



HPCE-512 - Repeatability

The core sciences behind the technology of the HPCE-512 are a mixture of chemistry, physics and biology. Experiments using typical analytes such as proteins have demonstrated the technology's capability in delivering significantly superior performance over conventional capillary electrophoresis (CE) systems.

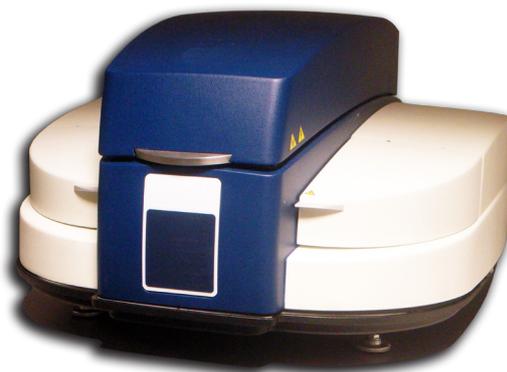
The key components of the instrument are the deployment of specific capillary conditioning regimes, the use of a 512-pixel photodiode detector array and the subsequent algorithmic signal processing of the acquired data.

This last parameter relies on data acquisition from each molecule as it traverses each pixel at specific time points. This allows a distance (z) - time (t) correlation called vertexing to be achieved as the system measures the time it takes for any one analyte to traverse the detector array so that the analytes' velocities are accurately measured.

Vertexing allows high quality data to be produced because vastly increased signal-to-noise ratios are acquired. This allows label-free analyte imaging that exceeds canonical techniques, as only signals that intersect the vertex are used. This is a powerful constraint for the removal of both uncorrelated random background noise and sources of correlated noise not associated with the vertex. This is quite different from conventional CE where the separated analytes are detected by a single point detection system as they traverse the lamp/detector zone. A major issue with capillary electrophoresis in single point detection systems is there is no straightforward way to visualise what is actually happening during the separation, with particular reference to the physiochemical state of the matrix and its movement through the system due to electroosmotic flow.

A key part of vertexing is the generation of an Equiphase map, enabling a very accurate picture of the physiochemical state of the capillary, the sieving matrix and the movement of analytes.

The Equiphase map is particularly important in creating repeatability metrics that ensure reliable data can routinely



and easily be acquired. *Figure 1* shows a standard protein ladder as old PAGE technology data (~36hrs) and label free data (24mins) produced by the HPCE-512 and the associated Equiphase map.

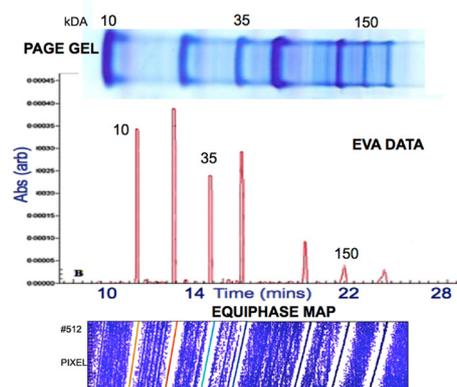


Figure 1. PAGE, HPCE-512 data and the Equiphase map of a standard protein ladder.

The Electroosmotic Flow issue

Capillaries consist of a fused silica inner with an outer polyamide coating for strength. The fused silica contains surface silanol groups (SiOH) that become ionized in the presence of common electrophoretic media so it is negatively charged under most common pH conditions.

The surface charge on the wall (zeta potential), results in the formation of an electric double-layer consisting of an adsorbed immobile layer of positive counter ions which is immobile even under the application of an electric field and a compact and mobile region with substantial cationic character.

Beyond this compact layer is a diffuse layer that is a neutral bulk electrolyte solution, where the zeta potential is no longer felt.

When a potential difference is applied to the capillary, the cations in the compact layer migrate in the direction of the cathode. The ions are solvated in the electrolyte, thus the bulk electrolyte buffer is also mobilised in the direction of the cathode. This fluid flow is called electroosmotic flow (EOF) and if not monitored by multi-point detection and controlled by capillary conditioning will lead to poor data, especially with respect to repeatability metrics (*Figure 2*).

