Evaluation of Capillary Electrophoresis Performance Through Resolution Measurements

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Abstract

The application of resolution measurements to an electrophoretic system can give a quantitative analysis of the health of that system. Capillary electrophoresis has become a routine method for the analysis of DNA and resolution measurements can be used to assess the resulting electropherogram. A number of methods are available to evaluate resolution and three methods are detailed in the current work. Parameters such as polymer concentration and column length were also examined in terms of resolution, and changes therein, if these parameters were modified.

Key Words: Capillary electrophoresis, DNA, resolution, forensic science, performance analysis

Introduction:

Capillary electrophoresis has become a popular method for the separation and analysis of DNA. The instrumentation currently in preferred use has a single capillary through which one sample is analyzed at a time and a new gel matrix replaced before the analysis of the next sample proceeds. A variety of gel matrices, both commercial and in-house prepared, along with variations in column length may be used to optimize the capillary electrophoresis process. (1) To evaluate these or other parameters that may affect an electrophoretic system, or to monitor the quality of an established system, one needs to objectively review the results obtained from the electropherogram. Many approaches exist that can accomplish this. Classical chromatographic methods have used theoretical plates to determine the efficiency of a chromatographic system (2) and these methods have been applied to assess capillary electrophoresis systems. (3) The term, theoretical plates, typically refers to the partitioning of solute between a fixed immobile matrix and a mobile phase which is different than what occurs in the analysis of DNA by capillary electrophoresis. In the typical analysis of DNA by capillary electrophoresis (CE), the DNA molecule is separated by a sieving mechanism based on fragment base sizes. An evaluation approach that may be more appropriate for CE than theoretical plates is to determine the resolution of the system. Probably the most important parameter to determine the health of the electrophoretic system,

resolution measures the ability of the system to separate components. Quantitative resolution measurements will permit the monitoring of the system over time, and allows for the comparison of data within and between laboratories.

A number of approaches are available to calculate resolution. Luckey et al. (4) uses an expression that relates the distance between two peaks and the widths of those peaks measured at half the peak height:

$$R = [2(\ln 2)]^{1/2}(t_2 \cdot t_1)/(W_{h1} + W_{h2}) \qquad \text{eq. 1}$$

This can be written in terms consistent with other equations described in this work as:

$$R = [2(\ln 2)]^{1/2}(\Delta X)/(W_{h1} + W_{h2}) \qquad \text{eq. 2}$$

In this equation, ΔX is the distance between the two peaks under consideration, W_{h1} is the width of peak one at half its height and W_{h2} is the width of peak two at half its height. Figure 1 demonstrates how to obtain W_h and ΔX. Whether in millimeters, time or scan number, measurements of ΔX and W_h must be in the same units.

Dividing the base difference (ΔM) between the two peaks under consideration by the resolution number obtained with equation #2 gives the resolution in bases (5):

$$R_b = \Delta M / R \quad \text{(base resolution)} \qquad \text{eq. 3}$$

Values of one or less determined from equation 3 mean that DNA fragments differing in length by a single base would be separated at the baseline. Values greater than one indicate the peaks will be merged. A value of 1.7 indicates that theoretical peaks would be merged at the 50% of their height.

Another approach to the calculation of resolution has been detailed by Heller et al. (6) and by Lerman and Sinha (7). Their method evaluates a single peak instead of two as described by Luckey. This approach allows for an easy comparison between different electrophoretic parameters or for a simple and effective evaluation of a system.

$$RSL = W_h / (dX/dM) \approx W_h / (\Delta X/\Delta M) \qquad \text{eq. 4}$$

Where RSL stands for resolution length, W_h is the peak width at half height for a single peak under consideration, ΔX is the distance between this peak and an adjacent peak, and ΔM is the difference in bases between the evaluated and the adjacent peak. At an RSL of one, theoretical peaks that differ by one base and are of similar height, merge at 50% of their height. (Heller, C. personal communication)

For routine forensic casework applications, our approach has been to monitor the resolution of the system through what we have termed a valley value, V_v. To determine the V_v, capillary electrophoresis of a sample containing DNA fragments that differ by one base is performed. The valley, or the point where the two peaks merge, is divided by the peak height of the larger peak.

$$V_v = V/H \qquad \text{eq. 5}$$

Where V is equal to the valley and H is equal to the peak height. Peaks that do not merge are considered completely resolved. This procedure works best when the two peaks are of similar height. The V_v can also be expressed as a percent by simply multiplying equation 5 by 100.

