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APPLICATION OF CGE TO VIRUS IDENTIFICATION

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SUMMARY

Protein profiling is an increasingly valuable tool for the characterization of protein populations and has been used to identify microorganisms, most often using 2-dimensional gel electrophoresis followed by mass spectrometry. We present a rapid method for the identification of viruses using microfluidic chip gel electrophoresis (CGE) of high-copy number proteins to generate unique protein profiles. Viral proteins were solubilized, fluorescently labeled and then analyzed using the μChemLab™ CGE system (~10 minutes overall). A Bayesian classification approach was used to classify the reproducible and visually distinct protein profiles of MS2 bacteriophage, Epstein-Barr, Respiratory Syncytial and Vaccinia viruses as well as discriminate between closely related T2 and T4 bacteriophage.

Keywords: Chip gel electrophoresis, Protein profiling, Virus identification, Laser-induced fluorescence (LIF)

1. INTRODUCTION

Protein profiling is a technique broadly applicable to characterizing microorganisms and has been described predominantly in the mass spectrometry literature for identifying bacterial and viral proteins (1-5). Protein profiling is very appealing for diagnostics, as it is applicable to a variety of organisms, including viruses, and does not require specialty reagents such as cell culture lines, antibodies and nucleic acid primers and probes typical of more common approaches such as cell culture, immunofluorescence assays and PCR.

Although typical methods for protein profiling employ 2D gel separation of proteins followed by mass spectrometric analysis of manually excised protein gel bands, methods using capillary electrophoresis can be useful for identifying isolated microbes, saving both time and labor (6, 7). The protein signatures for even the most complex viruses are relatively simple—for example, the genome of Variola major (smallpox) encodes 187 putative proteins (8) and Ebola encodes eight proteins (9)—and thus are within the peak capacities of most gel electrophoretic separations.

Chip-based analytical methods are rapidly maturing and now compare to capillary-based methods in terms of analytical merits such as speed and resolution (10-13). Chip-based analyses are also much easier to integrate with other processing steps such as PCR and protein digestion (14, 15). Several commercial chip-based electrophoresis systems are now available.

Here we describe a microfluidic protein profiling approach using protein solubilization coupled with microfluidic chip gel electrophoresis (CGE) for the identification of viruses (16). This method

is rapid (<10 minutes) and sensitive, and adaptable to sample preparation and analysis using either capillary- or chip-based gel electrophoresis instrumentation.

To accurately determine the molecular weight of viral proteins, we describe a two-color LIF detection system to simultaneously measure fluorescence from reference standards (650 nm) and analyte proteins (470 nm) in a sample. This method makes it possible to locate standard peaks without interference from either the sample or background peaks; likewise, standard peaks do not add to the sample protein peak profile. We utilize a Bayesian classification methodology to evaluate whether or not CGE protein profiles can be used to uniquely identify each virus in the context of the others.

2. MATERIALS

Prepare all solutions using ultrapure water (any commercial water purification system capable of producing high quality water such as 18 MΩ cm at 25 °C will suffice) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

2.1 SAMPLE PREPARATION COMPONENTS

1. Sample/lysis buffer: 5 mM boric acid and 5 mM sodium dodecyl sulfate (SDS) adjusted to pH 8.5 with 1 M sodium hydroxide. This solution should be made up fresh at least once every couple of weeks to maintain the pH (See **Note 1**).
2. Fluorescamine dye solution: 10 mM fluorescamine (Molecular Probes, Eugene, OR, USA) in dry acetonitrile. This should be made up fresh each day.
3. Molecular weight standards: alpha-lactalbumin, carbonic anhydrase, ovalbumin and bovine serum albumin (all Sigma, St. Louis, MO, USA); cholecystikinin flanking peptide (CCK) (Commonwealth Biotechnologies, Richmond, VA, USA), mouse IgG (Transduction Laboratories, San Jose, CA, USA), and 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) (Molecular Probes, Eugene, OR, USA).
4. Pre-labeling of molecular weight standards: Proteins should be labeled and purified from excess dye (AlexaFluor647) according to manufacturers' protocols (Molecular Probes, Eugene, OR, USA). Once labeled the protein standards should be made up in 50 mM Tris pH 7-8 (see **Note 2**) at 10x of the working concentration, typically 20 to 100 nM range depending on the sensitivity of CGE/LIF system, and then stored at -20°C or below in small aliquots to avoid repeated freeze-thaw cycles.

