

Pyrosequencing® Applications

Sharon Marsh

Summary

Genetics research has benefited tremendously from the release of the human genome sequence. Subsequent technology has been developed and adapted to accommodate the need for faster, easier throughput of genetic assays. Pyrosequencing® is a unique system that allows the analysis of genetic variations including single-nucleotide polymorphisms, indels and short repeats, as well as assessing RNA allelic imbalance, DNA methylation status, and gene copy number. Advances in methodology, including multiplex and universal primer applications, have reduced assay cost and improved throughput. This chapter briefly reviews some of the many applications for Pyrosequencing technology.

Key Words: Pyrosequencing; DNA; RNA; genetics.

1. Introduction

Since the release of a working draft of the human genome there has been a drive for technological advancements to improve the throughput and analysis of genetics research.

Low-throughput procedures such as restriction fragment length polymorphism and allele-specific PCR (1) are time consuming and laborious when multiple samples and/or multiple assays are to be used. Conversely, high-throughput procedures utilizing chips or bead arrays (2) are cheap per variant but are limited by the large amounts of sample needed and are consequently limited to studies such as whole-genome scanning where data on up to 500,000 polymorphisms are required. A range of medium-throughput technologies have emerged to fill the gap, each with their advantages and disadvantages (3). Pyrosequencing is one such technology that allows rapid and reliable genotyping to be performed in a 96-well plate format.

2. Principles of Pyrosequencing

The origins of Pyrosequencing have been described in Chapter 1. This innovative technique is based on sequencing by synthesis. The assay takes advantage of the natural release of pyrophosphate whenever a nucleotide is incorporated onto an open 3' DNA strand. The released pyrophosphate is used in a sulfurylase reaction releasing ATP. The released ATP can be used by luciferase in the conversion of luciferin to oxyluciferin. The reaction results in the emission of light, which is collected by a CCD camera and recorded in the form of peaks, known as a Pyrogram® (4–6) (**Fig. 1**). When a nucleotide is not incorporated into the reaction, no pyrophosphate is released and the unused nucleotide is removed from the system by degradation through apyrase. This four-enzyme process is performed in a closed system in a single well. The processing time is simple and relatively fast (~20 min/96-well plate) and the cost of the reaction is comparable to other medium-throughput technologies.

3. Applications

The advantages with the Pyrosequencing system lie with the range of applications the technology can be applied to (**Fig. 2**). Single-nucleotide polymorphisms (SNP), insertion/deletions (indels), short tandem repeats, pooled allele frequencies, human leukocyte antigen (HLA) typing, gene copy number, allelic imbalance in RNA, methylation status, and short sequencing stretches are among the numerous applications, many of which are described in this book. As short stretches of sequence are synthesized during the assay, novel polymorphisms close to the polymorphism in question have also been identified using this technique, where they may be missed or cause inaccurate genotype calls using other methods (7). The assay is applicable to almost any source of DNA or RNA (e.g., blood, saliva, cell line, plasma, serum, tissue, formalin-fixed and/or paraffin-embedded samples, and whole genome-amplified DNA). In addition, the use of a universal biotinylated primer and multiplex analysis of up to three different amplicons can be performed (8–10), reducing genotyping cost and time of throughput. No other system provides this range, throughput, and cost advantage. An up-to-date listing of publications involving Pyrosequencing technology can be found at <http://www.biotagebio.com/DynPage.aspx?id=8890&search=publications>.

3.1. Human Genetics

Historically, genetics research works back from a phenotype using family inheritance patterns and linkage analysis to narrow down regions associated with genetic disorders. However, once the genome regions have been assigned they often still span huge stretches of DNA and narrowing down further to identify the causative genes/haplotypes/variants can be an arduous process.

