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Pyrosequencing for Accurate Imprinted Allele Expression Analysis

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ABSTRACT

Genomic imprinting is an epigenetic mechanism that restricts gene expression to one inherited allele. Improper maintenance of imprinting has been implicated in a number of human diseases and developmental syndromes. Assays are needed that can quantify the contribution of each paternal allele to a gene expression profile. We have developed a rapid, sensitive quantitative assay for the measurement of individual allelic ratios termed Pyrosequencing for Imprinted Expression (PIE). Advantages of PIE over other approaches include shorter experimental time, decreased labor, avoiding the need for restriction endonuclease enzymes at polymorphic sites, and prevent heteroduplex formation which is problematic in quantitative PCR-based methods. We demonstrate the improved sensitivity of PIE including the ability to detect differences in allelic expression down to 1%. The assay is capable of measuring genomic heterozygosity as well as imprinting in a single run. PIE is applied to determine the status of *Insulin-like Growth Factor-2* (IGF2) imprinting in human and mouse tissues. J. Cell. Biochem. 116: 1165–1170, 2015.

KEY WORDS: IMPRINTING; PYROSEQUENCING; INTRON-CROSSING PRIMER; IGF2; EPIGENETIC

Genomic imprinting depends on the presence of differential epigenetic marks at maternal and paternal alleles. These marks are dictated during embryonic development and restrict gene expression to a single inherited allele. Thus far, there are 95 human and 122 mouse genes identified as imprinted (http://www.geneimprint.com/site/genes-by-species) and many more believed to occur based on genomic screens [Luedi et al., 2007]. Imprinted genes are involved in many aspects of development including fetal and placental growth, cell proliferation, and adult behavior. Consequently, disorders in imprinting have been linked to numerous human genetic diseases including Beckwith–Wiedemann, Prader–Willi, Angelman syndrome [Falls et al., 1999], and diabetes [Bennett et al., 1997]. Many cancers exhibit imprinting alterations including neuroblastoma [Caron et al., 1993], acute myeloblastic leukemia [Katz et al., 1992], Wilms′ tumor [Scrable et al., 1989], colorectal carcinoma [Zuo et al., 2011], breast [Ito et al., 2008], and prostate cancer [Jarrard et al., 1995].

Thus far, imprinted genes have been found to be regulated by a variety of mechanisms including epigenetic modifications of promoter sequences, boundary elements, silencers, and overlapping antisense transcripts. Differential promoter methylation is a common mechanism to silence one allele of an imprinted gene (Fig. 1A). Alternatively, allele-specific chromatin boundaries can dictate association between two different gene promoters and shared enhancer regions, such as at the *Igf2-H19* imprinted locus where differential methylation affects the binding ability of a chromatin insulator (Fig. 1B). Roughly 15% of imprinted genes are associated with antisense transcripts, mostly noncoding, which impact chromatin structure and DNA methylation. Several imprinted genes have differentially methylated regions (DMRs) that are methylated on the active allele, which proposes that these sequences contain silencers that are inactivated by methylation, perhaps by excluding repressive factors [Sasaki et al., 1992; Brandeis et al., 1993].

Current methods for evaluating allele-specific expression are listed in Table I but have a number of limitations. PCR followed by restriction endonuclease digestion is an older method [Wu et al., 1997; Ross et al., 1999], and the efficiency of restriction endonucleases is rarely complete. In addition, PCR amplification can result in the formation of mispaired heteroduplex DNA, which can inhibit cleavage at restriction sites [Langhans, 2009]. The use of

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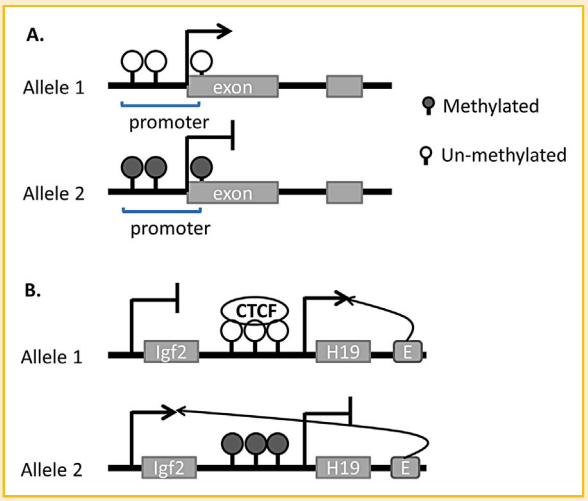

