

Pyrosequencing

A Powerful New Technology

Patients frequently react differently to drugs. An effective treatment for one person may have no impact on someone else, or may even cause adverse reactions. The main cause of these varying effects is the diversity of humans' genetic make-up. Understanding the individual differences in the human genome thus helps physicians select and tailor treatments for their patients. That is the promise of personalized medicine.

The foundation of personalized medicine is pharmacogenomics – the study of how a person's genetic inheritance affects the body's response to drugs. Pharmacogenomics enables physicians to customize therapies for effectiveness and efficiency, avoiding a trial-and-error approach.

Interest has piqued in the oncology community as of late regarding personalized medicine. Serious focus has been given to looking at populations of patients, and determining how to group them into sub-populations with similar characteristics in order to provide them efficacious treatment. One of the technologies gaining ground in this area of research is Pyrosequencing.

Pyrosequencing Technology – a Fast, Sensitive Reliable Tool in Cancer Research

Pyrosequencing technology, which is based on the principle of sequencing by synthesis (fig. 2), provides real-time sequence information. Unlike other technologies routinely used in molecular diagnostics (e.g., PCR), Pyrosequencing reads the actual target sequence. While PCR can only detect known sequences, Pyrosequencing can detect all known and unknown genetic variations in all DNA target regions in which mutations occur.

Mutation and methylation analysis by traditional Sanger sequencing only offers qualitative or semi-quantitative information. Quantitative Sanger sequencing requires time-consuming and

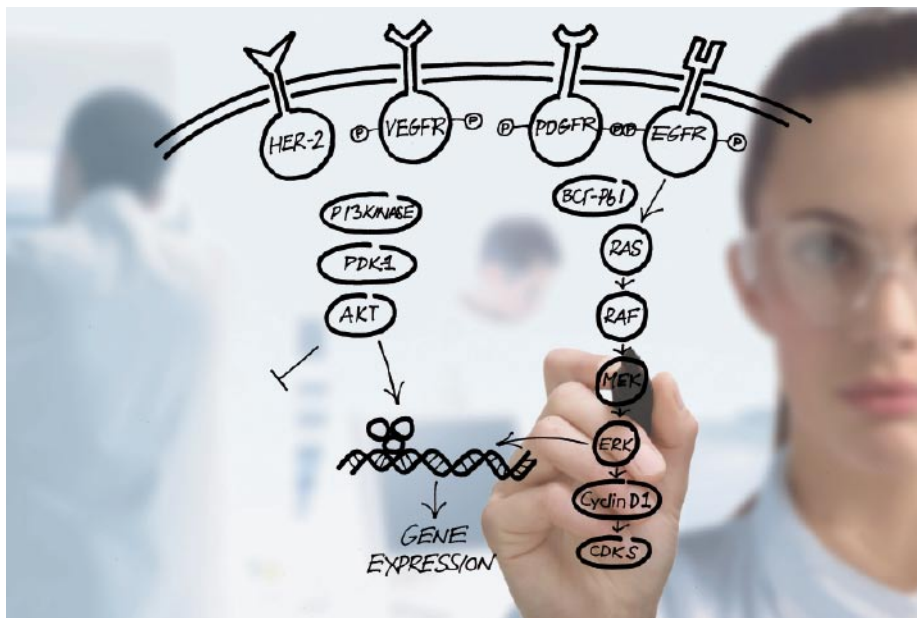


Fig. 1: EGFR Signaling pathway and KRAS

KRAS is regarded as the main effector of the EGFR signaling cascade and may represent a bottleneck in the signaling pathway. Mutations of KRAS will constitutively activate the pathway and render it resistant to upstream inhibitors such as EGFR inhibiting monoclonal antibodies.

labor-intensive cloning of PCR products prior to sequencing. Pyrosequencing, by contrast, offers easy, flexible sequencing (without cloning) combined with highly accurate quantification, in as little as 15 minutes.

Pyrosequencing technology is very applicable for cancer research. For example, it enables analysis of complex mutations in a single assay and facilitates characterization of cancer mutations which are contiguous and/or multi-variable. In addition, it enables easy detection of unknown mutations in hot spots. The technology is ideal for quantification of methylation at multiple, consecutive CpG sites. Researchers can easily quantify single nucleotide polymorphisms (SNPs), allowing them to characterize genetic alterations in tumors and identification of novel tumor markers. Pyrosequencing is important for loss of heterozygosity (LOH) quantification, which helps identify tumor suppressor genes. Most important, perhaps is that Pyrosequencing is in full concordance with traditional sequencing, yet easier and more cost effective than Sanger Sequencing.

One particular area of applicability that is receiving a great deal of attention

from cancer researchers is in regards to the KRAS gene.

KRAS and Cancer

KRAS is an oncogene which plays a crucial role in the development of tumors. Mutations in the RAS family of proto-oncogenes (comprised of HRAS, NRAS and KRAS) are very common, being found in up to 30% of all human tumors. Mutations in the KRAS gene result in a KRAS protein that is permanently active. Such oncogenic activation has been suggested to be involved in many aspects of the development and progression of cancer, including abnormal cell growth, proliferation, and differentiation, as well as increased invasion and metastasis (fig.1). Mutant KRAS is present in approximately 35–45% of metastatic colorectal cancers (CRC), in 15–50% of lung cancers and in 72–90% of pancreatic cancers. The mutations have been localized in various DNA sections, such as in codons 12, 13 and 61.