Changes in the gel matrix or variations in the capillary length can affect the resolution of the system. Two different gel matrices are commercially available, as are columns of two different lengths. Both gels and both capillaries have been evaluated here. The work presented examines the effect polymer concentration and column length has on resolution. Methods are also described that allow the investigator to easily monitor

resolution and perform routine quality control on the system and reagents used in the DNA electrophoresis process.

Materials and Methods

The capillary electrophoresis unit employed for this study was an ABI PRISM ™ 310 Genetic Analyzer (PE Corporation, Foster City, CA). Two capillary columns differing only in length were evaluated. Both columns (PE Corporation) were uncoated, 50 um ID, with one 47 and the other 61 cm in length. The read length, the distance from injection end to laser, was 36 and 50 cm for the 47 and 61 cm columns respectively. The separation mediums were the proprietary polymer products POP 4 or POP 6 (PE Corporation) as noted within the text. The run buffer was a 1:10 dilution of 310 Genetic Analyzer Buffer with EDTA (PE Corporation). An allelic ladder from the AmpFISTR™ Green I kit (PE Corporation) was prepared for CE analysis by mixing 1 microliter of the ladder with 0.5 microliter of GeneScan-350™ ROX internal lane standard (PE Corporation) and 12 microliters of deionized formamide (Amresco, Solon, OH). The AmpFISTR™ Green I kit contains primers that amplify the sex typing locus amelogenin, and three STR loci, THO1, TPOX, and CSF1PO. The allelic ladder supplied with this kit represents typical alleles for each locus.

Samples were routinely electrokinetically injected at 15 kV for 5 seconds, followed by electrophoresis at a constant voltage of 15 kV and a

constant temperature of 60° C. Times for the analysis varied as to column length or polymer employed. Prior to each injection the column was back flushed with new polymer by the instrument. The software employed was GeneScan® version 3.1, with the analysis parameters set to light smoothing. Only those electropherograms with peaks ranging from 150 to 5000 relative fluorescence units (rfu) were used in the analysis. From previous studies, peaks above 5000 rfu were found to not be within the linear range of the instrument. Peaks below 150 rfu were not evaluated due to a laboratory imposed lower cut off.

Resolution measurements were conducted using a digital caliper to obtain peak widths, heights and distance measurements between peaks from printed, similarly scaled electropherograms. The caliper was used to obtain very accurate measurements for the comparisons performed here. The GeneScan® software is perfectly suitable for routine resolution analysis. When appropriate, the base difference between peaks was expressed as bases and not base pairs since the system under consideration examines single strand DNA.

Results and Discussion

The commercial availability of different polymer solutions and columns of different length allows for an easy evaluation of these products for the

separation of DNA. A number of observations can be made from the experiments performed using these products. The peak spacing, the distance between adjacent peaks, increases as the polymer concentration or column length increases. The width of the peaks, measured at half height, broadened with the change from POP 4 to POP 6.polymer. Likewise, the increase in column length from 47 to 61 cm, while maintaining the POP 4 polymer, approximated the increase in peak width seen with the POP 6 polymer as shown in figure 2. Resolution values are dependent upon peak width and peak spacing. As noted above, both are affected by column length and polymer concentration. Figure 3 shows the dependence of resolution, RSL, on the length of column, polymer concentration and DNA chain length. When polymer concentration or column length is increased, the resolution of the system is improved. However, regardless of the system variable, the corresponding increase in peak spacing seen with increases in polymer concentration or capillary length is found to be limited by fragment length, which in turn affects resolution. The number of bases in the DNA fragment, is inversely proportional to resolution, i.e. as the fragment length increases the resolution of the system decreases (figure 3). A comparison of the resolution values obtained using equations 3, 4 and 5 are shown in table 1. The values detailed in table 1 were obtained using both amelogenin peaks, alleles 7 and 8 in THO 1, 9 and 10 in TPOX, and 10 and 11 in CSF1PO. The

first peak in each locus noted was used to determine W_h for the calculation of RSL and both peaks were used to calculate R_b. The term V_v was calculated using the 9.3 and 10 alleles in the THO 1 locus. This table allows one to compare resolution values obtained using the equations discussed above for the three different electrophoretic systems described. These values may also be useful for comparison to internal laboratory results or may suggest suitable systems to attain a resolution for a particular application.