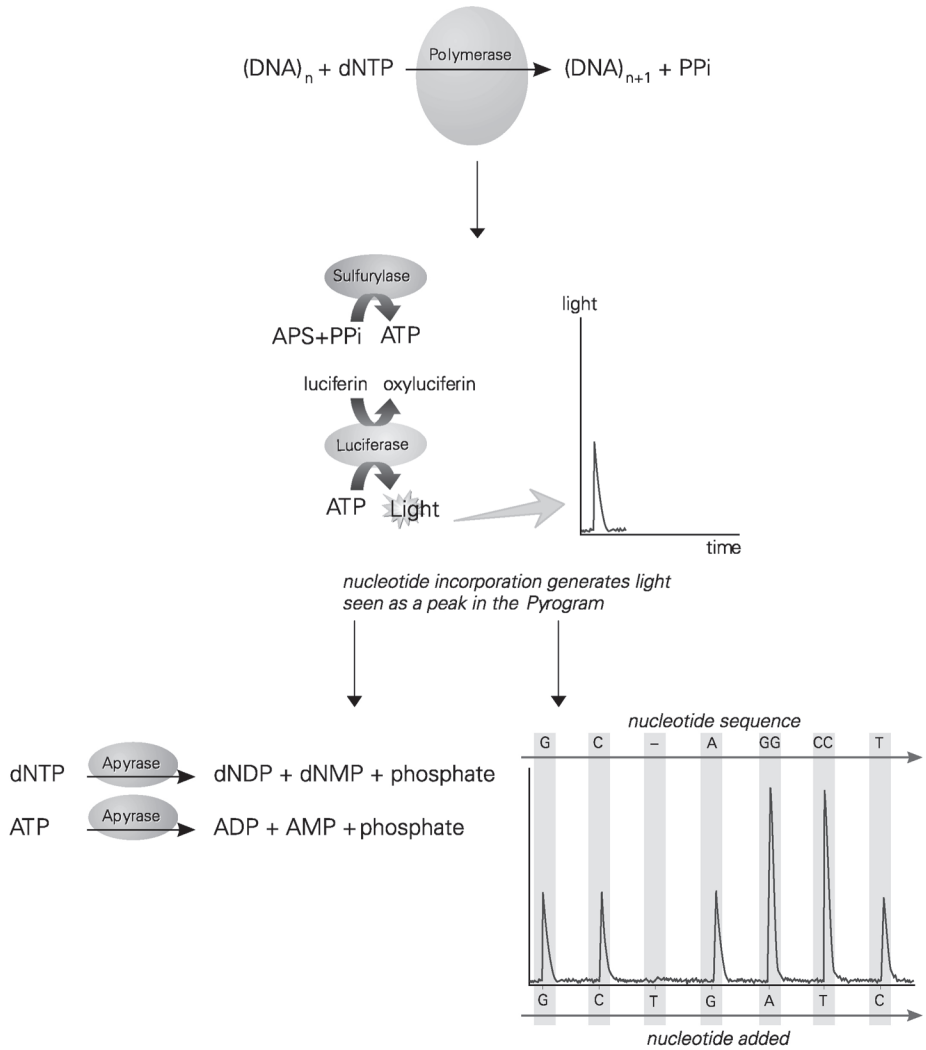


Fig. 1. The principles of Pyrosequencing®. (Reproduced with permission from **ref. 46.**)

Utilizing the Pyrosequencing allele quantification software, pooling individual samples allows a rapid and cost-effective process for screening allele frequencies in affected and control populations (**11**). For example, Permutt et al. used a case–control design to screen 91 SNPs from a 7.3-Mb region in pooled samples of 150 individuals with diabetes and 150 controls from the same population (Ashkenazi) (**12**). This allowed a direct comparison of allele frequencies between cases and controls and was performed in 182 assays rather than the

