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Single-tube *Ptprc* SNP genotyping of JAXBoy (CD45.1) and C57BL/6J (CD45.2) mice by endpoint PCR and gel electrophoresis

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Abstract

Allelic variation at the *Ptprc* gene, which encodes the pan-leukocyte marker CD45/Ly5, is commonly exploited to track hematopoietic reconstitution by flow cytometry in mixed bone marrow chimera transplant experiments. Historically, this was accomplished using bone marrow from C57BL/6 (*Ptprc*^{b}/CD45.2/Ly5.2) and congenic B6. SJL-*Ptprc*^{a}*Pepc*^{b}/Boy (*Ptprc*^{a}/CD45.1/Ly5.1) mice. Recently, the Jackson Laboratory directly CRISPR-engineered the *Ptprc*^{a} allele in C57BL/6J mice. This new isogenic strain, termed JAXBoy, differs from wild-type C57BL/6J mice by two nucleotides, compared to the biologically significant 37 megabase (Mb) SJL interval retained in B6. SJL-*Ptprc*^{a}*Pepc*^{b}/Boy/J mice. Currently, *Ptprc*/CD45 variants are identified by flow cytometry or allele-specific real-time PCR, both of which require specialized workflows and equipment compared to standard genotyping of endpoint PCR products by gel electrophoresis. Here, we employed allele-specific oligonucleotides in conjunction with differential incorporation of a long non-specific oligo 5′-tail to allow for simultaneous identification of the *Ptprc*^{a} and *Ptprc*^{b} alleles using endpoint PCR and gel electrophoresis. This method allows for integration of *Ptprc* genotyping into standard genotyping workflows, which use a single set of thermocycling and gel electrophoresis conditions. Importantly, the strategy of primer placement and tail addition described here can be adapted to discriminate similar single- or multi-nucleotide polymorphisms at other genomic loci.

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CRediT authorship contribution statement

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Declaration of competing interest

There are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mcp.2024.101962.

Keywords

Ptprc; CD45; JAXBoy; SNP; Genotyping; PCR

1. Introduction

The ability to track hematopoietic reconstitution by flow cytometry in mixed bone marrow chimera transplant experiments is fundamental to immunology and hematology research. This is typically accomplished using bone marrow from C57BL/6 (*Ptprc*^{b} encoding CD45.2/Ly5.2) and congenic B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy (*Ptprc*^{a} encoding CD45.1/Ly5.1) mice, as well as heterozygous CD45.1/CD45.2 (*Ptprc*^{a}/*Ptprc*^{b}) mice. In 2019, detailed genomic characterization of B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy mice sourced from different vendors, including the Jackson Laboratory (JAX), revealed that the *Ptprc* (*Protein tyrosine phosphatase receptor type c*) locus on chromosome 1 was part of a 27–92 Mb (JAX = 37 Mb) retained SJL genomic interval that impacted gene regulation and protein expression relevant to immune cells and their response to pathogens [1]. Moreover, transplant studies have demonstrated a functional defect in hematopoietic reconstitution from B6.SJL-*Ptprc*^{a} *Pepc*^{b}/Boy bone marrow compared to C57BL/6 [2]. In response to these important insights, Mercier et al. created the CD45.1^{STEM} mouse by knocking-in the *Ptprc*^{a} allele (K302E, AAA > GAA) into C57BL/6 N mice [2]. Similarly, in 2021 JAX CRISPR-engineered the *Ptprc*^{a} allele in C57BL/6J mice to generate C57BL/6J-*Ptprc*^{em6Lutzy}/J mice (known as “JAXBoy”), which are available for purchase (https://www.jax.org/strain/033076). The isogenic JAXBoy strain differs from wild-type C57BL/6J mice solely by two single nucleotide polymorphisms (SNPs) including the K302E mutation (AAA > GAA) along with an adjacent silent mutation at N303 N (AAC > AAT). Due to this small genetic difference between wild-type C57BL/6J (CD45.2) and JAXBoy (CD45.1) mice, *Ptprc*/CD45 variants are currently identified by flow cytometry or allele-specific real-time polymerase chain reaction (PCR) amplification; both of which require specialized workflows and equipment compared to standard genotyping of endpoint PCR products by agarose gel electrophoresis. Thus, designing primers to accommodate this widely used methodology will provide a useful alternative *Ptprc* genotyping method for researchers who already use endpoint PCR for genotyping other genes.

There are many different approaches to designing PCR-based assays for genotyping SNPs. One approach, commonly used in amplification refractory mutation system (ARMS) PCR and simple allele-discriminating PCR (SAP), involves incorporating an additional nucleotide mismatch next to the SNP site on each of the two allele-specific oligonucleotides (ASOs) to increase specificity for the two alleles [3, 4]. As the *Ptprc* alleles in JAXBoy and C57BL/6J already contain two polymorphic nucleotides separated by four non-polymorphic nucleotides ([A/G]AAAA[C/T]), we hypothesized that carefully designed ASOs targeting this region would function similarly to those designed for ARMS or SAP. Of note, we aimed to design a *Ptprc* genotyping assay that allowed setup of a single tube reaction (SAP requires separate reactions for each of the alleles) and produced only two bands (ARMS produces three bands) for unambiguous interpretation by gel electrophoresis. Moreover, differentiating two similarly sized amplicons using ARMS/-SAP requires the addition of fluorophore labels

and/or short GC-rich tails to the 5′-end of ASOs coupled with analysis by real-time PCR or separation by capillary electrophoresis [3,5–7].