The results detailed on figure 3 show that one base resolution could be expected with the POP 4 system for fragments up to approximately 250 bases. However this value is somewhat misleading. At an RSL of one, peaks differing by one base in size are expected to merge at half height, but this does not take into consideration the overlap of the peaks and the summing of this overlap. If peak deconvolution programs were used, single base resolution could be observed. The approximate merge point of two peaks differing by one base is described by V_v. The relationship between V_v and RSL is shown in figure 4. These RSL calculations were performed on the THO I 9 and 10 peaks, with V_v calculated from the THO I 9.3 and 10 peaks. The plot of these two assessments of resolution appears to be linear, and would indicate that either method to evaluate resolution would be acceptable. The calculation of V_v from the THO I peaks provides a value for the resolution in this limited proximity of the electrophoresis run, and

does not necessarily provide information concerning the entire electropherogram. However, in our experience, the assessment of V_v provides a good estimation of the resolution of the system, and can be applied as a means for quality control.

The V_v calculation is a quick and an intuitive method for evaluating the resolution of a system. However in those situations where one base variants are not available for the calculation of V_v resolution, the RSL method can be easily used. This can be accomplished through the direct calculation of appropriate allelic ladders run in conjunction with a series of electrophoresis runs, or through calculations performed on the electropherogram from a particular sample. For individual sample resolution calculations, either the observed alleles or the internal size standard can be used to evaluate resolution. To assess whether one could expect comparable resolution values using either the peaks observed in an allelic ladder or from the internal size standard, RSL determinations were performed on measurements taken throughout the electropherograms for both the Green I ladder and ROX internal size standard. The results from these calculations were graphed against each other, and resulted in a linear plot. Figure 5 shows the relationship between RSL for the Green I ladder and the ROX internal size standard. This graph demonstrates that nearly equivalent assessments of resolution can be obtained by evaluating ladder alleles or the ROX internal size standard. Calculating RSL using the size

standard would allow some consistency in the evaluation of samples that have variations in alleles and fluorescence intensities.

The quality of the reagents used in CE can drastically affect resolution. (8) Formamide is routinely used as a denaturant for the analysis of single strand DNA. Evaluating the quality of the formamide is often done through conductivity measurements but can also be accomplished through resolution measurements. The V_v measurement is a very sensitive indicator of formamide quality and can be obtained by injection of the Green I allelic ladder processed with the test formamide. A given lot of formamide can be assessed, frozen in aliquots, after this initial test is performed.

Resolution measurements can provide an evaluation of the health of the capillary electrophoresis system. Through resolution monitoring, one can determine if the electrophoretic run and all reagents used in the process have performed as expected. The number of procedures discussed here for the evaluation of system resolution should lend flexibility in implementation to any laboratory interested in tracking resolution for quality assessment purposes.

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Table 1

A comparison of the resolution values for RSL, R_b, and V_v obtained using the polymers POP 6 and POP 4 with the 47 cm length column or POP 4 with the 61 cm length column (denoted as POP 4L in the table).

Figure 1

Description of terms used in the calculation of resolution. The peak width (W_h) is measured at half peak height, thus if a peak has a height of 1000 units, W_h would be measured across the width of the peak at 500 units. The ΔX term is the distance between the two peaks in the same unit measurements used to determine W_h.

Figure 2

The variation of peak width (W_h) as a function of DNA fragment length in three different CE systems: 47 cm length column using polymer POP 4 (· · · ·), 47 cm length column using polymer POP 6 (— —), and 61 cm length column using polymer POP 4 (——) and noted as POP 4L in the legend. Increasing the polymer concentration or column length results in peak broadening as detailed. The measurements were obtained from the allele peaks in the Green 1 ladder.

