VARIAN

Helix System Technology and Theory

The Helix System is a bio-compatible HPLC designed for DNA fragment analysis. Initially used for the purification and analysis of oligonucleotides, HPLC expanded to double stranded DNA analysis when improvements were made in column technology. Today, HPLC can be used for:

- Oligonucleotide analysis and purification
- Sizing of double stranded DNA
- Detection of sequence variants by denaturing HPLC (DHPLC)

Improvements in column technology occurred in 1992, when ion paired reversed phase (IR-RP) columns were developed by Peter Oefner and Christian Huber.

What is Ion Paired Reversed Phase Chromatography (IP-RP)?

The Helix System uses ion pair chromatography to separate out DNA fragments.
In HPLC, a stationary phase contained in the column is used to retain samples, and a mobile phase is used to release the sample off the column. For DNA IP-RP applications, the stationary phase is comprised of hydrophobic media. The mobile phase is a mixture of an aqueous buffer with an organic co-solvent (acetonitrile) and a counter ion of opposite charge (triethylammonium amine – TEAA) from the sample molecule (DNA). The counter ion (TEAA) in the buffer is positively charged and forms pairs with the negatively charged phosphate groups of DNA, thus coating the DNA molecule in a hydrophobic layer. The number of TEAA molecules attached to the DNA is proportional to the length of the DNA fragment, therefore determining the degree to which the DNA is retained by the stationary phase. The coated DNA molecule is absorbed to the column matrix until it is exposed to a specific concentration of acetonitrile in the mobile phase. The application of an increasing acetonitrile gradient releases the sample in order of increasing length, with smaller fragments eluting first and larger fragments eluting later.

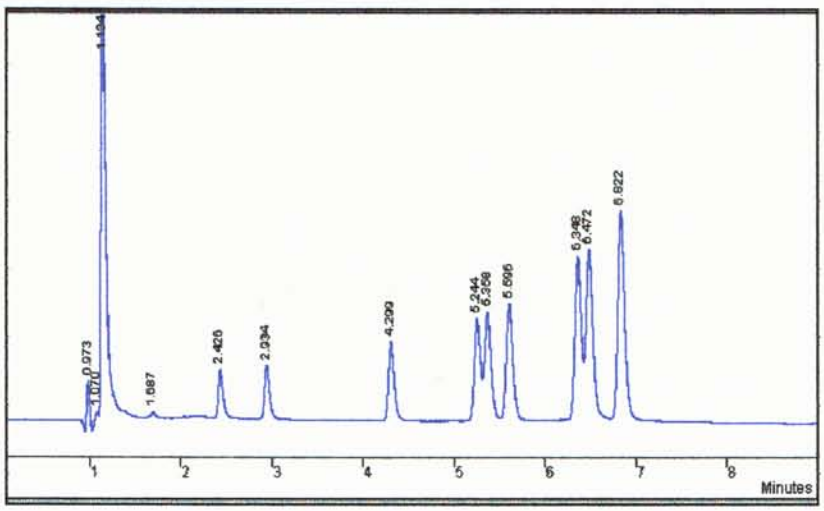
DNA coated with ion pairing agent (TEAA)

Hydrophobic column media

TEAA in the buffer coats the DNA molecule, making it hydrophobic, thus causing the molecule to "stick" to the hydrophobic column media.

DNA Fragment Sizing and Denaturing HPLC

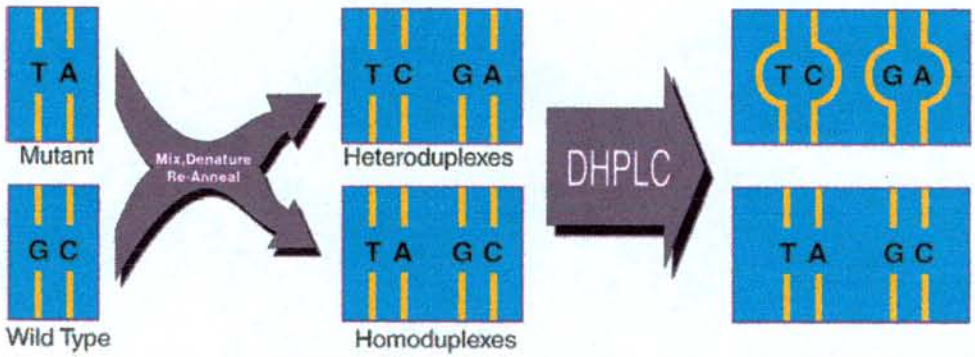
At non-denaturing conditions, DNA is fully double-stranded and standard IP-RP rules apply. DNA separation is based solely on fragment size and is independent of sequence. Sizing results from the Helix System are similar to results obtained from an agarose gel. Using a standard restriction digest such as pUC 18 *Hae*III, an unknown fragment size can be interpolated by comparing retention times. Also, PCR products can be checked for purity. A single well-defined peak at the expected retention time for the amplicon size indicates the presence of a clean PCR product.