2.2 CGE COMPONENTS

A. Chip washing solutions: 1 M NaOH, 1 M HCl and water
B. CGE buffer: Polyethylene glycol/polyethylene oxide sieving gel, in the 14-200 kDa range (Beckman, Fullerton, CA).
C. Microfluidic chip: The chip is 2.0 x 2.0 cm square, made of fused silica (see **Notes 3** and **4**), and incorporates a 100 nL, 34 μm deep sample loop (see **Note 5**), a 500 μm offset T-style injector (see **Note 6**), and a 10 cm long, 30 μm deep separation channel (Figure 1)

(see **Notes 7 and 8**). The sample loop connects to the separation channel via a 4 μm deep sample transfer channel (see **Note 5**). The relative pressure resistance of the four channel arms connecting the buffer, sample, sample waste and waste ports enable all channels to be filled when the fill port is pressurized. Shallow segments of channel (1 mm wide and 4 μm deep) at the flush/fill and buffer reservoir ports serve as on-chip weir-type particle filters to reduce the number of particles introduced into the channels (Figure 1a).

2.3 Electrophoresis apparatus

1. Fluid reservoirs: The microfluidic chip connects to fluid reservoirs and a syringe injection port via an O-ring sealed manifold plate (Figure 1b). Fluid reservoirs (~500 μL each) are located above the "via" holes at the buffer, sample, sample waste and waste ports (see **Note 9**).
2. Electrodes: Four electrodes are inserted into the buffer, sample, sample waste and waste fluid reservoirs that, when energized, provide the electric field for electrophoresis. The range of voltage applied to the electrodes is between 0 - 10 kV. Control of the voltages applied to the four electrodes, the currents measured, and current control is performed through a control board interface (see **Note 10**).
3. Fill port: A syringe connected to the fill port is used to introduce fluids (e.g., CGE buffer, cleaning solutions) into the channels on the chip.
4. LIF detector: Two lasers, a 5 mW 405 nm "blue" laser (Sanyo, Japan) and a 7 mW 635 nm "red" laser (Sanyo, Japan) are used to excite the dye-labeled protein fluorescence (see **Note 11**). The collinearly aligned laser beams are trained on the chip separation channel ~10 cm downstream from the injection point, in an epifluorescent configuration. To collect separate fluorescence emission signals from the sample and standards in real time, the red and blue lasers alternate at 10 Hz, while the detector operates continuously. Figure 2 illustrates typical two channel outputs.

3. Methods

3.1 Sample preparation and solubilization

1. Dilute purified stock samples of viruses at least 1:10 (or more depending on concentration of the starting stock sample) into the borate/SDS sample lysis buffer (see **Note 12**). Final concentrations of the proteins of interest in the viral sample should be between 10 and 100 nM to generate a strong signal with fluorescamine, and will depend on both the sensitivity of the CGE system used and the virus itself. Sample volumes should be at least 10 microliters in volume order to ensure enough sample to inject into the CGE system. Use of small plastic tubes with caps (e.g., Eppendorf tubes) for sample handling is advised to minimize protein losses through adsorption and volume losses through evaporation.
2. Heat the diluted sample at 95 ^{o}C for 5 minutes. Remove from heat and let cool to room temperature. Add one-tenth volume 10 mM stock fluorescamine dye solution to a final concentration of 1 mM fluorescamine. Immediately vortex the sample briefly to ensure thorough mixing. The sample may become cloudy upon dye addition, but will clear up

within seconds and may become pale yellow in color, indicating the dye has reacted (see **Note 13**).

3. For analysis by the two-color CGE/LIF system, add 1/10 volume amount of the AlexaFluor647-labeled internal standards to the fluorescamine-labeled viral sample. The sample is now ready for analysis by CGE.