Fig. 1. Mechanisms in imprinted genes. A, Differential silencing by CpG island or promoter methylation. B, Allele-specific regulation of neighboring genes by differential methylation of boundary elements within a CpG island. Regulatory factors, such as CTCF, bind to the unmethylated allele and block the access of upstream promoters to downstream enhancers, leading to transcriptional repression of the upstream gene.

polymorphic small tandem repeats (STR) is a reliable way to detect allele-specific expression; however, this assay can only be applied to a small subset of genes because STRs are uncommon in transcribed regions [Mansfield, 1993]. Another assay for allele-specific amplification utilizes multiple primers which target specific 3′ nucleotides. However, it is difficult to design primers which amplify with equal efficiency under identical reaction conditions [Pushnova and Zhu, 1998; Lambertini L et al., 2008]. Hot-stop PCR, an assay for linear quantification of allele ratios is PCR cycle independent, but requires a restriction endonuclease site that recognizes a polymorphism and radioactivity [Uejima et al., 2001]. DNA sequencing combined with Fluorescent primer extension and dideoxynucleotide assay (Flu-PE and SNuPE) have been accurately utilized [Yan et al., 2002; Sievers et al., 2005; Fu et al., 2008], but these are labor intensive assays. Recently, work with RNA- Seq has suggested that the number of imprinted genes is much closer to earlier estimates [DeVeale et al., 2012], however, this is a very costly approach for allele quantification. More recently, Pyrosequencing has been used to quantify allelic expression [Wang and Elbein, 2007; McKeown et al., 2014]. In this study, we evaluated the sensitivity and specificity of PIE to quantitate allele-specific expression associated with imprinting, and described the factors for robust quantification and the problems which may be encountered.

MATERIALS AND METHODS

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS (SNPS)

The assay requires the presence of a SNP, differing at the two alleles, which allows allele-specific expression to be identified. SNP data for regions throughout the genome is available at NCBI (http://www.ncbi.nlm.nih.gov/snp). We utilized a previously identified A/G polymorphism in *IGF2* located on exon 5 in the human transcript and exon 6 in the mouse. Approaches have evaluated allelic expression on these loci with both *Apa*I digestion [Fu et al., 2004], Flu-PE [Fu et al., 2008], and pyrosequencing [Yang et al., 2013]. Given the previous data on homozygous and heterozygous individuals for both loci, the sensitivity and specificity of the assays were able to be compared.

TISSUE

Human prostate tissues were obtained from radical prostatectomy patients after approval by the Institutional Review Board at the University of Wisconsin-Madison. C57BL/6 wild type mice are homozygous (G) at this SNP region, and C57BL/6 *castaneous* mice contain an (A) at this locus. The offspring (termed CI) of female C57BL/6 wild type mice crossed with *castaneous* males are heterozygous for genotyping (A/G), but only the paternal allele (A) is expressed if the *Igf2* imprint is maintained. Mouse tails were obtained from 21 to 24 day old mice and prostate tissues were obtained from 3 month and older mice. Animal protocols had been approved by Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

DNA, RNA EXTRACTION, AND CDNA SYNTHESIS

One microgram of genomic DNA (gDNA) was extracted using the DNeasy Blood & Tissue Kit (Qiagen). RNA was isolated from tissues using RNeasy kit (Qiagen) following the protocol supplied by the manufacturer. *Dnase* I was used to eliminate any contaminating genomic DNA. cDNA was synthesized with the Epitech Reverse Transcription Kit (Qiagen) using 400 ng of total RNA. Oligo-dTs (final concentration 2.5 μM) were used instead of the kit included RT Primer Mix.

If gDNA contamination of cDNA remains an issue, we have utilized the following approach: Typically, with shorter extension

TABLE I. Methods for Imprinting Analysis

Method description	References
PCR followed by restriction endonuclease digestion	Ross et al., 1999, Wu et al., 1997, Langhans, 2009
Polymorphic small tandem repeats	Mansfield, 1993
Allele amplification with primers specific for 3' matches	Lambertini L et al., 2008, Pushnova and Zhu, 1998
Hot-stop PCR	Uejima et al., 2001
Polymorphic restriction sites with real-time PCR	Weber M, 2003
Flu-PE and SNuPE	Fu et al., 2008, Sievers et al., 2005, Yan et al., 2002
RNA-Sequencing	DeVeale et al., 2012
Pyrosequencing	Wang and Elbein, 2007, McKeown et al., 2014

time contaminating gDNA should not be amplified. To ensure purity of cDNA amplification, the PCR products are checked by gel electrophoresis, the presence of a high molecular weight band indicates gDNA amplification. The lower band (amplified from cDNA) can be excised and purified using standard gel purification methods.