In this method-oriented study, we evaluated the use of ASOs in conjunction with differential incorporation of a long non-specific oligo 5′-tail (adapted from PCR amplification of multiple specific alleles [PAMSA]) for simultaneous identification of the highly similar *Ptprc*^{a} and *Ptprc*^{b} alleles using endpoint PCR and gel electrophoresis. In contrast to PAMSA, which employs a much shorter 5′-oligo tail and requires high percentage agarose or polyacrylamide gels to resolve SNP alleles [8–10], the method we describe herein allowed for integration of *Ptprc* genotyping into our standard genotyping workflowusing a single set of thermocycling and gel electrophoresis conditions. Importantly, the strategy of primer placement and 5′-tail addition described in this study can be adapted to discriminate similar multi-nucleotide polymorphisms (MNPs), as well as individual SNPs, at other genomic loci.

2. Materials and methods

2.1. Animals

Peripheral blood (PB) and tail tissue for this study were collected as part of ongoing experimental procedures approved by the Institutional Animal Care and Use Committee at the University of Colorado Anschutz Medical Center. C57BL/6J (JAX Stock # 000664) and C57BL/6J-*Ptprc*^{em6Lutzy}/J (known as “JAXBoy”; JAX Stock # 033076) mice were purchased from JAX. Heterozygous CD45.1/CD45.2 mice were bred by crossing JAX Stocks # 000664 and 033076.

2.2. Flow cytometry

Retro-orbital sinus blood collection was performed on adult male and female mice under isoflurane anesthesia. Blood was prepared for flow cytometry as described previously [11] and stained with anti-CD45.1 (PE-Cy7; clone A20; BioLegend) and anti-CD45.2 (FITC; clone 104; Thermo-Fisher) monoclonal antibodies for acquisition on a Agilent NovoCyte Penteon flow cytometry analyzer. Analysis was performed with FlowJo (BD Life Sciences).

2.3. Preparation of genomic DNA from mouse tail tissue

Genomic DNA (gDNA) was extracted from 1 to 2 mm tail tissue of 14-day-old male and female mice using the HotSHOT protocol [12] in a final volume of 0.4 mL and used in all PCR experiments unless otherwise noted. HotSHOT gDNA concentrations are not routinely captured by our laboratory and were not obtained for this study.

2.4. Real-time probe-based endpoint PCR

PCR oligos and allele-specific probes (ASP) were adapted from the JAX protocol for Stock # 033076 (sequences listed in Supplementary Table S1) and purchased from Integrated DNA Technologies (IDT). Small scale IDT PrimeTime (double-quenched) probes contained a FAM or HEX fluorophore on the 5′-end, an internal quencher (ZEN) in the middle, and a second quencher (Iowa Black FQ) on the 3′-end. Reactions were prepared with PrimeTime Gene Expression Master Mix with low ROX reference dye (IDT, Catalog # 1055772), 0.4 μM each oligo, 0.15 μM each ASP, and 2 μL HotSHOT gDNA in a final volume

of 20 μL (Supplementary Table S2). Reactions were amplified on an Applied Biosystems QuantStudio Flex 6 real-time PCR system (Thermo-Fisher) based on cycling parameters adapted from JAX, IDT, and Thermo-Fisher (Supplementary Table S3). Data were analyzed on the instrument and exported into a Microsoft Excel spreadsheet. A high-resolution graphical representation of the exported data was created using GraphPad Prism software.

2.5. Endpoint PCR followed by agarose gel electrophoresis

PCR oligos (desalted standard), including ASOs, were purchased from Thermo-Fisher (sequences listed in Supplementary Table S1). Reactions were prepared in 0.2 mL 8-Strip PCR UltraFlux Tubes (Light Labs, Catalog # A4006Z) with Dream*Taq* Hot Start Green PCR Master Mix (Thermo-Fisher, Catalog #K9022), 0.5 μM each oligo (except 0.25 μM each oligo for the internal control [IC] amplicon or as noted in Supplementary Table S5), and 2 μL HotSHOT gDNA in a final volume of 10 μL (Supplementary Tables S4 and S5). JumpStart RED*Taq* 2X ReadyMix (Sigma, Catalog #P0982) and Econo*Taq* PLUS GREEN Master Mix (Lucigen, Catalog # 30033) were also evaluated where indicated. All reactions were run on an Applied Biosystems MiniAmp Plus Thermal Cycler (Thermo-Fisher) using touchdown cycling parameters [13] adapted from JAX (Supplementary Table S6) or standard cycling parameters (Supplementary Table S10) where indicated. PCR amplicons were separated on 1.5 % agarose (Light Labs, Catalog # A-1705) gels in 1X TAE buffer (G-Biosciences, Catalog #R023) supplemented with 0.5 μg/mL ethidium bromide (Thermo-Fisher, Catalog # 15585011) for 45 min at constant voltage (150V). Gel images were acquired using an Omega Fluor Plus Gel Doc System (Gel Company, Inc.). Amplicons were sized using the SmartCheck 100 bp DNA ladder (Accuris, Catalog # PR4010). Additional methods are described in Appendix A.