3.2 CGE METHOD

1. Degas the CGE buffer/gel by placing in an ultrasonic bath.
2. Prepare the chip for use by inserting the chip into the instrument/manifold such that the appropriate fluid reservoirs are aligned and attached to the chip fluidic ports (see **Note 14**). To prepare a dry chip for first-time use, the chip should be manually pressure flushed with the following solutions for 5 minutes each in this order: water, 1 M NaOH, 1 M HCl, water, then CGE buffer/gel. A small amount of fresh CGE buffer/gel should be pressure flushed through the separation channel prior to injecting each sample to minimize carryover and to ensure the best reproducibility.
3. Fill the reservoirs with CGE buffer/gel, and apply voltages to the reservoirs to verify electrical continuity. Currents should be monitored both initially and during separations for several reasons (see **Note 10**):
4. While preparing the chip, turn on the lasers and allow them to warm up. Monitor the fluorescence signal background during initial continuity testing. The baseline should be low and become stable over time. Bubbles and any precipitates migrating down the channel during conditioning will show up as sharp spikes in the fluorescence baseline.
5. When the system is operating stably, inject the viral sample, or a suitable blank, using the syringe injection port on the instrument. This injection will fill the sample loop on the chip (see **Note 15**).
6. Begin the electrokinetic injection of the sample onto the chip by starting the "Inject" voltage programming mode. Typical injection voltage settings will be S: 0V; SW: 990V; B: 400V ; W: 450V. Note that for these separations the polarity will need to be "reversed" so that negatively charged SDS-coated proteins will migrate toward the detector placed at the anode.
7. Inject the sample for ~10-30 seconds, or until the sample has reached the injection cross with the separation channel. The amount of time for electrokinetically injecting the sample may be determined by trial and error. Too-short injection times will lead to small or no peaks. Too-long injection times generally don't result in larger peaks after a certain point (if the injection volume is defined well (see **Note 16**).
8. Switch the voltage programming mode to "Run" and begin data collection of the fluorescence signal. Typical separation voltage settings will be S: 300V; SW: 300V; B: 0V; W: 4500V (see **Note 16**). See Figure 3 for an example of overlaid traces comparing the similar bacteriophages T2 and T4.

3.3 DATA ANALYSIS

1. The μChemLab™ device generates two signals: the electropherogram of the known reference standards (the "Red" channel) and the electropherogram of the unknown analyte

(the "Blue" channel). The standards are used to account for day-to-day and run-to-run drift in the system and can be used to identify the molecular weights of the viral protein peaks. Peak recognition algorithms are used to detect the standard peaks in the "Red" channel data, and the viral protein peaks in the "Blue" channel data (**see Note 17**).

2. The standard peaks are then matched against a reference set of standard peaks used by the database, and the migration times of the standard peaks in the sample are adjusted to match the reference migration times.
3. The correction based on known analytes is then applied to the unknown analytes to facilitate identification. The adjusted viral protein migration times are compared against the database to determine whether or not there is a match to a virus in the database.
4. To determine if there is a database match, a Bayesian classification methodology is used. This method is well suited to handle noisy data, and can provide a measure of confidence in the classification. The technique uses Bayesian inference as the basis for machine learning, pattern matching, and agent classification required for virus detection (16) (see **Note 18**).
5. To apply the Bayesian method, a sizeable database of electropherograms is needed. We found that data taken from 126 CGE runs of six viruses (T2, T4 and MS2 bacteriophage; Epstein-Barr, Respiratory Syncytial and Vaccinia viruses) can be used to train and test the classification algorithms. For each virus, 4-5 distinguishing protein peaks are identified, and the distance between them characterized from the training data (**see Note 19**). Successful classification with non-training data was 66/69 or 95% with no false positives. (**see Note 20 and 21**).
6. The reference standards, which represent a protein molecular weight ladder, can also be used to determine the molecular weight of the viral proteins using a cubic fit determined empirically (**see Note 22**).

4. NOTES

1. The best sensitivity by CGE/LIF is seen when the sample buffer is a relatively low ionic strength (at least 10-fold more dilute than the running buffer); this is due to sample stacking/preconcentration effects. Because the field strength in the low ionic strength sample buffer is much higher than it is in the running buffer, during electrokinetic injection proteins migrate out of the sample buffer rapidly and stack up at the interface with the running buffer. Peaks are sharper and the proteins more concentrated, yielding more sensitive detection. Buffers below ~5mM should not be used due to poor buffering capacity.
2. Prelabeled protein standards should be made up in primary amine-containing buffers such as Tris after preparation. The Tris will act as a scavenger of any residual reactive fluorescamine when the protein standards, already labeled with AlexaFluor647 are added to the fluorescamine-labeled viral protein sample, preventing double labeling of the standards.
3. Fabrication of the microfluidic separation chips is described in detail elsewhere (17). Briefly, chips were fabricated using standard photolithographic, wet etch and bonding techniques. Three microchannel depths of 4, 30, and 34 μm are isotropically etched in

base wafers. Fluid access holes are drilled into a cover wafer before it is visually aligned and thermally bonded to the base wafer. The combined wafers are diced into individual 2 x 2 cm chips.