Studies have shown that KRAS mutation testing can better define which CRC patients will benefit from treatment with epidermal growth factor receptor (EGFR) inhibiting monoclonal antibodies, such as Amgen's Vectibix (panitumumab) and Im-

clone/Bristol-Myers Squibb/Merck KgaA's Erbitux (cetuximab). One such study from the Belgian University in Leuven, suggested that the KRAS mutation status is a prognostic biomarker predicting the outcome of EGFR therapies. In this study, approximately 40% of all CRC-patients had mutated K-ras genes. The trial data indicated that such patients will not benefit from, and in some cases even experience negative reactions to EGFR antibodies, while patients without specific mutations are likely to benefit from this drug treatment.

In response to these studies, European regulators adopted the indication for Vectibix (panitumumab) to include only patients whose tumors carry the unmutated KRAS gene. In November 2008, the U.S. National Comprehensive Cancer Network (NCCN) issued new guidelines for treatment of CRC which recommended that only patients with tumors characterized by the unmutated KRAS gene shall be treated with EGFR drugs. This organization of 21 cancer centers furthermore recommended that oncologists should generally determine the KRAS gene status of all patients diagnosed with CRC prior to any treatment.

A Molecular Assay for the Cancer Biomarker KRAS

Qiagen recently introduced the first Pyrosequencing-based molecular assay for KRAS. The PyroMark KRAS Kit is CE-marked for in-vitro diagnostic use in Europe and currently used in second-line treatment of metastatic CRC together with Erbitux or Vectibix. The kit enables researchers to detect and quantify mutations in codons 12, 13 and 61 using Pyrosequencing technology on the benchtop PyroMark Q24 MDx instrument.

Fragments spanning either codons 12 and 13 or codon 61 are amplified by PCR and then sequenced through the defined region to detect and quantify the common mutations. The assay design even enables detection of additional, rare mutations in these codons. The surrounding sequence context serves as a built-in quality control of the results.

Researchers can realize faster, more accurate detection of mutations using Pyrosequencing technology versus traditional PCR methods.

A Promising Future for Pyrosequencing in Cancer Research

Billions of dollars are spent each year on prescriptions for drugs and other thera-

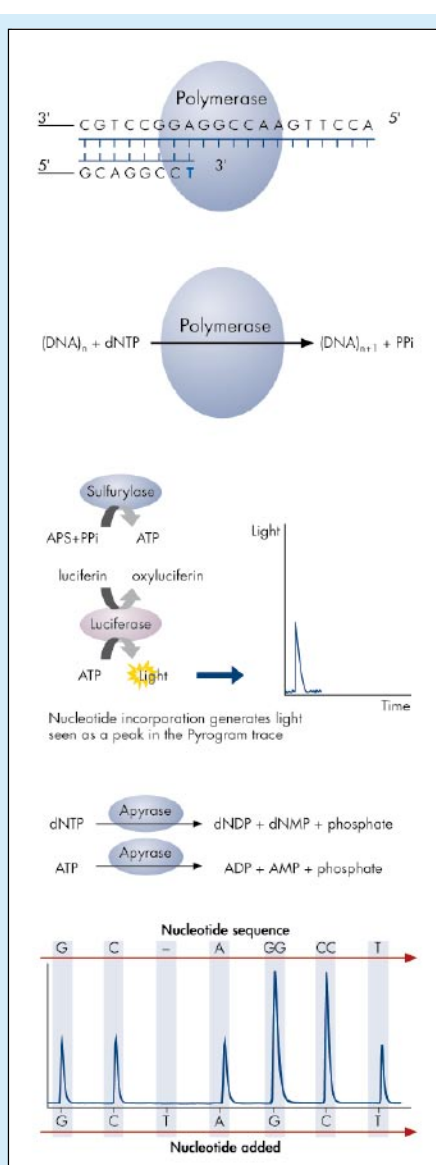


Fig. 2: Principle of Pyrosequencing
The Pyrosequencing reaction undergoes a sequence of various steps for each nucleotide sequenced. The Technology is based on the principle of sequencing by synthesis and provides quantitative data in sequence context.

pies that ultimately turn out to be ineffective or even harmful for particular individuals. Estimates suggest that in Germany alone, up to 58,000 deaths are caused by incorrectly prescribed drugs. In the United States, studies have shown that the most frequently prescribed medications are effective in fewer than 60% of patients. Without significant improvements in the ability to target the right treatment to the most appropriate patients, these adverse effects are likely to intensify and become more frequent in an aging population, with treatment costs rising as a result.

Research regarding the KRAS gene and its influence in cancer and personalized cancer treatment itself is only in its infancy. As more researchers begin to use the technology, we will begin to over-

Step 1

A sequencing primer is hybridized to a single-stranded PCR amplicon that serves as a template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin.

Step 2

The first deoxyribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

Step 3

ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram®). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.

Step 4

Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

Step 5

Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alpha-thio triphosphate (dATP-S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

come the challenges of treatment by maximizing the efficacy and safety of therapies for both specific groups and individual patients.

References

- [1] Institute of Clinical Pharmacology, University of Hannover, 7.3.2003
- [2] Harvard Business Review, October 2007

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