3. Results

3.1. Flow cytometric phenotyping of CD45 isoform expression on PB leukocytes

In lieu of genotyping gDNA extracted from animal tissues (e.g., tail snips), flow cytometric phenotyping of surface CD45 protein expression on leukocytes from PB (or other hematopoietic tissue) can be performed. This is commonly employed by labs to confirm CD45.1/CD45.2 heterozygosity in competitor donor mice to be used in forthcoming competitive transplant studies and to follow donor chimerism after transplant. Fig. 1 shows representative flow cytometric results of PB leukocytes from homozygous JAXBoy (CD45.1) and C57BL/6J (CD45.2) mice, and heterozygous (CD45.1/CD45.2) mice.

3.2. Real-time probe-based endpoint PCR MNP genotyping of Ptprc from gDNA

In lieu of flow cytometric characterization, real-time probe-based endpoint PCR can be used to genotype these animals. In fact, this is the only genotyping method described and recommended for the JAXBoy mouse. To assess the reproducibility of this JAX assay in our own lab, we performed real-time probe-based endpoint PCR using HOTShot gDNA from a small number of tail snips from homozygous and heterozygous mice. As expected, the assay correctly genotyped all nine test samples (Fig. 2).

3.3. Endpoint PCR MNP genotyping of Ptprc followed by agarose gel electrophoresis

To examine the utility of using gel electrophoresis of endpoint PCR products for genotyping *Ptprc* alleles (in place of the aforementioned methods), we designed and tested primers in several stages. We first designed four different forward (FWD; F) primers (Ptprc_a_F1, _F2, _F3, and _F4) specifically targeting one or both of the adjacent SNPs at K302E (AAA > GAA) and N303 N (AAC > AAT) of the JAXBoy *Ptprc*^{a} allele (Fig. 3A and Supplementary Table S1). We paired these FWD primers with four different reverse (REV; R) primers (Ptprc_R1, _R2, _R3, and _R4) targeting a downstream non-polymorphic sequence in the *Ptprc* gene and performed touchdown PCR (16 total FWD + REV combinations) to test specificity of the different primer pairs for the *Ptprc*^{a} allele. Each of the 16 FWD + REV primer combinations produced a correctly sized amplicon (see Supplementary Table S7) using gDNA template from JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}) animals (Fig. 3B, top). However, each of the two FWD primers targeting only the K302E SNP (Ptprc_a_F3 and Ptprc_a_F4) also produced a similarly sized amplicon using gDNA from C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}) mice, indicating lower specificity for the *Ptprc*^{a} allele (Fig. 3B, bottom). Further studies were carried out with the Ptprc_a_F1 primer (targeting both nearby SNP positions), as this primer (paired with any of the REV primers) was specific to the *Ptprc*^{a} allele and produced the strongest amplicons on gel electrophoresis.

Based on the results obtained for *Ptprc*^{a} testing, we modified the sequences of the Ptprc_a_F1 and F2 primers to specifically target the *Ptprc*^{b} allele instead (Fig. 3A and Supplementary Table S1). We paired each of these two new FWD primers (Ptprc_b_F1 and Ptprc_b_F2) with the same four REV primers as above and performed touchdown PCR (8 total FWD + REV combinations) to test specificity of the different primer pairs for the *Ptprc*^{b} allele. Whereas the Ptprc_b_F1 primer produced stronger amplicons on gel electrophoresis, the Ptprc_b_F2 primer was more specific to the *Ptprc*^{b} allele (Fig. 3C).

Each of the Ptprc_a_F1 and Ptprc_b_F2 primers produced amplicons with all four universal REV primers; however, the R1 and R3 pairings appeared to be the most productive (Fig. 3B–C). Therefore, we exploited this result in designing individual SAP-based genotyping assays [3] for the *Ptprc*^{a} (JAXBoy) and *Ptprc*^{b} (C57BL/6J) alleles, such that each PCR assay would produce different product sizes (178 and 238 bp, respectively) to further aid in distinguishing the two assays from each other on agarose gels. Both individual genotyping assays were effective at distinguishing homozygous JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}) or C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}) and heterozygous (*Ptprc*^{a}/*Ptprc*^{b}) mice (Fig. 3D). Nevertheless, this approach requires separate genotyping assays and is, therefore, more time-intensive and requires more reagents/consumables.

To increase genotyping efficiency, we next tested a strategy to combine simultaneous *Ptrpc*^{a} and *Ptrpc*^{b} genotyping into a single-tube assay format based on the principles of PAMSA [8–10]. Using the Ptprc_b_F2 primer as a base, we first added three non-specific oligo tail sequences of varying lengths (50, 70, or 81 nucleotides) to the 5′-end of the primer and tested it with the Ptprc_R3 primer in touchdown PCR with gel electrophoresis (Fig. 4A). Each of the FWD + REV primer combinations produced a correctly sized amplicon using gDNA template from C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}) but not from JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}) animals (Fig. 4B and Supplementary Table S7), as expected.