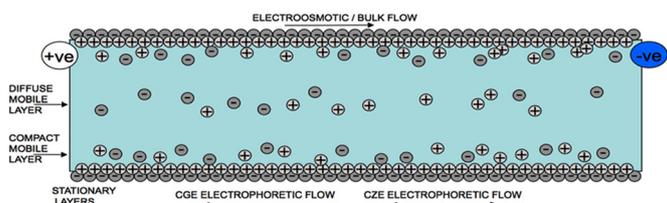


Figure 2. Electroosmotic flow

The HPCE solution

Multi-point detection allows the distance and time-based data sets to be displayed immediately post-analysis as an Equiphase map. The EOF can be monitored for specific separations by observing the behaviour of analyte bands or buffer components. This allows capillary conditioning and other parameters (separation length, pH, temperature, buffers etc.) to be optimised to generate significantly superior data in terms of repeatability as well as resolution and quantification. Inconsistent EOF causes problems for traditional CE systems as they all rely on single point detection and cannot visualise the problem. The multi-point approach of the HPCE-512, however, overcomes these problems.

HPCE Analysis Software – GST and EVA

The Generalised Separation Transform (GST) is designed to retain maximum information on spectra shape as well as quantification. GST is employed as a front-end analysis, the algorithm maximizes signal-to-noise in an approach that retains shape information of the analyte peak.

GST utilizes a method of combining electropherograms before detecting signal peaks. Once transformed, the single-diode electropherograms are signal-averaged to form a histogram. This analysis is very fast, simple and can produce a single dataset that looks like a traditional electropherogram. All original data is retained and it can be shown that the GST approach produces a complementary and unbiased measure of the protein concentration and a powerful validation of the EVA methodology (Equiphase Vertexing Algorithm - see below).

Several details must not be overlooked when considering the multipixel averaging approach to signal-to-noise improvement. In each consecutive single-diode electropherogram, a distinct analyte peak will have a slightly longer migration time as each successive diode is further from the injection point. This means that to signal average,

the signals cannot simply be summed at a particular time point for all the single electropherograms.

Furthermore, the single electropherograms cannot simply be time-shifted relative to one another, as each analyte possesses a distinct velocity (shown by linear slopes superimposed over analyte peaks). Thus, only one analyte will be correctly averaged for any specific velocity correction. To correctly signal average the single diode electropherograms, the last diode (#512) is considered the base electropherogram and intense signal processing procedures are applied to correctly average across all the pixels (*Figure 3*).

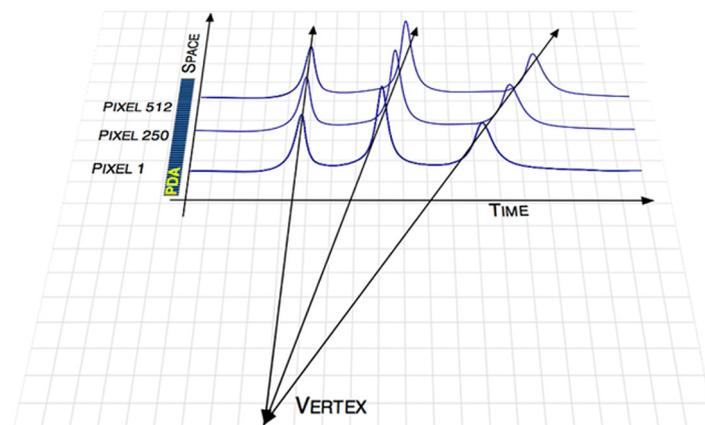


Figure 3. Vertexing and peak finding. The sloped line shows how a vertex is generated. To correctly signal average the last right hand peak is used as the baseline.

