expression could further enhance the protein down-regulation observed in T cells in chronic inflammation, rs1052231 has been investigated as a susceptibility factor in autoimmune disease (i.e., SLE and rheumatoid arthritis), for which there is conflicting evidence.[10,16,20,24,25]

To facilitate rs1052231 analysis, we have designed a genotyping assay based on polymerase chain reaction with confronting two-pair primers (PCR-CTPP). With this method, the two SNP alleles are detected simultaneously in a single, standard PCR, allowing fast and simple genotype assessment of large collections of DNA samples.[26–28] For PCR-CTPP, four primers are used, which, in combination, amplify three overlapping gene regions in rs1052231 heterozygotes. External primers FP1 and RP2 (Table 1) define the area including the SNP and should produce, in all samples, the longest amplicon. The latter is thus used as an internal positive control that ensures integrity of each reaction (Figure 1). Each of the other two primers (FP2 and RP1) is specific for one of the rs1052231 alleles and, in combination with its confronting external primer (RP2 and FP1, respectively), generates an allele-specific amplicon of unique size, only in DNAs carrying that allele. External primers are chosen at different distance from the internal ones so that the three amplicons migrate as three distinct bands in agarose-gel electrophoresis. That way, depending on the sample genotype, two or three different PCR products are generated, allowing a straightforward allele assignment (Figure). The primer sequences and PCR-CTPP conditions are shown in Table 1.

TABLE 1 Primers and conditions for *CD247*-rs1052231 T > A genotyping by PCR-CTPP

Primer name	Sequence 5′→3′	Final concentration (μM)
FP1 (CD3ZF + 769)	gcggagcaagaggagg	0.20
RP1 (CD3ZRa + 844)	ctgggcagttataggtcccat	1.33
FP2 (CD3ZFt + 844)	caggaagacccaacact	0.66
RP2 (CD3ZR + 994)	gcaaaataggaaggctttagca	0.20

Notes: CD247-rs1052231 T > A genotyping consists of a single PCR-CTPP per sample. External primers, FP1 and RP2, recognize conserved *CD247* sequences: no polymorphisms with minor allele frequency $\geq 3 \times 10^{-4}$ reported in the Genome Aggregation Database r3.1.1 (gnomAD, $N \approx 148{,}000$), and in Trans-Omics for Precision Medicine Program (TOPMed, $N \approx 132{,}000$). On the other hand, the annealing site of FP2, specific for rs1052231 T, includes rs1052230 C (underlined). Although the minor allele rs1052230 G is fairly common (~0.15 in the aforementioned databases), it is detected almost exclusively in association with rs1052231 A—no examples of the mixed motif rs1052230 G-rs1052231 T were seen in ~400 sequenced alleles (our unpublished observation), nor were they reported in the ~500 families assessed in Reference 10. Therefore, rs1052230 should affect neither FP2 performance nor rs1052231 genotyping with this method. The bases currently shown for rs1052230 and rs1052231 in public SNP frequency databases correspond to the antisense strand (e.g., the rs1052231 A minor allele is designated "T," whereas rs1052230 G is called "C"). Each reaction includes ~100 ng of genomic DNA in 15 μl of PCR buffer (67 mM Tris–HCl, pH 8.8, 16 mM $[NH_4]_2SO_4$, 2 mM $MgCl_2$, 0.01% Tween-20 and 0.1 mM deoxyribonucleotide triphosphates) with 0.6–1.0 U of Taq polymerase (Bioline, London, UK), and four oligonucleotide primers. The optimal concentration of each primer was adjusted empirically, so that they produced bands of similar intensities. Such concentrations may need local adjustment because of differences in oligonucleotide providers and batches; performance of the primer mixes should also be verified regularly by using reference samples of known genotype in each assay. PCR was carried out in ABI2720 thermocyclers (Applied Biosystems, Foster City, California) and its conditions were 2 min at 96°C, then 35 cycles of 10 s at 96°C, 10 s at 65°C and 30 s at 72°C. Ten microliters of the amplification products were electrophoresed in ~2.5% agarose gels with a migrating distance of approximately 3–4 cm and revealed with ethidium bromide.

For optimization and validation of this method, we studied 52 samples, obtained after informed consent, whose genotypes we had previously assigned by means of Sanger direct sequencing (details available upon request). Correlation between the results obtained by both methods was complete, thereby demonstrating reliability and robustness of the presented PCR-CTPP genotyping assay.

To facilitate the local adjustment and validation of our method in other laboratories, we further used it to

characterize a panel of worldwide available reference cell lines. This panel comprises 50 cell lines representing all three possible rs1052231 genotypes (T/T, A/T, and A/A), thus allowing access to control DNAs (Table 2).