Having determined that the Ptprc_b_F2T3 primer with the 81-nucleotide tail performed adequately, we evaluated it together with Ptprc_a_F1 and Ptprc_R3 to accurately distinguish homozygous from heterozygous mice. In this initial test, homozygous JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}) and C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}) mice were all correctly identified (Fig. 4C, left). However, interpretation of the results for each of the heterozygous mice (*Ptprc*^{a}/*Ptprc*^{b}) was not as clear (Fig. 4C, left). Taken together, these data suggested that the *Ptprc*^{b} amplicon was preferentially amplified under these initial conditions (0.5 μM each primer; 1.0 molar ratio; touchdown PCR). To equalize the amplification efficiencies of the two amplicons in heterozygous samples, we varied the molar ratio of the two FWD primers in the PCR reaction [14,15]. This experiment showed that using a higher molar ratio of Ptprc_a_F1 to Ptprc_b_F2T3 primers allowed for accurate genotyping of all three *Ptprc* genotypes and was consistently best at a ratio of 1.33–1.67 (Fig. 4C). A ratio of 1.33 (0.5 μM Ptprc_a_F1; 0.375 μM Ptprc_b_F2T3; and 0.5 μM Ptprc_R3) was selected for use in our genotyping workflow as it was much more consistent across multiple runs (Fig. 4D and data not shown). We found that this ratio of FWD primers could also be used to qualitatively estimate *Ptprc*^{a} or *Ptprc*^{b} chimerism across a large dynamic range (~5–95 %) in PB gDNA samples (Supplementary Fig. S1A). However, increasing the molar ratio of FWD primers to 1.67 did enhance the amplification and detection of the less efficiently produced *Ptprc*^{a} amplicon (Supplementary Fig. S1B).

As an alternative approach to optimizing the ratio of FWD primers for touchdown PCR, we also evaluated standard PCR cycling parameters (i.e., fixed annealing temperature) using equal molar concentrations of primers (0.5 μM each primer; 1.0 molar ratio). These data demonstrated that accurate genotyping of all three *Ptprc* genotypes was also achieved by using standard PCR cycling parameters with annealing temperatures between 50 and 59 °C (Supplementary Fig. 2A). Nevertheless, increasing the molar ratio of FWD primers to 1.33 with standard cycling parameters led to more balanced production of the *Ptprc*^{a} and *Ptprc*^{b} amplicons in heterozygous samples (Supplementary Fig. 2B).

In addition, we demonstrated that our simultaneous allelic discrimination assay for *Ptprc* is compatible with alternative *Taq* polymerase master mixes (e.g., JumpStart RED *Taq* or Econo *Taq* PLUS Green) using either touchdown or standard PCR cycling conditions (Fig. 4D and Supplementary Fig. 2C).

3.4. Endpoint PCR SNP genotyping of Ptprc followed by agarose gel electrophoresis

Lastly, we evaluated the feasibility of adapting our single-tube MNP genotyping approach to interrogate a solitary SNP, focusing on the SNP at K302E (AAA > GAA) of the *Ptprc* locus as proof of concept (Supplementary Fig. 3A). However, our earlier data showed that primers (e.g., Ptprc_a_F3) targeting only one of the two adjacent *Ptprc* SNPs were more promiscuous than those targeting the entire MNP (Fig. 3A). Based on the ARMS/SAP/PAMSA methods [3,4,9], we incorporated destabilizing mismatched nucleotides at the 3′ penultimate position (A > [T/C/G]) of *Ptprc*^{a} and *Ptprc*^{b} specific primers (Ptprc_a_F3[b/c/d] and Ptprc_b_F3 [b/c/d]; Supplementary Fig. 3A and Supplementary Table S8) and observed a reduction in off-target *Ptprc*^{b} and *Ptprc*^{a} SNP specificity, respectively (Supplementary Fig. 3B). We then evaluated internally mismatched F3 primers containing the 81-nucleotide 5′-

tail sequence (Ptprc_a_F3[c/d]T3 and Ptprc_b_F3[c/d]T3) and found that standard cycling conditions, using increasingly higher annealing temperatures, resulted in improved (but imperfect) specificity compared to using touchdown PCR (Supplementary Fig. 3C and data not shown). Finally, we assessed different primer combinations using varied ratios of the two FWD primers (to increase specificity of the long-tailed primer) and standard cycling conditions to accurately distinguish homozygous from heterozygous mice (Supplementary Fig. 3E–F and data not shown). From these trials, we identified a combination of primers (Ptprc_a_F3cT3, Ptprc_b_F3d, and Ptprc_R3) and set of conditions (56 °C annealing temperature and 0.75 molar ratio of FWD primers [F3d: F3cT3]) that provided optimal amplicon intensity and specificity for all three *Ptprc* genotypes (Supplementary Fig. 3E–F).

4. Discussion

In this study, we have shown that carefully designed ASOs could be used in a single genotyping assay utilizing endpoint PCR followed by agarose gel electrophoresis for the simultaneous detection of two *Ptprc* alleles that differ by two non-adjacent SNPs ([A/G]AAAA[C/T]). We found that the placement of these ASOs was important, as primers that included only one of the two nearby SNPs (e.g., 5′–N_{19}[A/G]–3′ instead of 5′–N_{14}[A/G]N_4[C/T]–3′ or 5′–N_{13}[A/G]N_4[C/T]N–3′) were not sufficiently specific. However, with careful design (inclusion of destabilizing mismatched nucleotides adjacent to the SNP) [3,4,9] and extensive optimization of cycling conditions and molar ratios of ASOs, we also demonstrated the feasibility of genotyping an isolated SNP. The successful implementation of our single-tube genotyping assay for *Ptrpc* alleles provides an ASO design framework to discriminate similar MNPs (or SNPs) at other genomic loci using endpoint PCR genotyping assays in place of real-time probe-based PCR methods (which our lab does not routinely utilize for genotyping).