ASSAY DESIGN

Two sets of primers were designed and used in subsequent PCR and Pyrosequencing. The first primer set is designed for regular PCR amplification using the online-free software primer3 [Untergasser et al., 2012]. The optimal design covers the SNP region, crosses an intron, amplifies both cDNA and gDNA, and the intron between the exons should be long enough to differentiate the amplicons between gDNA and cDNA by gel electrophoresis. Amplification of both cDNA and gDNA can be achieved by designing exon-primed intron crossing (EPIC) primers, in which the forward and reverse primers flank at different exons. Additionally, the preferable amplicon size should not exceed 2 kb, as this will allow both gDNA and cDNA to be amplified under the same reaction conditions with the only alteration of PCR extension time.

The second primer set is designed as pyrosequencing primers surrounding the SNP in the region of interest using the PyroMark Assay Design 2.0 software (Qiagen). Some considerations need to be taken: (1) Amplicons should be 150–250 bps, with either forward or reverse primer biotinylated. (2) To compare the ratios, the result must provide only two peaks upon Pyrosequencing, this can be achieved by manual alteration of dispensation in the PyroMark software. (3) An effort should be made to avoid multiple quantified bases adjacent to each other, so that the peaks are well defined and equally estimated. This is achieved by adjusting the pyrosequencing primer binding site. All primer sequences are listed in Table II.

FIRST AND NESTED PCR TO GENERATE PRODUCTS

Two different approaches were used to generate PCR products used for Pyrosequencing and the specificity of each approach was compared (Fig. 2). In both approaches, one round of PCR was performed followed by one round of nested PCR. The first approach was a 2-step PCR using (EPIC) primers. To do this, a large fragment (1.3 kb for human IGF2 and 700 bp for mouse Igf2) was amplified using 2 μl of cDNA or 40 ng of gDNA, using HotStar Taq master mix (Qiagen) and 0.2 μM of each primer. The amplification program was as follows: Initial denaturation of 95°C for 15 min; 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 90 and 45 s for human and mouse IGF2, respectively. PCR products from the initial PCR were then used for nested PCR using 0.5 μl of 1:10 dilution of the 1st PCR product and the Pyrosequencing primers flanking SNP regions. Both reverse primers featured 5′ biotin modifications and were HPLC purified, the PCR reaction mixtures are the same as the 1st PCR except the final concentration for each primer was 0.3 μM. Cycling conditions were 15 min of enzyme activation at 95°C followed by 30–40 cycles of 30 s at 95°C, 30 s at 56°C, and 25 s at 72°C.

The second approach used the Pyrosequencing primers for both the first PCR and the subsequent nested PCR (materials and conditions same as above). PCR products were checked on 2% agarose gels prior performing Pyrosequencing. A single strong PCR band with minimal primer–dimer formation is required in order to obtain a reliable result.

PYROSEQUENCING

For both approaches, 5 μl of biotinylated PCR products were mixed with streptavidin sepharose beads, shaken at 1400 rpm for 10 min. The streptavidin–biotin-bound PCR products are then captured with the probes of the Pyrosequencing Vacuum Prep Tool and rinsed in rounds of 70% ethanol, 0.2 M NaOH, and 10 mM Tris, pH 7.6; each wash for 10 s. The vacuum is turned off and the beads were released in 12 μl of the annealing solution containing 0.5 μM of pyrosequencing primer in 96-well PyroMark plate. The plate was denatured for 2 min at 80°C, cooled down, and loaded on PyroMark™MD Pyrosequencing System (Qiagen) following the manufacturer's instructions. The status of each locus was analyzed using PyroMark™MD software 1.0 (Qiagen).

To evaluate imprinting status gDNA and RNA from normal and cancerous human prostate tissues was pyrosequenced simultaneously. Genomic DNA was examined to confirm heterozygosity. The cDNA results were normalized according to the ratio of alleles obtained from gDNA, loss of imprinting (LOI) is represented as expression % of recessive allele. For each sample, three independent PCR products were generated and pyrosequenced in duplicate format. Statistical analysis was performed by *t*-test, $P < 0.05$ was considered significant. For each run, these controls are recommended: The PCR product without sequencing primer, the biotinylated primer and sequencing primer without DNA, and the sequencing primer alone.