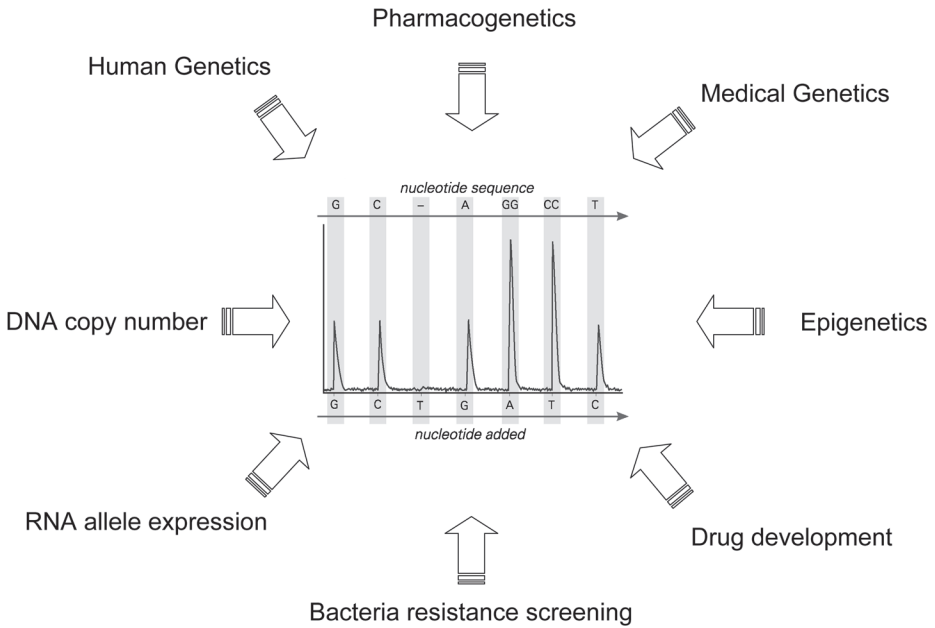


Fig. 2. Applications of Pyrosequencing®. Pyrogram® adapted from Fig. 1.

27,300 that would be necessary to screen all of the individuals for each polymorphism separately. Studies such as this can allow a rapid screening process to narrow down the regions that should be subsequently genotyped at a higher density in individual samples.

3.2. Pharmacogenetics

Pharmacogenetics research, the search for inherited variability in drug response, relies on rapid, accurate genotyping technologies. There are many examples of the use of Pyrosequencing for pharmacogenetics research in the recent literature (13–27). Although the majority of DNA variation is in the form of individual DNA nucleotide differences (SNPs), other variants including tri-allelic polymorphisms, tandem repeats, and indels have been shown to be clinically relevant. For example, a functional dinucleotide (TA) repeat in the *UGT1A1* gene has been associated with severe toxicity in cancer patients treated with the chemotherapy drug, irinotecan (28). The majority of people have six copies of the TA repeat. The *UGT1A1**28 polymorphism corresponds to seven copies of the repeat, and this is the allele associated with toxicity. This polymorphism can be identified by direct sequencing, but the analysis is time consuming and the assay can be expensive. An accurate Pyrosequencing assay

has been developed that screens this polymorphism in a 96-well plate format and significantly reduces analysis time (24).

The ability to multiplex reactions is also a boon to pharmacogenetics research. The multidrug transporter gene, *ABCB1* (MDR1) has three commonly studied polymorphisms. As these are all usually screened in patient samples, assessing all three in one multiplexed assay (29) can reduce time, cost, and importantly, reduces the amount of patient DNA required.

Copy-number polymorphisms can also have pharmacogenetic relevance. *CYP2D6* is another metabolizer of commonly used drugs. Gene duplication events lead to polymorphic *CYP2D6* gene copy numbers between individuals. Amplification of the *CYP2D6* gene is associated with increased enzyme levels and consequently increased *CYP2D6*-mediated metabolism. Time-consuming and labor-intensive methods such as long-range PCR can be used to quantify the number of *CYP2D6* genes in a patient. However, the development of a Pyrosequencing assay (Chapter 12) to quantify gene copy numbers (30) allows rapid and reliable data to be generated. For *CYP2D6* the assay takes advantage of a known pseudogene (*CYP2D8P*) and the allele quantification software to compare the ratio of peak heights and determine the number of *CYP2D6* genes present (31).

Along with identifying clinically relevant polymorphisms, an added complication can be found where an imbalance in allele expression is seen in the RNA expression of a gene. For example, in patients heterozygous for a functional polymorphism, if the deleterious allele is over-represented in the RNA compared with the wild-type allele, this could significantly affect patient outcome. Over-representation of specific alleles has been demonstrated in *CYP3A5*, a gene responsible for the metabolism of many commonly prescribed drugs. A polymorphism in the 3'-untranslated region of the *CYP3A5* gene is over-represented in cDNA from individuals whose DNA is heterozygous for the polymorphism (32). This is ultimately owing to tight linkage with a SNP in the promoter region of the gene causing increased expression (32). Consequently, the need to look to RNA for allele expression may provide more information than simply assessing the DNA for the presence of the functional allele. Chapter 13 details a method for assessing allelic imbalance, which could be readily applied to studies such as these. Consequently, assessment of RNA allele expression and DNA polymorphism in the status of the same individual can be readily performed on the same technology platform.