The method described here is adapted from several different methods, chiefly PAMSA [8–10]. Most critical was the addition of a non-specific long 5′-oligo tail to one of the ASOs to aid in differentiating otherwise identically sized amplicons by gel electrophoresis. Many published studies we identified from the literature favored using small 5′-oligo tails that required higher percentage agarose or polyacrylamide gels for resolving PCR amplicons. As an alternative, we hypothesized and showed that longer tails (>50 nucleotides) produced unambiguous results when using 1.5 % agarose gels in 1X TAE buffer. However, this approach led to amplification bias of the larger amplicon using touchdown cycling conditions, likely related to the much higher melting temperature of the long-tailed *Ptprc*^{b} primer compared to the shorter *Ptprc*^{a} primer in the earlier rounds of PCR (when *Ptprc*^{b} template includes amplicons containing the incorporated tail sequence). To overcome this challenge, we varied the molar ratio of the two FWD primers (i.e., reduced molar concentration of the long-tailed primer with higher melting temperature) or used standard (i.e., fixed annealing temperature) cycling conditions [14,15]. Reducing the concentration of the long-tailed *Ptprc*^{b} primer also improved the sensitivity of our single-tube assay for detecting the *Ptprc*^{a} allele at low chimerism percentages (5–10 %).

In addition to providing a framework for the design and implementation of a single-tube genotyping assay for MNPs (and theoretically small indels), as proof-of-concept, we also

successfully adapted this same approach (with important changes) for genotyping only one of the two adjacent *Ptprc* SNPs in JAXBoy mice. Nonetheless, optimizing the conditions conducive to unbiased and specific genotyping of one SNP was considerably more difficult than was for the MNP assay. Central to this was the necessity to incorporate 3′ penultimate mismatched nucleotides into *Ptprc*^{a} and *Ptprc*^{b} specific primers to reduce off-target *Ptprc*^{b} and *Ptprc*^{a} SNP specificity, respectively [3,4,9]. These primers markedly decreased amplicon production using touchdown cycling conditions, particularly after addition of the long (81-nucleotides) tail to each of the FWD primers. The choice of mismatched nucleotide in the ASO also made a significant difference in whether an amplicon was even produced (in our case, A > T was substantially less productive) [3,10]. Thus, if practical to do so, we advocate testing all three possibilities (e.g., A to T, C, or G) when using this approach. This strategy allowed us to identify and use different mismatched nucleotides (C or G) in the two ASOs, which may help minimize complementarity between one ASO and the product of the other [10]. Although not tested, moving the mismatched nucleotide one or two positions upstream (5′) may have improved amplicon efficiency and/or specificity [10], and should also be considered. Notably, standard cycling conditions were required to optimize the SNP assay, with higher annealing temperatures leading to reduced bias for the larger amplicon and decreased off-target amplification from the long-tailed primer. And in contrast to the MNP assay, decreasing the molar ratio of short to long FWD primers (in conjunction with higher fixed annealing temperatures) equalized the amplification efficiencies of the two differently sized amplicons.

Overall, our approach to MNP and SNP genotyping of the *Ptprc*^{a} and *Ptprc*^{b} alleles by single-tube endpoint PCR followed by agarose gel electrophoresis allowed for seamless integration of *Ptprc* genotyping into our own standard genotyping workflows, which use a single set of thermocycler and gel electrophoresis conditions, and represents a more cost- and time-effective strategy for our lab (and presumably others) to screen for, or confirm, heterozygous *Ptprc*^{a}/*Ptprc*^{b} mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

Data will be made available on request.

Abbreviations

ARMS	amplification-refractory mutation system

ASO	allele-specific oligonucleotide
ASP	allele-specific probe
FWD/F	forward (primer)
gDNA	genomic DNA
IC	internal control
Mb	megabase
MNP	multi-nucleotide polymorphism
NTC	no template control
PAMSA	PCR amplification of multiple specific alleles
PB	peripheral blood
PCR	polymerase chain reaction
REV/R	reverse (primer)
SAP	simple allele-discriminating PCR
SNP	single nucleotide polymorphism
TD	touchdown

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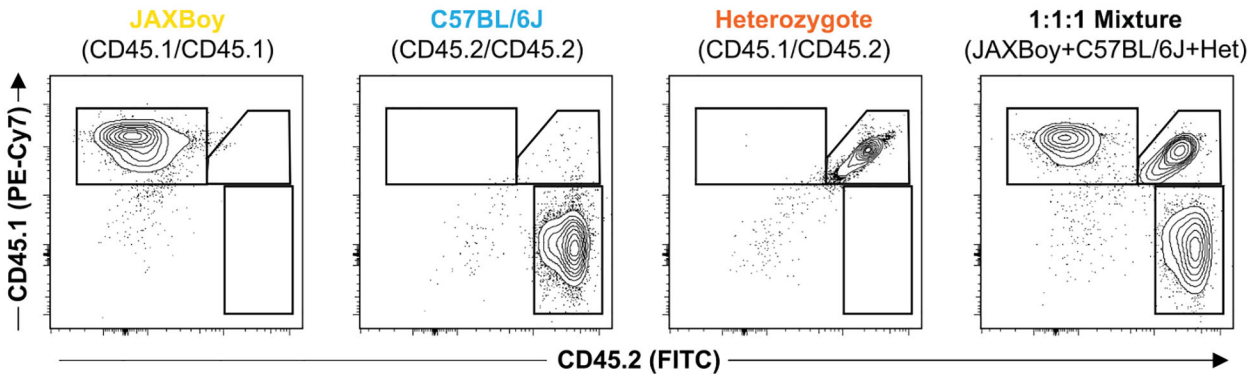

Fig. 1.