This process is generalised to include not just the analyte peaks, but also every data point on the base electropherogram. This allows all points on the weighted electropherogram to be signal averaged, subsequently leading to a single complete signal averaged electropherogram histogram.

Essentially, the GST algorithm is designed to be a noise filter. As the noise component is as likely to be positive as it is to be negative compared to the background voltage, only the real analyte velocities will be accumulated in the histogram, while the integrated noise in a given range of velocities grows much more slowly. This leads to an excellent signal-to-noise ratio.

The Equiphase Vertexing Algorithm (EVA) is the core of the HPCE analysis package. This analyses the electropherogram from each pixel and reduces it to a set of distance-time points corresponding to detected absorption peaks (the Equiphase map). The resulting set of signals clearly shows the sample bands moving through the system and whether or not they are steady and untouched by anomalous EOF effects. They also show any irregularities in the gel, e.g. bubbles or buffer components, as signals that move with anomalous velocities or direction. This has proved to be a useful monitor of run quality as the signals can be associated with, and used to improve knowledge of, the injection vertices.

In each readout period the bands passing the detector appear as a drop in voltage. If these are plotted in a space-time diagram, background signals will appear as randomly distributed points, while the analyte fragments passing through the area covered by the detector will produce defined sloped lines which represent the velocity of the fragment. As all the bands from a common sample injection start at the same point in space-time, these lines will have a common vertex.

Equiphase Map

The Equiphase Vertexing Algorithm (EVA) searches for and calculates the size and position of analyte peaks. The raw electropherograms are processed to reduce them to a set of absorption peaks and a distance-time Equiphase map is built from detected absorption peak heights from each pixel. This map shown in *Figure 4A*, clearly shows the movement of 2 macromolecules through the system, which appear as sloped lines.

Bands are subsequently detected by looking for significant signals in a line of constant velocity in the Equiphase map. At each time slice, EVA calculates the constant velocity of a band that would be visible in the last pixel of the PDA, the base electropherogram. The algorithm then defines a box (*Figure 4B*) in the Equiphase plane centred on the constant velocity line and the signal points for each pixel are summed, giving the final output of the EVA processing.

EVA then uses the points in the Equiphase map to find the vertex associated with the sample bands. Once the vertex has been defined in time and distance, the algorithm assigns each point in the Equiphase map to it.

The use of EVA relative to GST

Both GST and EVA algorithms are designed to maximize the resolution (the algorithmic or “virtual” resolution) and repeatability of the data. The standard quantification is based on peak area, with peak height available as an alternative measure.

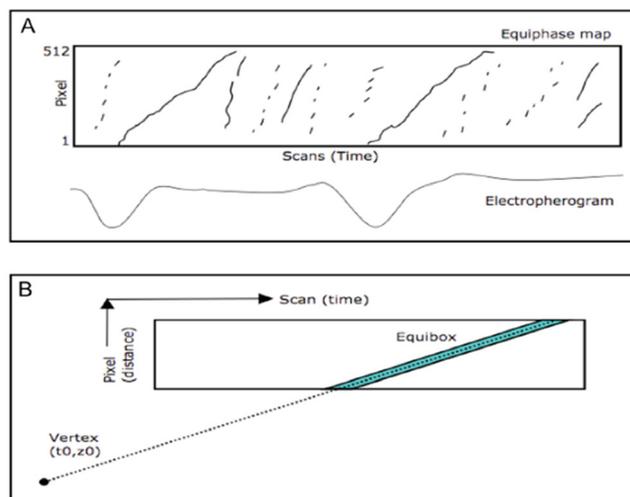


Figure 4. Equiphase map (A) and the Equibox band detection (B).

Through peak detection, EVA allows for better resolution relative to GST. This is due to the fact that GST can smudge peaks if an analyte velocity does not appear constant throughout its separation. This can be due to optics, diffusion, or other phenomena such as matrix migration. On the contrary GST retains peak shape, as there is no prior peak detection present in the algorithm. Since all raw and processed data are kept, other analyses can subsequently be performed.