The method presented here enables genotype assignation of dozens to hundreds of samples in one or a small number of assays of only a few hours, using DNA amounts of, approximately, 100 ng, conventional thermocyclers, and appropriate electrophoresis chambers. The small size of all PCR products generated in this assay (less than 300 bp) should facilitate analysis of partially degraded or poor-quality DNAs. However, in severely degraded samples, the greater internal positive control band might disappear, circumstance in which the result should be disregarded because of the risk of missing the intermediate band that marks one of the alleles.

In terms of risk of contaminating reagents or samples with PCR products, CTPP is inferior to qPCR, in which reaction tubes need not be opened after amplification. In turn, PCR-CTPP, like PCR with sequence-specific primers (SSP), requires only an electrophoretic analysis after amplification; this is in contrast with methods like PCR-restriction fragment length polymorphism (RFLP) or DNA sequencing, which require further enzymatic reactions before electrophoresis. Several advantages of the method described here are shared with real-time PCR assays, used by other authors for rs1052231 analysis,[24] but reagents and equipment costs are notably lower for PCR-CTPP. Costs are also lower in comparison with more time-consuming methods like Sanger sequencing and PCR-RFLP.[10,16]

To sum up, we present an accurate, time-efficient, and cost-effective PCR-CTPP method for assessment of the *CD247*-rs1052231 T > A SNP, which should facilitate the study of its relevance in immunity and human health. In particular, the controverted behavior of this SNP minor allele as biomarker of autoimmune disease underlines the importance of the availability of simple and robust methods for genotype assignment.

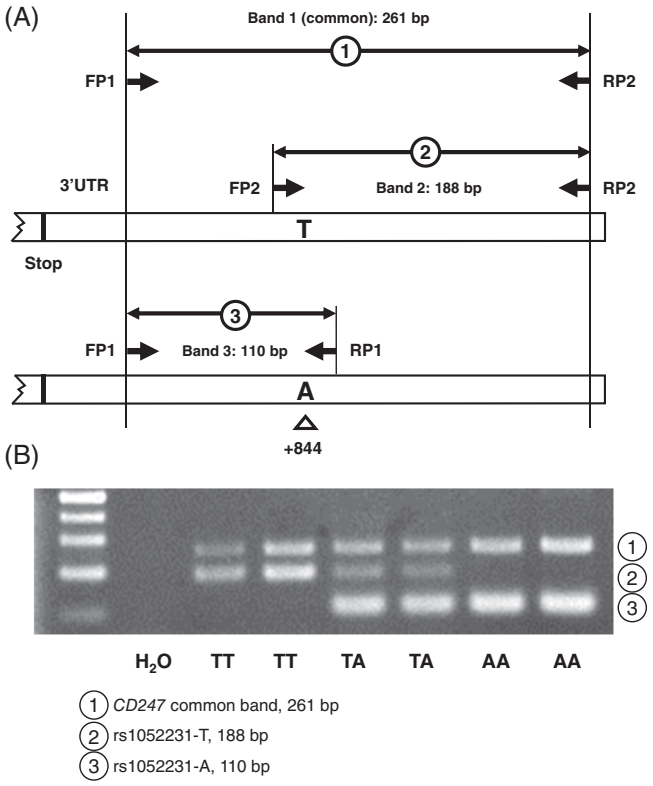

FIGURE 1 PCR-CTPP for *CD247* rs1052231-T/A genotyping. (A) General design—primers and amplicon sizes (not drawn to scale; modified from Vilches et al.[28]). (B) Results obtained using six DNA samples with different genotypes

TABLE 2 *CD247* rs1052231genotypes in a reference cell line panel

Cell lines					Genotypes
NKL	YT	HEK-293T	K562	AMAI	*TT*
BH	BM16	BM21	BOLETH	BRIP	
COX	DBB	E4181324	GB92	HOM-2	
HOR	HSB27	IBW9	JHA/CL	KT12	
LUY	MGAR	MOU	MZ070782	OLGA	
PAR	PE117	PF97387	PHL	PITOUT	
RML	RSH	TEM	WT8	WT24	
WT51					
JURKAT	NK92	DUCAF	JESTHOM	LE023	*TA*
NDSWW	SPO010	SWEIG	T7527	TAB089	
W0049	WDV	YAR			
DEU					*AA*

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

Karima Al-Akioui-Sanz designed and performed experiments, analyzed and interpreted data, and wrote the manuscript. Manuela Moraru supervised experiments, interpreted data, and wrote the manuscript. Carlos Vilches conceived and designed the study, directed research, and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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