4. Fused silica was chosen as the chip substrate because it demonstrates desirable material characteristics including favorable near UV optical properties, well-characterized electrokinetic surfaces, and easy surface property modification and cleaning.
5. The combination of a relatively low-back-pressure sample loop and a shallow channel connecting the sample loop to the separation channel that acts as a pressure restrictor, enables samples to be manually pressure injected into the chip via syringe without flooding the nearby separation channel with sample. This feature was found to be advantageous for being able to robustly introduce sample after sample without having to create a unique sample reservoir for each sample. Low to no sample carryover is possible with this design.
6. The offset T-injection design allows for a trade-off between greater sample size and the resulting sensitivity and the protein profile resolution. Offset lengths more than a few percent of the overall separation length will negatively impact resolution.
7. The channel length of 10 cm was found to be a good compromise between resolution and analysis time. Longer separation lengths are possible; we have successfully fabricated and tested 15 and 20 cm channels. Better resolution with these longer channels is possible; however, it is important to keep the V/cm comparable even with the longer channels or the separations could become prohibitively slow.
8. The 10 cm separation channel is coiled to fit on the 2cm x 2cm chip using low-dispersion turns to minimize dispersion and maintain resolution; the channel is 65 μm wide at the top which is very narrow relative to the 5 mm minimum loop radius, ensuring that the distance travelled at the inner edge is not significantly different that that travelled at the outer edge. In addition, equal numbers of right and left hand turns compensate for those slight differences.
9. One of the challenges associated with small (sub-milliliter) reservoir volumes is the possibility for buffer depletion. During electrophoresis, ions will migrate toward their respective electrodes and out of the originating buffer volumes. The result over time can be changes in the pH of the reservoir buffers and altered electrophoresis; consequently, the buffer reservoirs should be refreshed periodically (after several hours of continuous operation).
10. An electrophoresis apparatus that measures current is invaluable; currents are very diagnostic of electrophoresis status and can give a great deal of information about the quality of the separation, injection and whether particulates or air bubbles have compromised the analytical separation. For example, a break in current (zero current) or reduced current can indicate air bubbles are present in the channels. This can be remedied by flushing the chip with fresh degassed gel. Also, erratic currents or reduced or high currents can indicate a short in the electrical circuit. In severe cases, sometimes this can be seen by a spark or arcing from an electrode or reservoir. This can be remedied by ensuring leak-proof fluid connections to the chip, drying wet external portions of the

chip, looking for any cracks in the chip itself, and ensuring electrodes are not too close to each other to cause an arc.

11. The 405 and 635 nm wavelengths were chosen to match the fluorescent dyes used in these assays; a variety of other laser diodes at other wavelengths are commercially available and could be used. Likewise, if a commercial chip based fluorescent system is used, the dyes can be changed to be suitable for that system, provided the dyes are available in a primary amine reactive version (e.g. NHS esters). We have chosen to use fluorescamine for the fluorophore because of the rapid labeling kinetics and the lack of fluorescence until reacted with protein (i.e. fluorogenic). However, fluorescamine is not the brightest of dyes, and for high sensitivity applications it may be desirable to use alternative dyes. For non-fluorogenic dyes, a large unreacted dye peak in the electropherogram will be evident if the excess dye is not removed by size exclusion chromatography prior to analysis.
12. Protein profiling of other types of organisms (e.g. bacteria, spores) requires modification of the solubilization protocols to lyse the cells and reproducibly solubilize proteins prior to fluorescent labeling (18). Bacteria generally employ the same protocol as used for viruses; however, they will generally require a greater amount of SDS in the lysis buffer (1% SDS) to solubilize the significantly larger number of proteins in expected in vegetative bacteria compared to viral particles. Spores are quite resistant to lysis and solubilization due to the large number of heavily cross-linked proteins traditionally present in the spore coats. Addition of 50mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) in the lysis buffer effectively solubilizes the cross-linked proteins. TCEP is preferred over β-mercaptoethanol or dithiothreitol since it interferes less with the protein labeling step. Regardless, the bulk of the TCEP will still need to be removed from the lysed samples using disposable Micro Bio-SpinTM P6 or P2 size exclusion cartridges (Bio-Rad Laboratories, Hercules, CA) equilibrated in sample buffer. Organisms like bacteria and spores will be expected to generate more complicated protein profiles with a significant probability of overlapping peaks; CGE analysis would benefit from better resolution. An alternative separation gel with a smaller fractionation range (e.g. 10-100 kDa) may help, if available. Alternatively, if analysis time is not critical, longer separation channels (e.g. 15 or 20 cm) using the same sieving matrix can be used.
13. Fluorescamine is a highly reactive dye, and reacts with primary amine groups (lysines) on proteins. Fluorescamine also hydrolyzes rapidly in water. The reaction with amines is over in seconds; water hydrolysis is 10 times slower, but is also over in seconds. By using a large excess of dye, this method leads to extensive labeling of the proteins, which enhances detection sensitivity by CGE/LIF. The fluorescamine is added above the solubility limit in buffer; the initial precipitation step appears to protect the fluorescamine somewhat from the competing hydrolysis reaction in the aqueous buffer. As more dye reacts with the protein, more fluorescamine goes into solution, resulting in a more efficient labeling. Samples labeled with fluorescamine should be analyzed the same day for best results.
14. We have found this manifold approach convenient to facilitate swapping out chips with our integrated set-up (Figure 1b). However, other chip systems can be used and in fact,