3.3. Epigenetics

Hypermethylation of CpG islands located within or close to the 5' region of genes is associated with inhibition of gene expression. This can be particularly

important in tumors where hypermethylation can “switch off” tumor-suppressor genes and/or DNA damage repair genes, which can lead to sensitivity or resistance to chemotherapy drugs (33). Assessment of methylation status in CpG islands can be easily performed using bisulfate modification and comparing the conversion of cytidine to thymidine with the allele quantification Pyrosequencing software (Chapter 8). The procedure for detecting methylation using Pyrosequencing (34) was recently applied to determine the methylation status in oral squamous cell carcinoma (35), leading to the identification of *cytoglobin* as a novel putative tumor suppressor gene (35).

3.4. Medical Genetics

The use of Pyrosequencing to rapidly and accurately type HLA loci (Chapter 10) is of valuable clinical importance (36–41). The matching of patient and donor for HLA significantly reduces the chance of rejection following organ transplantation.

Other medical applications for Pyrosequencing include the typing of mutations (Chapter 9) conferring antibiotic resistance to bacteria or to differentiate between multiple bacterial or viral strains (42–44) and for screening for target phage ligands for novel drug development (Chapter 11) (45). These protocols demonstrate the utility of Pyrosequencing for clinical assessment and drug development applications.

The technique also has far-reaching applications to fields outside of human/medical genetics. Chapter 12 describes the use of gene copy number detection in animal studies where Pyrosequencing technology can be utilized to determine X and Y copy numbers in cattle sperm sorting, or coat color variation gene copies in pigs, demonstrating that the same technique and protocols can be applied to a wide variety of disciplines.

3.5. Conclusion

The availability of a multipurpose instrument for genetic analysis is a major boon to researchers. Pyrosequencing is an established, reliable technique that can be applied to all areas of genetics. Applications of Pyrosequencing technology can be found in widely diverse research fields from plant biology to immunology. The following chapters describe in-depth protocols and troubleshooting tips covering many of these applications.

Acknowledgments

The author is funded in part by the Pharmacogenetics Research Network (U01 GM63340), R21 CA113491 and R21 CA102461.

References

1. Rose, C. M., Marsh, S., Ameyaw, M. M., and McLeod, H. L. (2003) Pharmacogenetic analysis of clinically relevant genetic polymorphisms. *Methods Mol. Med.* **85**, 225–237.
2. Suarez, B. K., Taylor, C., Bertelsen, S., et al. (2005) An analysis of identical single-nucleotide polymorphisms genotyped by two different platforms. *BMC Genet.* **6**, S152.
3. Freimuth, R. R., Ameyaw, M. -M., Pritchard, S. C., Kwok, P. -Y., and McLeod, H. L. (2004) High-throughput genotyping methods for pharmacogenomic studies. *Current Pharmacogenomics* **2**, 21–33.
4. Ronaghi, M. (2001) Pyrosequencing sheds light on DNA sequencing. *Genome Res.* **11**, 3–11.
5. Ronaghi, M., Uhlen, M., and Nyren, P. (1998) A sequencing method based on real-time pyrophosphate. *Science* **281**, 363–365.
6. Marsh, S., King, C. R., Garsa, A. A., and McLeod, H. L. (2005) Pyrosequencing of clinically relevant polymorphisms. *Methods Mol. Biol.* **311**, 97–114.
7. Lanfear, D. E., Marsh, S., and McLeod, H. L. (2004) Caution with beta1-adrenergic receptor genotyping. *Clin. Pharmacol. Ther.* **76**, 185–186.
8. Aydin, A., Toliat, M. R., Bahring, S., Becker, C., and Nurnberg, P. (2006) New universal primers facilitate Pyrosequencing. *Electrophoresis* **27**, 394–397.
9. Pacey-Miller, T. and Henry, R. (2003) Single-nucleotide polymorphism detection in plants using a single-stranded pyrosequencing protocol with a universal biotinylated primer. *Anal. Biochem.* **317**, 166–170.
10. Pourmand, N., Elahi, E., Davis, R. W., and Ronaghi, M. (2002) Multiplex Pyrosequencing. *Nucleic Acids Res.* **30**, e31.
11. Wasson, J., Skolnick, G., Love-Gregory, L., and Permutt, M. A. (2002) Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by pyrosequencing technology. *Biotechniques* **32**, 1144–1150.
12. Permutt, M. A., Wasson, J., Love-Gregory, L., et al. (2002) Searching for type 2 diabetes genes on chromosome 20. *Diabetes* **51**, S308–S315.
13. Skarke, C., Grosch, S., Geisslinger, G., and Lotsch, J. (2004) Single-step identification of all length polymorphisms in the UGT1A1 gene promoter. *Int. J. Clin. Pharmacol. Ther.* **42**, 133–138.
14. Jin, M., Gock, S. B., Jannetto, P. J., Jentzen, J. M., and Wong, S. H. (2005) Pharmacogenomics as molecular autopsy for forensic toxicology: genotyping cytochrome P450 3A4*1B and 3A5*3 for 25 fentanyl cases. *J. Anal. Toxicol.* **29**, 590–598.
15. Henningsson, A., Marsh, S., Loos, W. J., et al. (2005) Association of CYP2C8, CYP3A4, CYP3A5, and ABCB1 polymorphisms with the pharmacokinetics of paclitaxel. *Clin. Cancer Res.* **11**, 8097–8104.
16. Lee, S. S., Kim, K. M., Thi-Le, H., Yea, S. S., Cha, I. J., and Shin, J. G. (2005) Genetic polymorphism of CYP2C9 in a Vietnamese Kinh population. *Ther. Drug Monit.* **27**, 208–210.