As well as the above advantages, these signal-processing steps allow the unique ability to monitor what is happening in the capillary itself, “visualising” the electroosmotic flow and using that data to optimise the separation conditions, e.g. pH, field strength and capillary conditioning, with the related improvements in data quality, especially repeatability, resolution and quantification accuracy.

EOF detection and control

Case study 1 – DNA separations to demonstrate matrix decay

Case study 1 (*Figure 5*) shows how use of the Equiphase map was used to monitor the condition of a DNA sieving matrix. Here a 25bp DNA ladder, which contains a 1800 bp fragment, was run through the same sieving matrix 11 times. The first run shows strong well-defined peaks on the left hand panel electropherogram, the upper right panel shows the corresponding Equiphase map.

Each red line is made from the many data points acquired by the multi-point photo diode array and shows strong and straight traces. Although these lines look parallel, when extrapolated downwards these lines will eventually meet at the vertex, as can be seen by the shallower slope of the 1800bp track at 17 minutes.

The electropherogram of run 11 of shows weak and undefined peaks. If this was a QA/QC process, the analyst may be concerned that the quality of the product was seriously compromised and erroneously dispose of the batch.

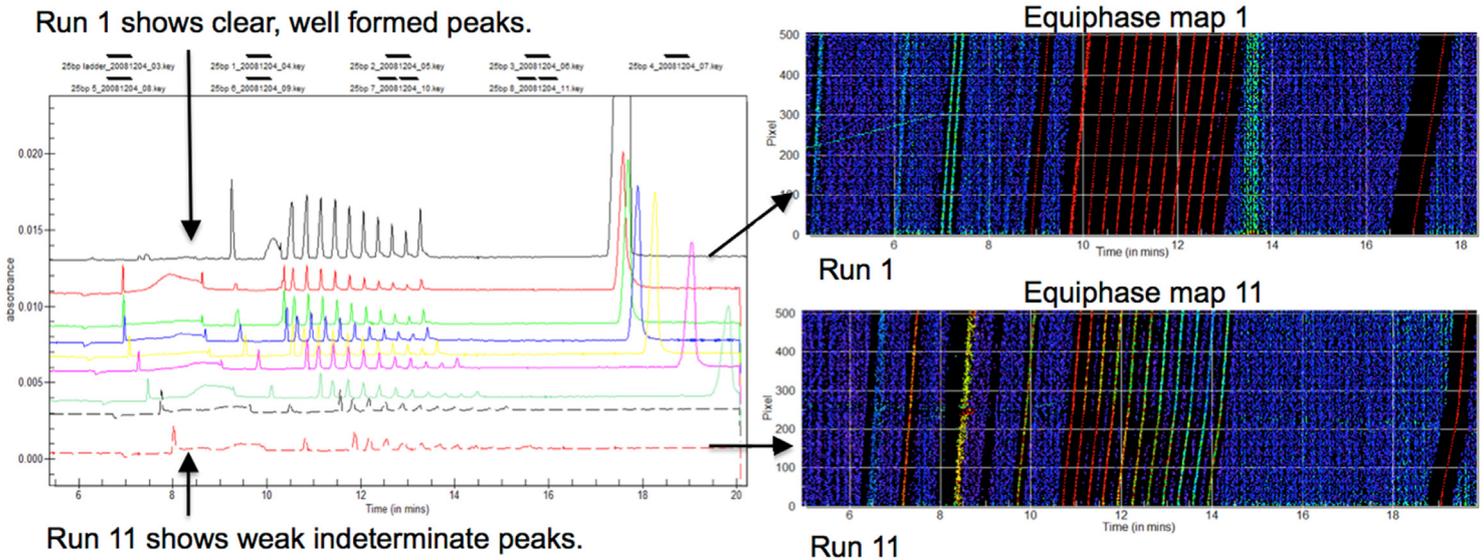


Figure 5. Case study 1, Electropherograms (Left panel) and Equipphase maps (Right two panels) for successive runs of a 25bp DNA ladder.