SENSITIVITY AND SPECIFICITY OF THE PYROSEQUENCING ASSAY

A mixing experiment was used to quantify the minimum detection of PIE. Two sets of gDNA or RNA from mouse tails were used which were homozygous for different nucleotides at the SNP locus (i.e. G/G and A/A). A total of 40 ng DNA or cDNA was mixed in differing ratios ranging from 1% to 100% at a particular nucleotide (i.e., 25% G/G DNA and 75% A/A DNA). Pyrosequencing was performed as described. The sensitivity was also detected using human prostate samples with gDNA homozygous at the *IGF2* SNP locus.

TABLE II. Primer Sequence

		1st PCR	Nested PCR
Human IGF2	Forward Reverse Pyrosequendng	5′-ATCGTTGAGGAGTGCTGTTTCC 5′-GAGCCAGTCTGGGTTGTTGC	5′-AGTCCCTGAACCAGCAAAGAG 5′-biotin-TCGGATGGCCAGTTTACC S′-AGCAAAGAGAAAAGAAGG
Mouse igf2	Forward Reverse Pyrosequendng	5′-CTCTCAGGCCGTACTTCCGGAC 5′-GCGCCGAATTATTTGATTT	5′-TTCCATCACGTCCCACACTA 5′-biotin-TGAATATATAATTTGGGGGGTGTC 5′-AAGGGGATCTCAGCA

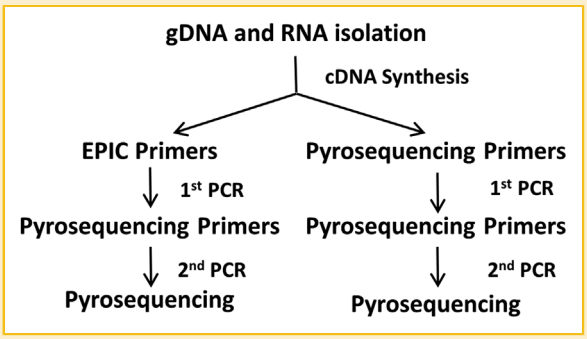

Fig. 2. Schema of two approaches generating PCR products for Pyrosequencing.

Wild type Cast-Bl6 mice retain imprinting and are heterozygous at the analyzed SNP locus. Genomic DNA and RNA isolated from the same tails of these mice were used to evaluate the specificity of PIE.

RESULTS

PIE PERFORMANCE

As described in the methods, we used two approaches to generate PCR products (Fig. 2). The PCR products from CI mouse tails, when using EPIC primers for the first PCR and Pyrosequencing primers for the nested PCR, showed expression from only one allele. However, when using the same CI mouse tails with primers inside a single exon for both first and nested PCR (Pyrosequencing primers), the cDNA exhibited ~20% expression of the recessive allele, which is silenced by imprinting. Therefore, the PCR products generated with EPIC primers are employed for all experiments described below. Also, we varied from 30 to 40 the number of cycles for the nested biotinylated amplicon. The cDNA was not well amplified when using <32 cycles. PCR products were amplified using 35 cycles for subsequent experiments as it was found that using 35–40 cycle amplification did not affect allelic ratios.

Figure 3A shows the performance of Pyrosequencing assay on Igf2 SNP using mouse tail gDNA with known homozygosity or heterozygosity at this locus. The homozygous tissues accurately showed one peak and the heterozygous showed equal heights from the peaks. The same results were obtained from human *IGF2* SNP assay using human prostate gDNA with known homozygosity or heterozygosity at the locus (data not shown).