Representative flow cytometric phenotyping of surface CD45 isoform expression on PB total leukocytes from JAXBoy, C57BL/6J, and heterozygous (JAXBoy × C57BL/6J) mice. A contrived mixture of all three populations is also presented (1:1:1 ratio).

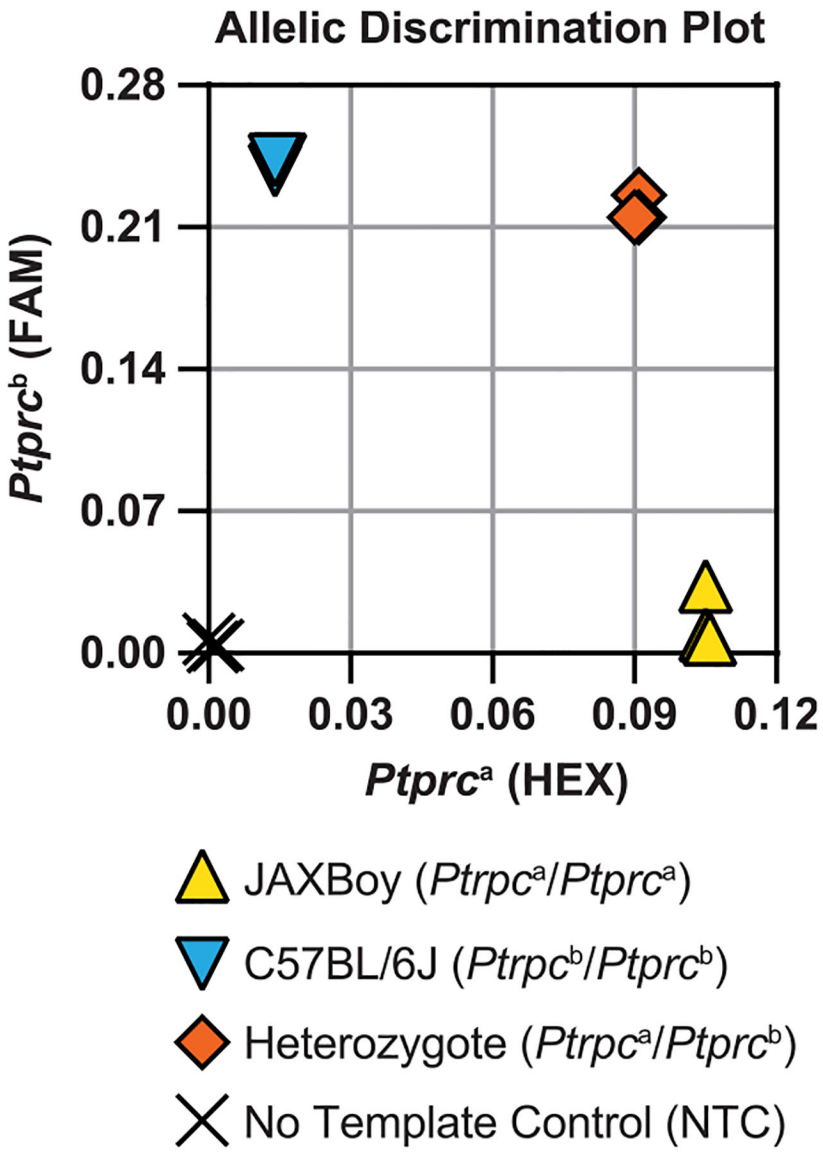

Fig. 2.

Real-time probe-based endpoint PCR MNP genotyping of *Ptprc* alleles in JAXBoy, C57BL/6J, and heterozygous (JAXBoy × C57BL/6J) mice. N = 3 mice per genotype. Assay conditions adapted from the JAX genotyping protocol (see also Supplementary Table S2–S3). GraphPad Prism software was used to graph data acquired and analyzed on the QuantStudio Flex 6 real-time PCR instrument.