A pUC 18HaeIII restriction digest at a non-denaturing temperature.

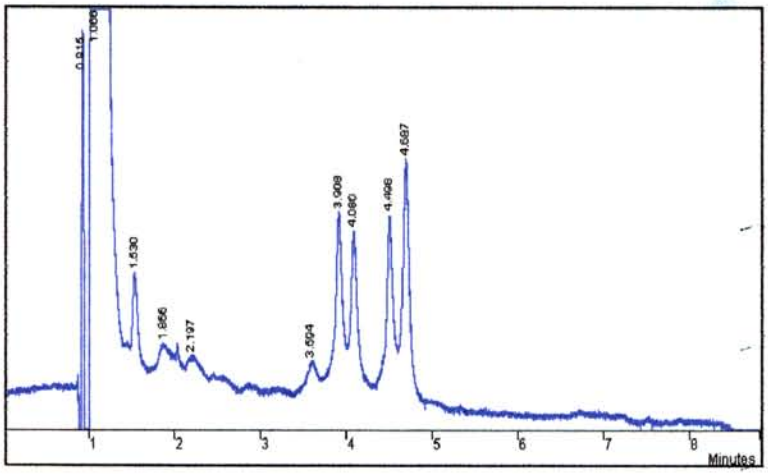
Unlike DNA fragment sizing, denaturing HPLC (DHPLC) occurs at higher temperatures, where DNA is partially denatured. The following steps are taken in DHPLC, which is used for single nucleotide polymorphisms (SNPs) and mutation detection.

1. A heterozygous fragment of DNA, containing a SNP, is amplified by PCR.
2. The amplicon undergoes a post-PCR denaturation/reannealing step in which the sample is gradually cooled from 95 °C to 65 °C. Four species are formed: two that contain a sequence mismatch (i.e. T/C and G/A) and two that are matched (i.e. T/A and G/C).
3. At a partially denaturing temperature, the less stable amplicons will form an opening in the double stranded structure. The more unstable, the larger the opening, or "bubble." In the following example, the order of stability, starting from the least stable, is G/A, T/G, T/A, G/C.



The wild type amplicon is homozygous (GC/GC – not shown) and the "mutant" amplicon is heterozygous (TA/GC). Four species are formed; two contain a sequence mismatch and two are matched.

4. In DHPLC, the least stable elute first and the most stable elute last. The chromatogram will show up to four peaks (2 heteroduplex peaks and 2 homoduplex peaks). Depending on the amplicon, homoduplex as well as heteroduplex species can co-elute, thus forming 2 or 3 peak patterns rather than 4. A single –peak pattern at a partial denaturing temperature indicates that the sample does not have a sequence mismatch, and does not contain a SNP or mutation.



Dys271 mutation standard with an A/G SNP. The first two peaks are heteroduplex, mismatched species. The last two peaks are homoduplex, matched species.

Overall, DHPLC is reported to have greater than 95% sensitivity in detecting SNPs and mutations (O'Donovan *et al.*, 1998). The degree of sensitivity obtained relies primarily on DHPLC temperature optimization. Generally, each amplicon will have a specific partial denaturing temperature at which SNPs/mutations will be detected. The optimal partial denaturing temperature can be predicted by entering the sequence in Stanford's Melt Program found at http://insertion.stanford.edu/meltdoc.html. If the sequence is unknown, the optimal temperature is found empirically.

For more information on determining PCR product purity and DHPLC temperature mapping, refer to our Standard Operating Procedures (SOP).