Figure 3

Resolution, RSL, as a function of DNA fragment length as polymer concentration or column length is varied. The legend used in this figure is the same as that detailed in figure 2. Resolution improves as polymer concentration or column length increases. However resolution decreases as fragment length increases.

Figure 4

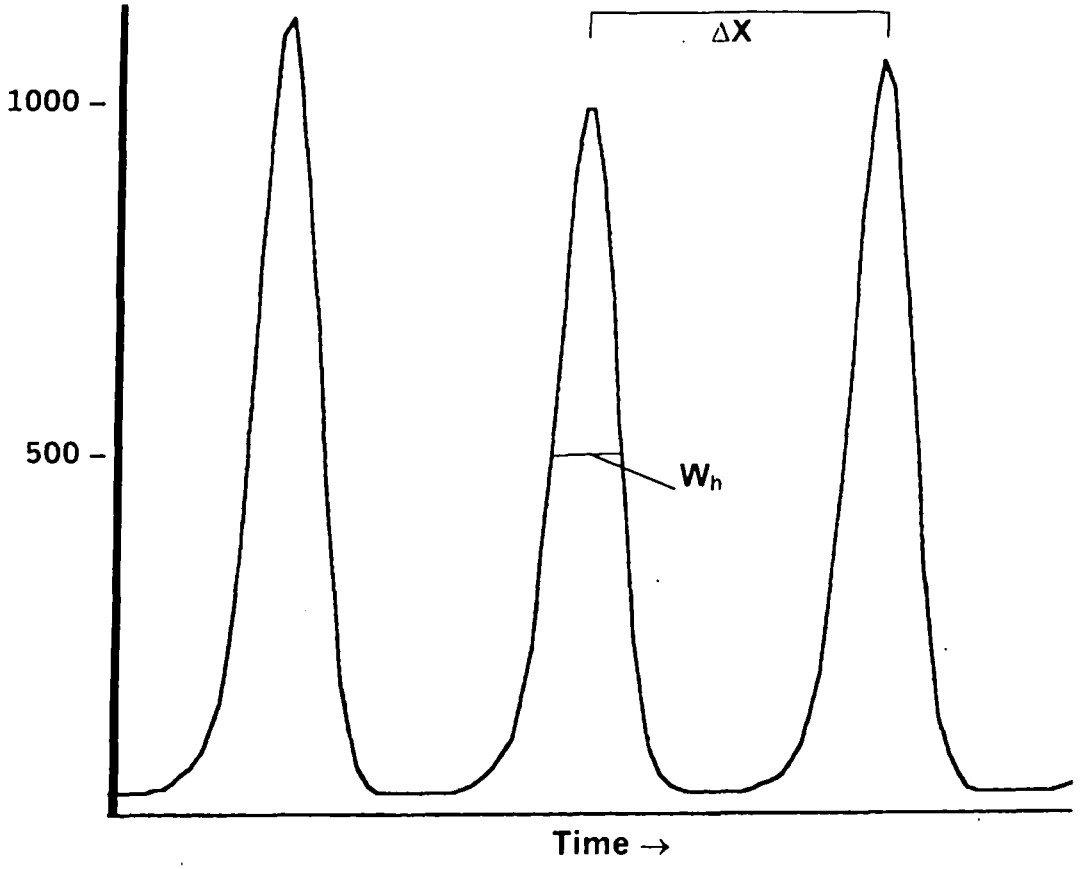
Valley value measurements expressed in percent as compared to resolution, RSL. Included in this data are results from POP 6 and POP 4L analyses. The majority of the data were analyses employing POP 4 with a 47 cm column.