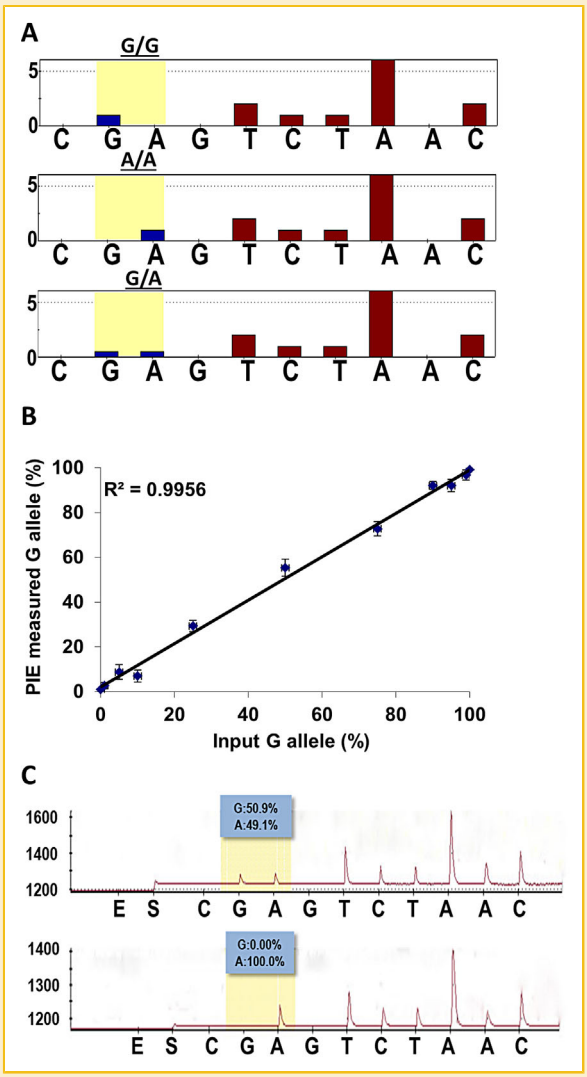

Fig. 3. A, PIE performance on Igf2 SNP using mouse tail gDNA. The homozygous mouse accurately showed one peak for both nucleotides, the heterozygous mouse showed equal heights from the two peaks. B, Sensitivity of PIE. PIE precisely reflected the theoretical ratio of the 2 alleles from the mouse cDNA homozygous for either allele. The trendline showed perfect linearity ($R^2 = 0.9956$), and 1% changes were able to be detected. The graphs were generated from three independent experiments and the data shown as Mean ± SD. C, Specificity of PIE. Pyrosequencing measured equals amounts of alleles in genomic DNA from heterozygous mice and only demonstrated expression from one allele using mRNA when the intron-crossing primers were used.

SENSITIVITY AND SPECIFICITY OF PIE

Human and mouse tissues were used to determine the sensitivity of PIE applied to the *IGF2* locus. Figure 3B shows the quantification of the actual allele expression by PIE from mouse tail cDNA precisely reflects theoretical expression, as the R-squared value for the trendline is 0.996. The sensitivities of PIE using mouse and human gDNA showed higher R^2 values than those generated from cDNA [Yang et al., 2013]. In both assays, PIE was able to detect 1% differences in allelic ratios. This Figure represents an average of three independent experiments run in duplicate. Technical replicates obtained from independent experiments show standard deviations ranging from 0.73% to 2.77%, indicating a robust assay with negligible inter-PCR and sequencing variation.

The specificity of PIE was determined using cDNA and gDNA isolated from wild type CI mouse tail snips (heterozygous at the SNP locus). As shown in Figure 3C (top), using PCR products generated with EPIC primers, gDNA showed equal percentages (50.9% and 49.1%) from both alleles. Analysis of cDNA accurately showed expression solely from the dominant allele with no expression from the imprinted allele (A – 100%; G – 0%).

IGF2 LOI DETECTED IN HUMAN PROSTATE TISSUES BY PYROSEQUENCING

We then analyzed the imprinting status of IGF2 using PIE in 5 normal and 12 tumor prostate tissues. Theoretically, the genomic ratio of 2 alleles is 50:50 in a SNP heterozygous tissue, but in actuality many samples consistently show 40:60 allelic imbalance in the genomic DNA [Wang and Elbein, 2007]. This was potentially due to a PCR artifact since it was not seen in the mouse assay. Therefore, we used both DNA and RNA from each sample for the pyrosequencing assay and normalized the RNA samples to the genomic DNA. The prostate gDNA showed 42–60% monoallelic expression for our samples (Table III). After normalization by the genomic allele ratio, the normal prostate cDNA showed expression of IGF2 from the recessive allele of 3–8.8%, while prostate tumor tissue showed progressive expression of the recessive allele up to 28%, $P < 0.05$. These normal prostate samples were from patients who had no evidence of prostate cancer and maintain imprinting. A loss of *IGF2* imprinting in a 'field defect' alters non-tumor prostate tissue in men with cancer [Fu et al., 2008].