17. de Jong, F. A., Marsh, S., Mathijssen, R. H., et al. (2004) ABCG2 pharmacogenetics: ethnic differences in allele frequency and assessment of influence on irinotecan disposition. *Clin. Cancer Res.* **10**, 5889–5894.
18. Aquilante, C. L., Lobmeyer, M. T., Langaee, T. Y., and Johnson, J. A. (2004) Comparison of cytochrome P450 2C9 genotyping methods and implications for the clinical laboratory. *Pharmacotherapy* **24**, 720–726.
19. Lanfear, D. E., Marsh, S., Cresci, S., Spertus, J. A., and McLeod, H. L. (2004) Frequency of compound genotypes associated with beta-blocker efficacy in congestive heart failure. *Pharmacogenomics* **5**, 553–558.
20. Eriksson, S., Berg, L. M., Wadelius, M., and Alderborn, A. (2002) Cytochrome p450 genotyping by multiplexed real-time dna sequencing with pyrosequencing technology. *Assay Drug Dev. Technol.* **1**, 49–59.
21. Ahluwalia, R., Freimuth, R., McLeod, H. L., and Marsh, S. (2003) Use of pyrosequencing to detect clinically relevant polymorphisms in dihydropyrimidine dehydrogenase. *Clin. Chem.* **49**, 1661–1664.
22. Garsa, A. A., McLeod, H. L., and Marsh, S. (2005) CYP3A4 and CYP3A5 genotyping by Pyrosequencing. *BMC Med. Genet.* **6**, 19.
23. Marsh, S., Xiao, M., Yu, J., et al. (2004) Pharmacogenomic assessment of carboxylesterases 1 and 2. *Genomics* **84**, 661–668.
24. Saeki, M., Saito, Y., Jinno, H., et al. (2003) Comprehensive UGT1A1 genotyping in a Japanese population by pyrosequencing. *Clin. Chem.* **49**, 1182–1185.
25. Fukunaga, A. K., Marsh, S., Murry, D. J., Hurley, T. D., and McLeod, H. L. (2004) Identification and analysis of single-nucleotide polymorphisms in the gemcitabine pharmacologic pathway. *Pharmacogenomics J.* **4**, 307–314.
26. King, C. R., Yu, J., Freimuth, R. R., McLeod, H. L., and Marsh, S. (2005) Inter-ethnic variability of ERCC2 polymorphisms. *Pharmacogenomics J.* **5**, 54–59.
27. Rieder, M. J., Reiner, A. P., Gage, B. F., et al. (2005) Effect of VKORC1 haplotypes on transcriptional regulation and warfarin dose. *N. Engl. J. Med.* **352**, 2285–2293.
28. Innocenti, F., Undevia, S. D., Iyer, L., et al. (2004) Genetic variants in the UDP-glucuronosyltransferase 1A1 gene predict the risk of severe neutropenia of irinotecan. *J. Clin. Oncol.* **22**, 1382–1388.
29. Lotsch, J., Skarke, C., and Geisslinger, G. (2003) Simultaneous screening for three mutations in the ABCB1 gene. *Genomics* **82**, 503–510.
30. Pielberg, G., Day, A. E., Plastow, G. S., and Andersson, L. (2003) A sensitive method for detecting variation in copy numbers of duplicated genes. *Genome Res.* **13**, 2171–2177.
31. Soderback, E., Zackrisson, A. L., Lindblom, B., and Alderborn, A. (2005) Determination of CYP2D6 gene copy number by pyrosequencing. *Clin. Chem.* **51**, 522–531.
32. Wojnowski, L. and Brockmoller, J. (2004) Single nucleotide polymorphism characterization by mRNA expression imbalance assessment. *Pharmacogenetics* **14**, 267–269.
33. Teodoridis, J. M., Hall, J., Marsh, S., et al. (2005) CpG island methylation of DNA damage response genes in advanced ovarian cancer. *Cancer Res.* **65**, 8961–8967.