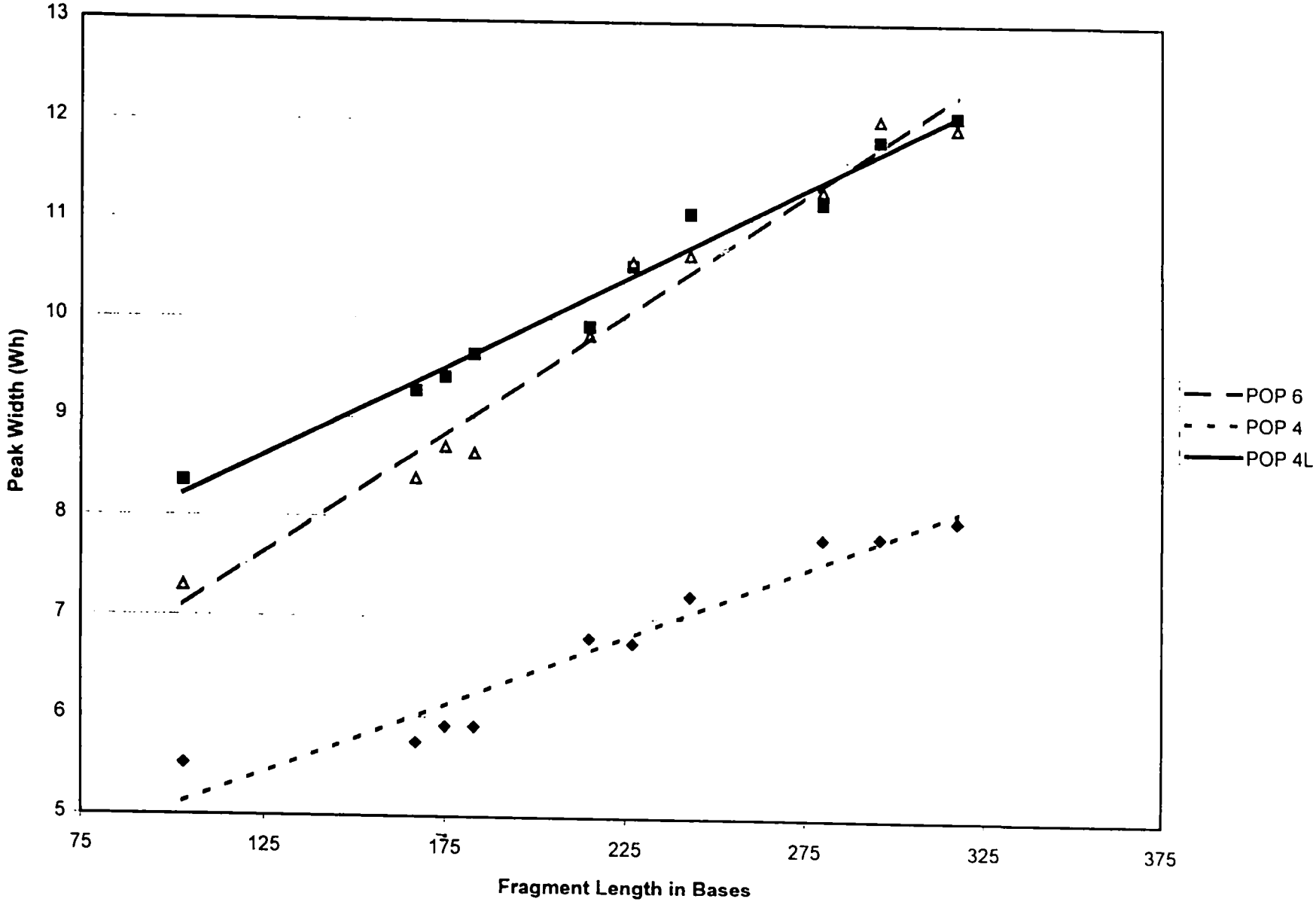
Figure 5

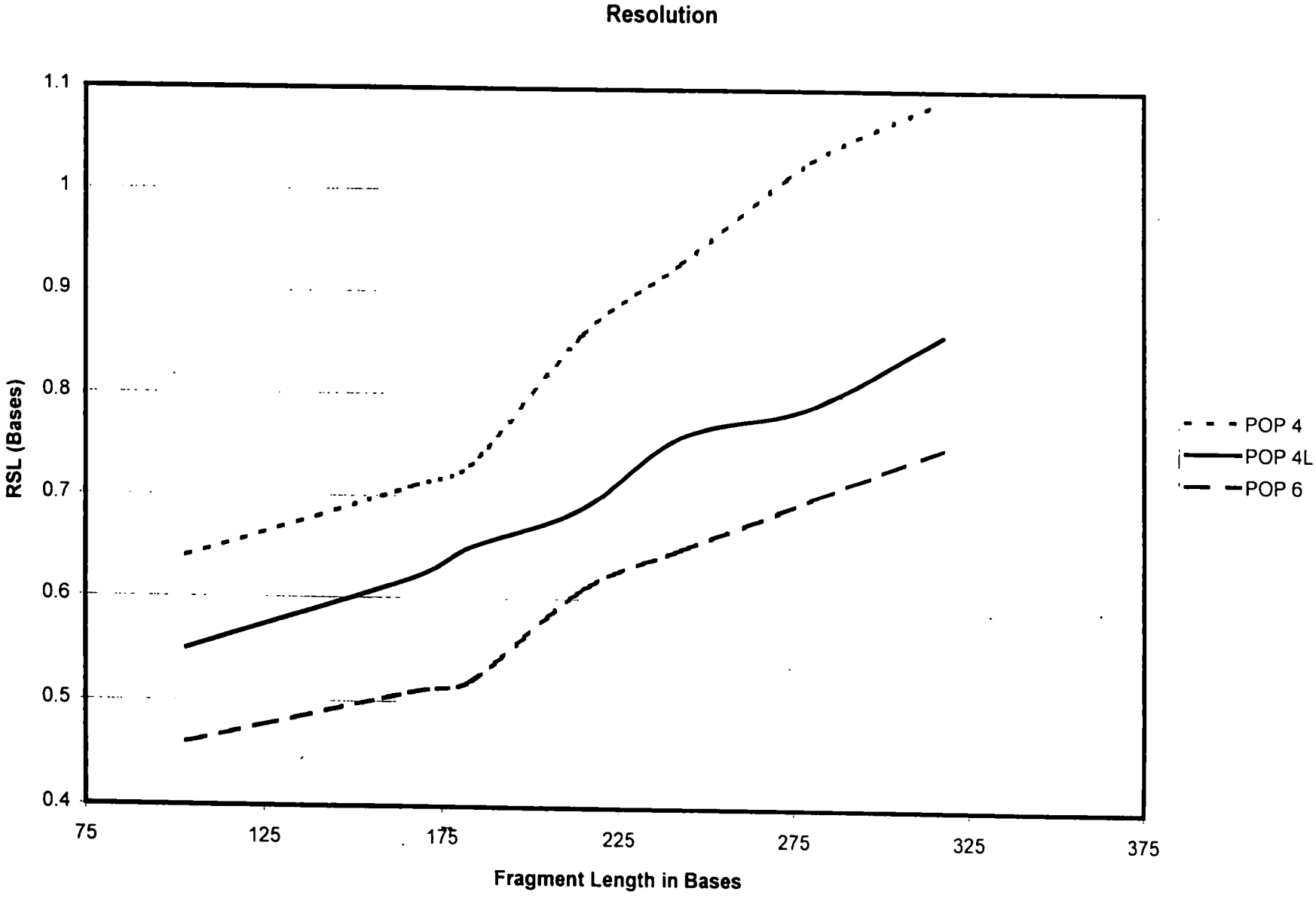
Resolution measurements as a function of fragment length for the ROX internal standard and for the Green 1 ladder. General overlap between the two data sets indicates that similar resolution measurements could be obtained using either the ROX peaks or an allelic ladder peaks for the measurement of RSL.