these methods can be adapted to capillary based separations using lab built or commercial systems, provided comparable LIF detection is available.

15. Although the on-chip sample loop volume is only 100 nL, the volume actually syringe injected should be a few microliters to ensure sweeping of the sample loop volume several times over, as well as fully flushing any connector and dead volumes associated with the injector (e.g., in the capillaries connecting the injector to the chip).
16. "Pinched" injection ensures that best resolution and peak shape possible for a given injection size. With offset T designs, as with many microfluidic structures, the electric field actually extends beyond the T itself. Depending on a variety of factors, this can result in injections volumes many times the actual dimensions of the offset itself resulting in very large peaks but at the expense of resolution. Application of a small voltage in the separation channel towards the center of the offset during injection (resulting in small negative currents) can constrain the injection to the region of the offset (see Figure 1c). One should be cautious; overly large pinch voltages will actually decrease the sensitivity. Likewise during the separation, residual fluorescent proteins in the sample transfer channel can leak into the separation channel resulting in rising baselines; small voltages applied to S and SW directed away from the channel (resulting in small positive currents) will prevent this (see Figure 1c).
17. Peak detection algorithms can be developed (16), or commercial software packages are available.
18. In the Bayesian methodology, characteristic signatures of known agents are expressed in terms of their electropherogram attributes, in this case the relative distances between key peaks. The likelihood functions for observing these attributes were then determined from training data for all agents under consideration. To classify an unknown analyte, we compute the ratios of the posterior probability of it being a specific agent to the probability of it being each of the other agents in the training set. Note that by definition this limits the identification to agents that the classification algorithm was trained on. However, the framework also evaluates the hypothesis that the unknown analyte is an "other" agent, outside the training set (and therefore not identified). The logs of the posterior probability ratios, here referred to as Bayes factors, provide the probabilistic inference, based on the data and prior information, of the identity of the unknown analyte. The magnitude of the Bayes factor provides a measure of confidence in the classification. Generally, a probability ratio of 100 to 1 is considered decisive for one hypothesis versus another. Therefore, as $e5 \approx 150$, we consider a Bayes factor > 5 as evidence in favor of one agent versus another.
19. We developed a database of 126 CGE runs of six viruses as follows: T2 (11 runs), T4 (18 runs), MS2 (41 runs), EBV (13 runs), RSV (14 runs), and Vaccinia (29 runs). Of these 126 runs, 57 were used as training data: MS2, 17/41; RSV, 5/14; Vaccinia, 12/29; EBV, 8/13; T2, 6/11; and T4, 9/18. To generate data for the database, an autosampler connected to the input of the μChemLab™ device was found to be helpful.
20. The three failures are easily explained by problems during the electrophoretic analysis (e.g., low sample concentration or injection problems) or peak detection by the preprocessing tool. The failures are of the detection type, where the classification

algorithm detected "other". There were no false alarms (detecting a virus when none is present). Note that for the T2 and T4 data, the classifier did not identify either phage as its closely related neighbor, indicating the algorithm is effective even for closely related species.

21. The successful classification rate for the testing samples is very good despite the very limited size of the experimental data available to build and test this classification approach. Also the classification as described here is based on a single type of attribute (elution time); additional attributes such as peak width or amplitude could be considered to further increase classifier robustness.