DISCUSSION

Pyrosequencing for Imprinting Expression (PIE) is a novel assay to determine allelic expression ratios of imprinted genes. The use of intron-crossing primers enables the detection of small differences in allelic expression while eliminating non-specific detection seen with Flu-PE and as a result of heteroduplex formation in other assays. Some researchers suggest using a large number of cycles (over 50) of short duration to generate the biotinylated amplicon, which has been found to be optimal for generation of highly specific products of small size [Wang and Elbein, 2007]. However, our test it is not dependent on PCR cycles as long as a single strong PCR band with minimal primer–dimer is observed, as utilizing more than 35 cycles in the nested PCR had no effect on the allelic ratio detected. In addition this assay eliminates the need for an endonuclease digestion site within the SNP region of interest, enabling a broader range of applicability to detect transcribed polymorphisms. PIE is a highly reproducible assay with a standard deviation <3% between independent experiments.

One potential limitation for the PIE assay is the use of EPIC primers to generate first PCR products, in contrast to using primers within the same exon. Intron crossing primers minimize recessive allele amplification that can result from genomic DNA contaminaiton. This DNA contamination may also happen in other assays (Table I) when measuring mRNA expression using primers within the same exon. In addition, researchers have reported using EPIC-PCR reduces null allele artifacts compared to the use of polymorphic small tandem repeats (STRs) [Chenuil et al., 2010; Lardeux et al., 2012]. We have also observed that in human prostate tissues with heterozygosity at the tested IGF2 locus allelic ratios range from 40 to 60% in gDNA consistent with other pyrosequencing reports [Yan et al., 2002; Wang and Elbein, 2007]. The reason for this is unclear, but it is likely assay specific. At the mouse *Ifg2* locus, we detected 47.5 to 50% for one allele (data not shown). Whatever method (Table I) is used to detect allelic imbalance, some correction should be made for uneven amplification not related to transcription by testing gDNA. One advantage of PIE is that the assay is able to test gDNA and cDNA samples at the same time. However, a limitation is that this creates a potential hurdle in requiring intron-crossing primers for the 1st PCR amplification.

For the mouse and human PIE assays we were able to design primers fitting the criteria as outlined in the methods. We initially tried to design an assay for another SNP in human IGF2, but since the SNP is located in the middle of very long exon (4kb) we were unable to design the primers crossing different exons. In the case where it is impossible to design the intron crossing primers, a standard curve may be constructed by mixing cDNA from individuals homozygous for each allele in ratios of 100, 99, 95, 90, 75, 50, 25, 10, 5, 1, and 0% using the same assay. The curve is expected to be linear, the cDNA data will generate a linear regression formula, and all tested samples can be normalized with the formula.

TABLE III. IGF2 Imprinting Status in Human Prostate Tissues Detected by PIE

	cDNA	gDNA			cDNA
	(%) G allele	(%) G allele	(%) A allelel	G/A	Normalized (%) Gallele
N1	5.8	50.1	49.9	1.00	5.8
N2	7.6	46.3	53.7	0.86	8.8
N3	2.5	45.1	54.9	0.82	3.0
N4	6.2	54.7	45.3	1.21	5.1
N5	3.3	42.6	57.4	0.74	4.4
Mean ± SD					5.4 ± 2.1
T1	17.2	46.8	52.3	0.89	19.2
T2	19.2	44.1	55.9	0.79	24.3
T3	21.1	52.1	47.9	1.09	19.4
T4	12.7	60.1	39.9	1.51	8.4
T5	13.6	47.8	52.2	0.92	14.9
T6	9.2	45.1	54.9	0.82	11.2
T7	11.5	44.2	55.8	0.79	14.5
T8	21.8	43.7	56.3	0.78	28.1
T9	17.7	45.3	54.7	0.83	21.4
T10	10.6	46.7	53.3	0.88	12.1
T11	16.2	53.5	46.5	1.15	14.1
T12	16.1	46.8	53.2	0.88	18.3
Mean ± SD					17.2 ± 5.7

N, normal prostate tissue; T, tumor prostate tissue

Pyrosequencing is low cost, accurate, rapid, and gives high throughput. PIE is efficient, reliable, and can be easily scaled to accommodate a large number of samples. We believe PIE delivers several advantages over current methods to determine allelic ratio expression.

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