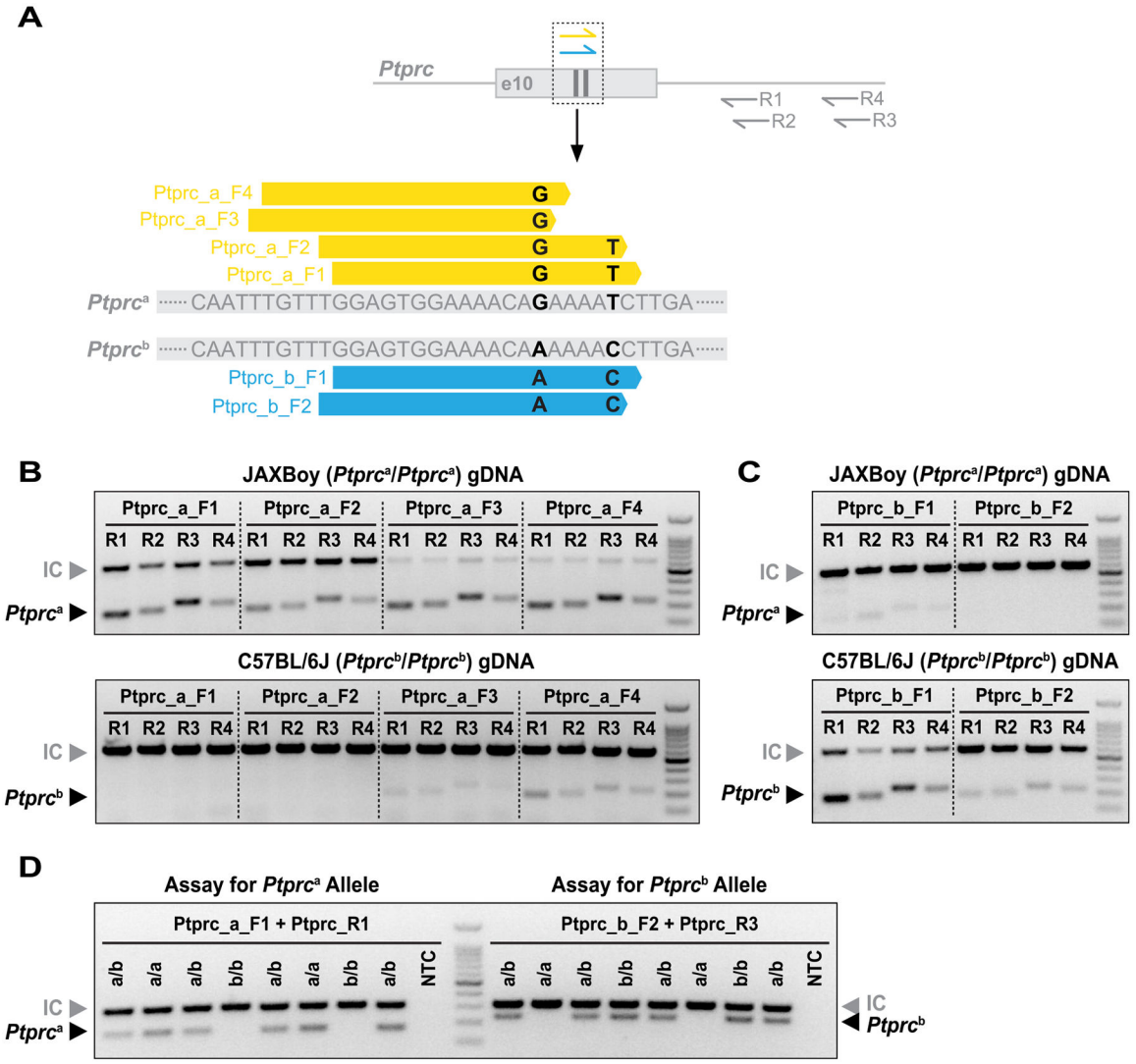

Fig. 3.

Optimization of MNP genotyping of individual *Ptprc* alleles by gel electrophoresis of endpoint PCR products. (**A**) Map and sequence detail of the *Ptprc* locus showing approximate primer locations. (**B**) *Ptprc*^{a} primer specificity testing. Four FWD primers specific to the *Ptprc*^{a} allele (Ptprc_a_F1, _F2, _F3, and _F4) were each tested with four universal REV primers (Ptprc_R1, _R2, _R3, and _R4) using either JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; top) or C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; bottom) gDNA. (**C**) *Ptprc*^{b} primer specificity testing. Two FWD primers specific to the *Ptprc*^{b} allele (Ptprc_b_F1, and _F2) were each tested with four universal REV primers (Ptprc_R1, _R2, _R3, and _R4) using either JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; top) or C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; bottom) gDNA. (**D**) Individual genotyping assays for *Ptprc*^{a} (left) and *Ptprc*^{b} (right) alleles. Each genotyping assay was tested against a panel of JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; a/a), C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; b/b), and heterozygous (*Ptprc*^{a}/*Ptprc*^{b}; a/b) gDNA samples. Amplification of the *R26* locus (**B**–**C**) or *Il2* locus (**D**) produces either a 603 bp or 324 bp internal control (IC) amplicon, respectively. NTC, no template

control. See also Supplementary Table S4–S7 for touchdown PCR assay setup, conditions, and expected amplicon sizes used in **B**–**D**.