However, by monitoring the Equipphase map, HPCE technology can prevent this sort of error, as it can plainly be seen that conditions in the capillary have changed. In the lower right Equipphase map, the lines are weak, have lower colour intensity and deviate from the straightness seen in the upper panel.

This feature of the Equipphase map shows that something has gone wrong with the separation conditions, possibly the matrix structure has broken down or the electroosmotic flow has altered. In the case of this separation, it might be the former and the user will be trained to stop the run and replenish the buffer, as well as perform any particular capillary conditioning steps. In dedicated standard operational protocols for specific separations the Equipphase map can also be used to tune the conditioning steps to optimise the parameters of the experiment.

Capillary conditioning

In the case of CE, surfaces are treated to generate a uniform silanol population, a process some refer to as activation, or more commonly as conditioning. This is automatically programmed on the HPCE-512 system and generally consists of solvent, acid and alkaline washes, (Methanol, NaOH, HCl etc) the duration, type and number of which will separation specific. Deactivation in CE is accomplished by the addition of coatings and dynamic additives that eliminate EOF or unwanted analyte adsorption and can also be used in the HPCE-512 system.

Case study 2 – DNA separations in coated and uncoated capillaries

As part of a separate EOF research project the use of a capillary pre-coating was employed where the capillary was flushed with 10% Polyvinylpyrrolidone (PVP) for 5 minutes prior to filling with a separation matrix.

The separation of a 25bp DNA ladder was performed in semi-diluted solutions of Polyethyleneoxide (PEO) with and without a 10% PVP dynamic coating to assess the self-coating ability of PEO and separation stability. Figure 6 illustrates Equipphase maps generated from the separation of a 25bp ladder (Promega) in 4% PEO (Mn 300,000) in the absence (A) and presence (B) of 10% PVP.

Separation between the 25bp fragments occurs in both cases. However, in the absence of PVP an EOF is clearly observed as the red and green traces on the left hand side of panel A that are moving in the direction of the EOF. These particulates present in the buffer are insoluble contaminants and stabilizers present in PEO (e.g. butylated hydroxytoluene). The DNA traces are boxed and enlarged for both experiments. Figure 7 shows the GST data for the ladder run with the PVP dynamic coating.