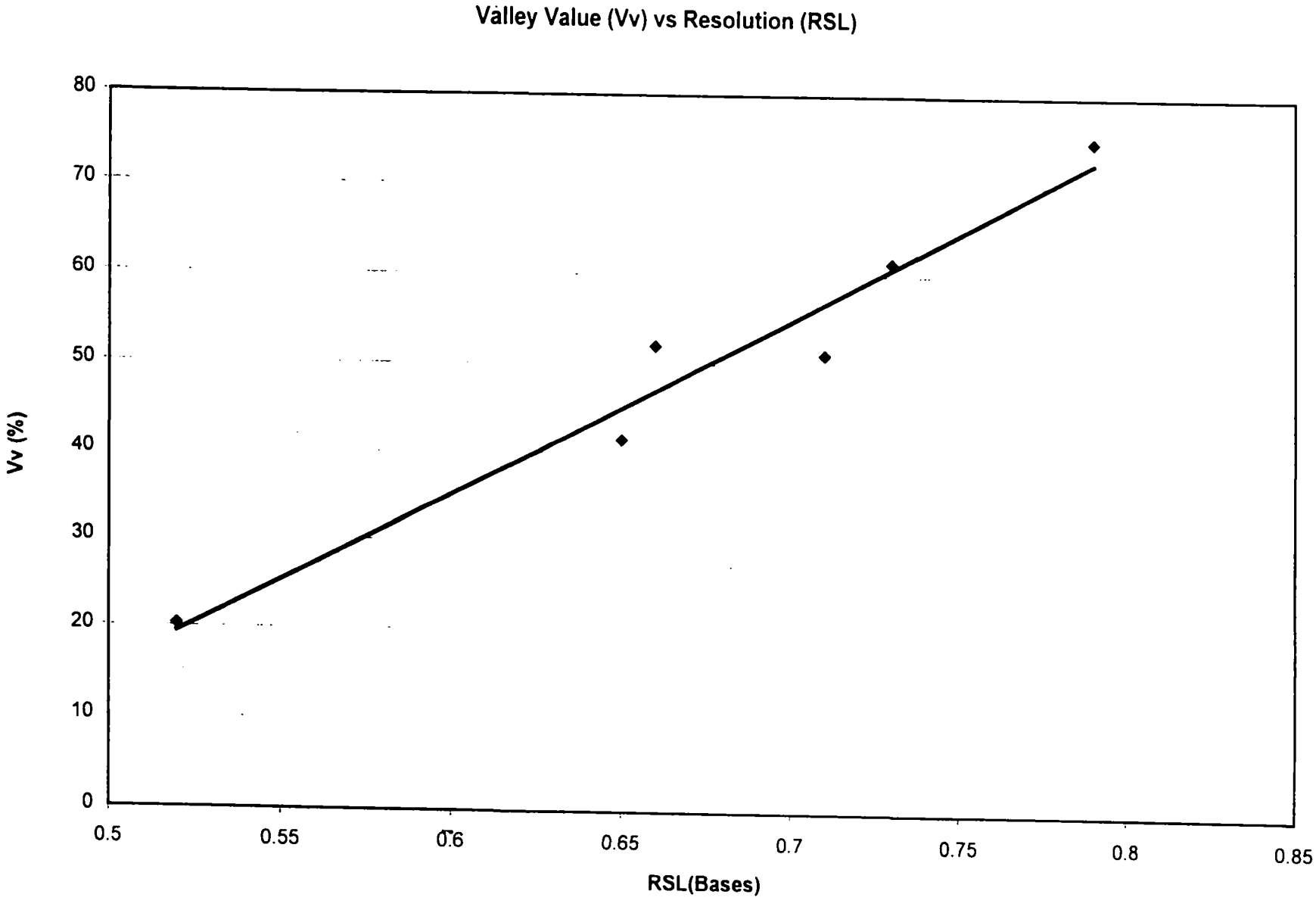
Resolution values for the loci contained in the Green 1 ladder

LOCUS	POP 4	POP 4	POP 4	POP 6	POP 6	POP 6	POP 4L	POP 4L	POP 4L
	R_b	RSL	V_v	R_b	RSL	V_v	R_b	RSL	V_v
Amelogenin	1.04	0.61		0.80	0.46		0.94	0.55	
THO 1	1.13	0.67	51%	0.90	0.53	20%	1.06	0.63	42%
TPOX	1.40	0.82		1.09	0.65		1.24	0.73	
CSF1PO	1.64	0.96		1.25	0.74		1.42	0.84	


ΔX
1000 –
500 –
W_h
Time →


13
12
11
10
9
8
7
6
5
Peak Width (Wh)
75
125
175
225
275
325
375
Fragment Length in Bases
POP 6
POP 4
POP 4L


Resolution
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
RSL (Bases)
75
125
175
225
275
325
375
Fragment Length in Bases
POP 4
POP 4L
POP 6


Valley Value (Vv) vs Resolution (RSL)
Vv (%)
80
70
60
50
40
30
20
10
0
0.5
0.55
0.6
0.65
0.7
0.75
0.8
0.85
RSL(Bases)