34. Tost, J., Dunker, J., and Gut, I. G. (2003) Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *Biotechniques* **35**, 152–156.
35. Shaw, R. J., Liloglou, T., Rogers, S. N., et al. (2006) Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. *Br. J. Cancer* **94**, 561–568.
36. Entz, P., Toliat, M. R., Hampe, J., et al. (2005) New strategies for efficient typing of HLA class-II loci DQB1 and DRB1 by using Pyrosequencing. *Tissue Antigens* **65**, 67–80.
37. Wu, H., Khanna, D., Park, G., et al. (2004) Interaction between RANKL and HLA-DRB1 genotypes may contribute to younger age at onset of seropositive rheumatoid arthritis in an inception cohort. *Arthritis Rheum.* **50**, 3093–3103.
38. Ringquist, S., Alexander, A. M., Styche, A., Pecoraro, C., Rudert, W. A., and Trucco, M. (2004) HLA class II DRB high resolution genotyping by pyrosequencing: comparison of group specific PCR and pyrosequencing primers. *Hum. Immunol.* **65**, 163–174.
39. Ramon, D., Braden, M., Adams, S., Marincola, F. M., and Wang, L. (2003) Pyrosequencing trade mark: A one-step method for high resolution HLA typing. *J. Transl. Med.* **1**, 9.
40. Hochberg, E. P., Miklos, D. B., Neuberg, D., et al. (2003) A novel rapid single nucleotide polymorphism (SNP)-based method for assessment of hematopoietic chimerism after allogeneic stem cell transplantation. *Blood* **101**, 363–369.
41. Ringquist, S., Alexander, A. M., Rudert, W. A., Styche, A., and Trucco, M. (2002) Pyrosequence-based typing of alleles of the HLA-DQB1 gene. *Biotechniques* **33**, 166–165.
42. Innings, A., Krabbe, M., Ullberg, M., and Herrmann, B. (2005) Identification of 43 *Streptococcus* species by pyrosequencing analysis of the rnpB gene. *J. Clin. Microbiol.* **43**, 5983–5991.
43. Pai, R., Limor, J., and Beall, B. (2005) Use of pyrosequencing to differentiate *Streptococcus pneumoniae* serotypes 6A and 6B. *J. Clin. Microbiol.* **43**, 4820–4822.
44. Sinclair, A., Arnold, C., and Woodford, N. (2003) Rapid detection and estimation by pyrosequencing of 23S rRNA genes with a single nucleotide polymorphism conferring linezolid resistance in Enterococci. *Antimicrob. Agents Chemother.* **47**, 3620–3622.
45. Rahim, A., Coutelle, C., and Harbottle, R. (2003) High-throughput Pyrosequencing of a phage display library for the identification of enriched target-specific peptides. *Biotechniques* **35**, 317–324.
46. Marsh, S., King, C. R., Garsa, A. A., and McLeod, M. L. (2005) Pyrosequencing of clinically relevant polymorphisms. In: *Pharmacogenomics: Methods and Protocols* (Innocenti, F., ed.), Humana Press, Totowa, NJ, pp. 97–114.



<http://www.springer.com/978-1-58829-645-0>

Pyrosequencing Protocols

Marsh, S. (Ed.)

2007, XII, 196 p., Hardcover

ISBN: 978-1-58829-645-0

A product of Humana Press