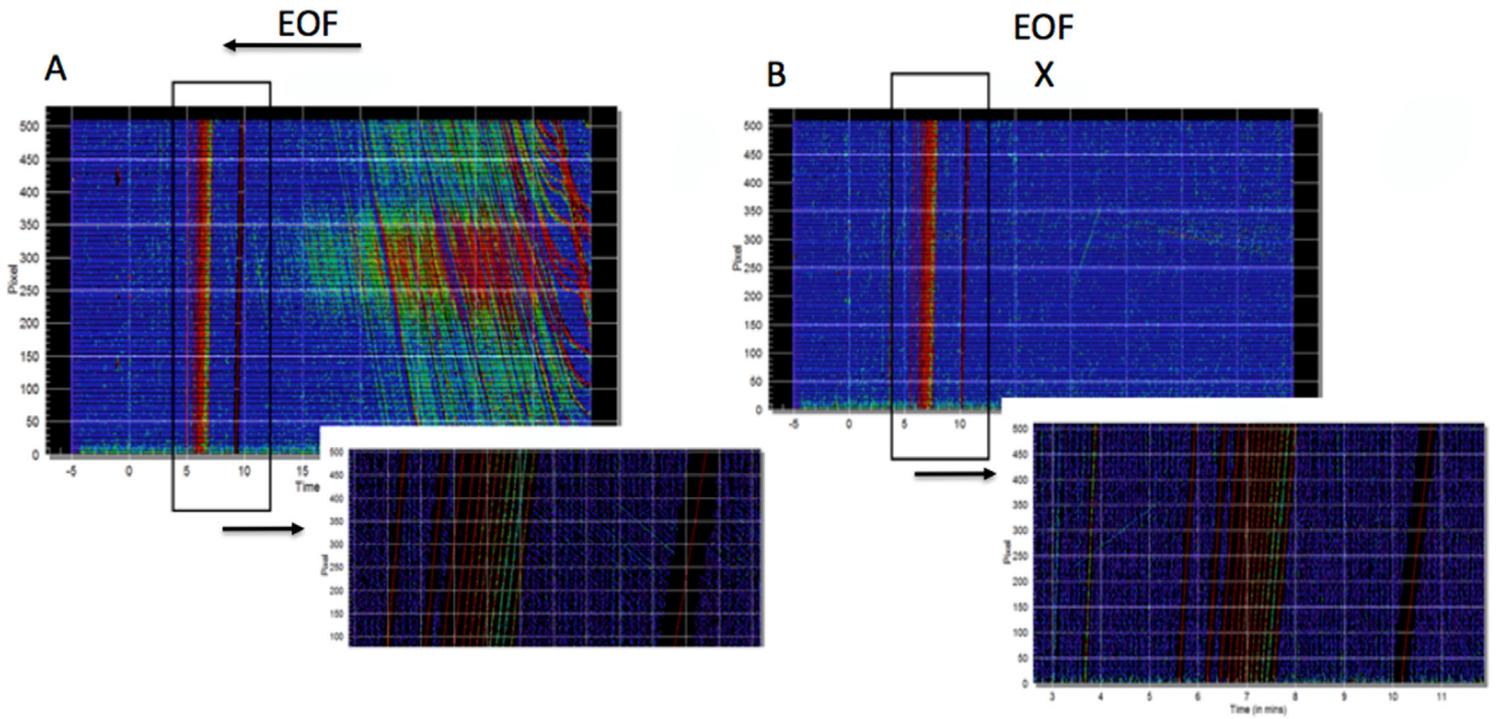


Figure 6. The control of EOF by capillary coating. The DNA bands are moving with the EOF to the right hand side.

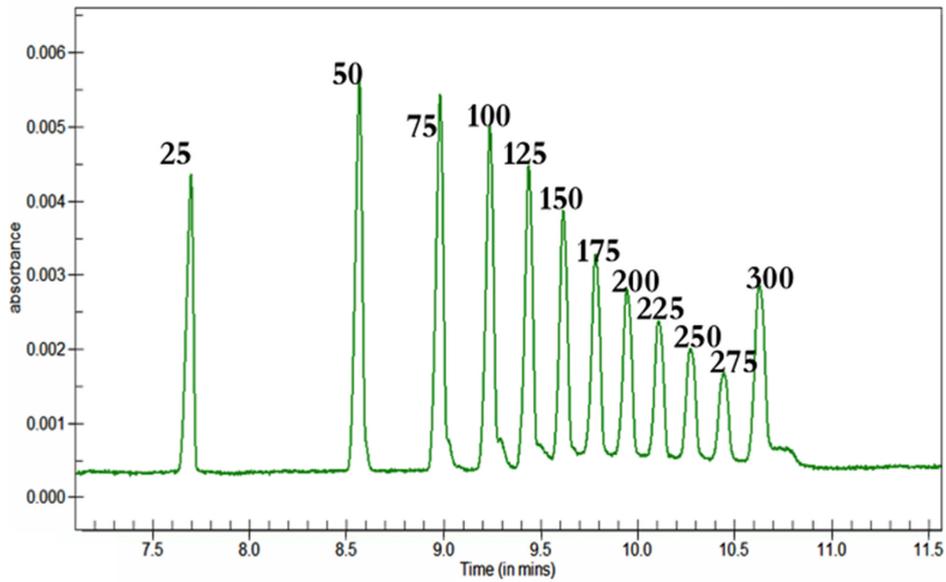


Figure 7. The GST readout of the 25 bp DNA ladder.