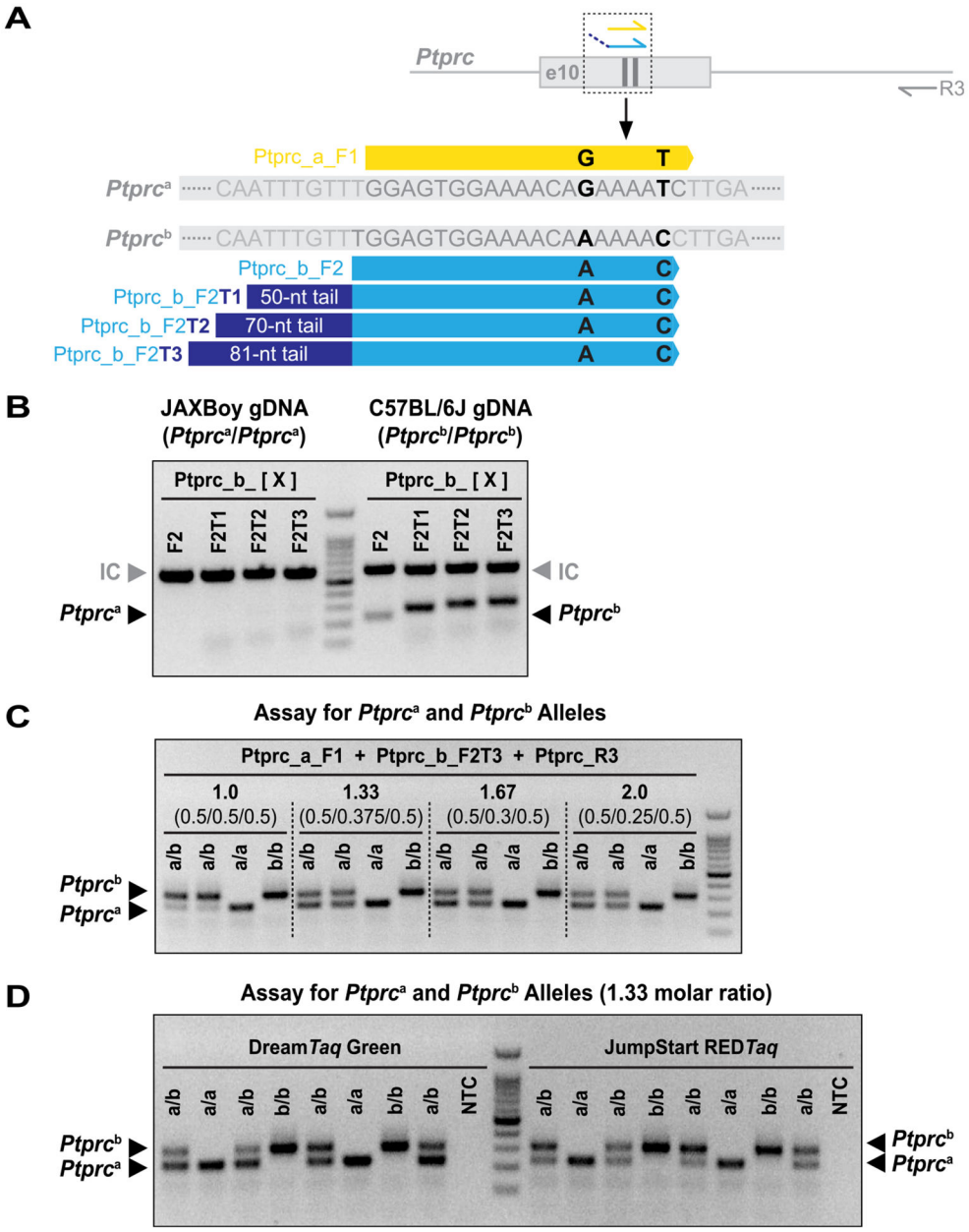

Fig. 4.

Optimization of long-tailed genotyping of the *Ptprc*^{b} allele by gel electrophoresis of endpoint PCR products. (**A**) Map and sequence detail of the *Ptprc* locus showing approximate primer locations. (**B**) Long-tailed *Ptprc*^{b} primer specificity testing. Four FWD primers specific to the *Ptprc*^{b} allele with non-specific 5′-oligo tails of varying lengths (Ptprc_b_F2 [no tail], _F2T1, _F2T2, and _F2T3) were each tested with a universal REV primer (Ptprc_R3) using either JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; left) or C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; right) gDNA. Amplification of the *R26* locus produces a 603 bp internal control (IC) amplicon. (**C**) Optimization of single-tube simultaneous MNP genotyping reactions for *Ptprc*^{a} and *Ptprc*^{b} alleles. Four different molar ratios (1.0, 1.33, 1.67, and 2.0) of FWD primers (Ptprc_a_F1 to Ptprc_b_F2T3) were tested with the Ptprc_R3 primer in single-tube

PCR reactions against a panel of JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; a/a), C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; b/b), and heterozygous (*Ptprc*^{a}/*Ptprc*^{b}; a/b) gDNA samples. Final molar concentrations of the three primers (F1/F2T3/R3) is shown in parenthesis below the F1:F2T3 molar ratio. (**D**) Single-tube simultaneous genotyping reactions for *Ptprc*^{a} and *Ptprc*^{b} alleles is compatible with different *Taq* Hot Start 2X Master Mix formulations. PCR reactions were prepared using Dream *Taq* Green (left) or JumpStart RED *Taq* (right) 2X Master Mixes with 1.33 molar ratio of FWD primers and tested against a panel of JAXBoy (*Ptprc*^{a}/*Ptprc*^{a}; a/a), C57BL/6J (*Ptprc*^{b}/*Ptprc*^{b}; b/b), and heterozygous (*Ptprc*^{a}/*Ptprc*^{b}; a/b) gDNA samples. NTC, no template control. See also Supplementary Table S4–S7 for touchdown PCR assay setup, conditions, and expected amplicon sizes used in **C**–**D**.