22. The following set of proteins was used to generate a calibration curve of molecular weight versus migration time for CGE using the μChemLab™ instrument in addition to the dye HPTS (0.3 kDa): CCK peptide (1.1 kDa), α-lactalbumin (14.2 kDa), carbonic anhydrase (29.5 kDa), ovalbumin (45 kDa), bovine serum albumin (65 kDa) and IgG (150 kDa). Seven separate measurements, each containing the dye and the six protein standards, were used to generate the calibration curve. For these measurements the device was operated in constant current mode and the resulting migration times were linearly corrected using the peaks corresponding to HPTS and IgG as standards. The fit, although completely empirical, can be used to infer molecular weights of proteins in complex mixtures. The fit in the figure is given by:

$$\mathrm{MW} = \left[1 + \tanh\left(\alpha\ (t - T0)\right)\right]\left\{A + B\left(\frac{t}{\tau}\right) + C\left(\frac{t}{\tau}\right)^2\right\}$$

where t is the migration time in seconds and the coefficients are: $\alpha = 0.015\ \mathrm{sec}^{-1}$, T0 = 170 sec, A = 46 kDa, B = -146 kDa, C = 117 kDa, and $\tau = 218.85$ seconds (16). The coefficients for the fit are obtained by least squares minimization of the residual from fitting the above analytic form to the measurements (total of 49 points used to fit the 5 free parameters – note that τ is used as a normalization parameter and can be absorbed into the coefficients of the polynomial and thus is not a free parameter) . All values were weighted equally. The function in front of the quadratic in the above equation accounts for the very weak dependence of molecular weight on migration times for species whose molecular weights are below the gel sieving range (i.e., <14 kDa).

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FIGURES

Figure 1: a) Layout of the microfluidics chip used showing the low dispersion turns, particle traps and the offset-T injection; b) chip installed in the compression manifold; c) fluorescent image of the offset-T region of the chip during a "pinched" injection and just after switching to the Run or separation mode.

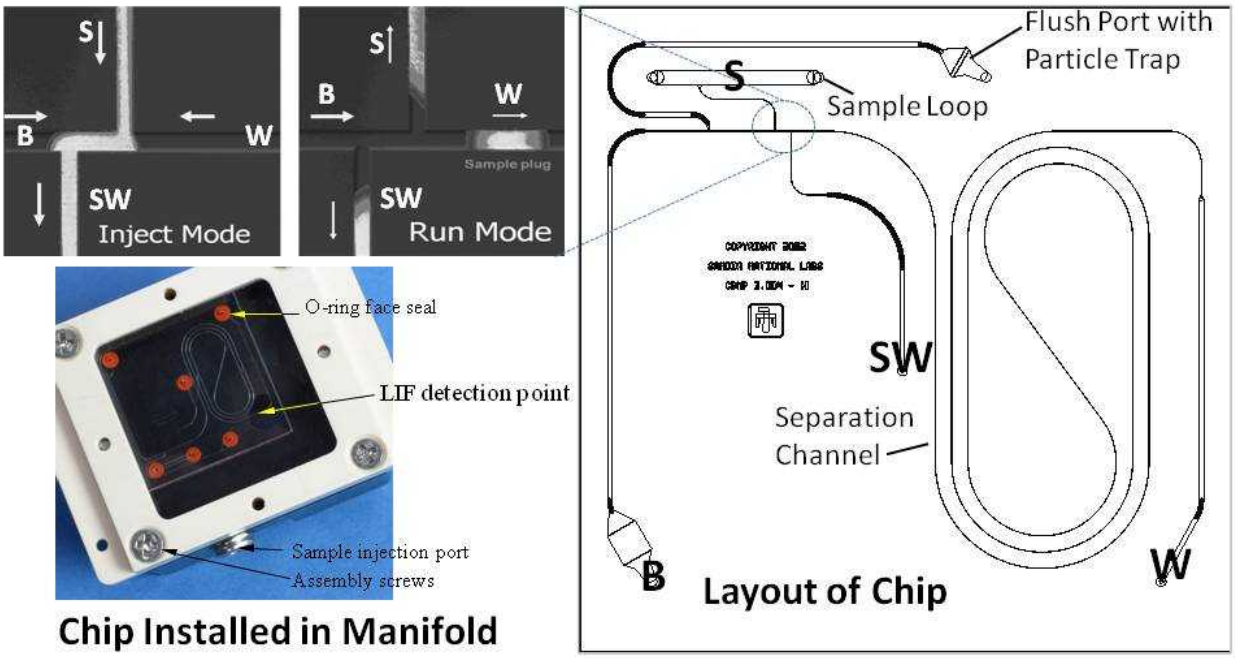

Figure 2: Typical two-color output from the system showing viral proteins labeled with fluorescamine in the Analytical or “Blue” channel and the prelabeled protein standards in the Reference or “Red” channel.

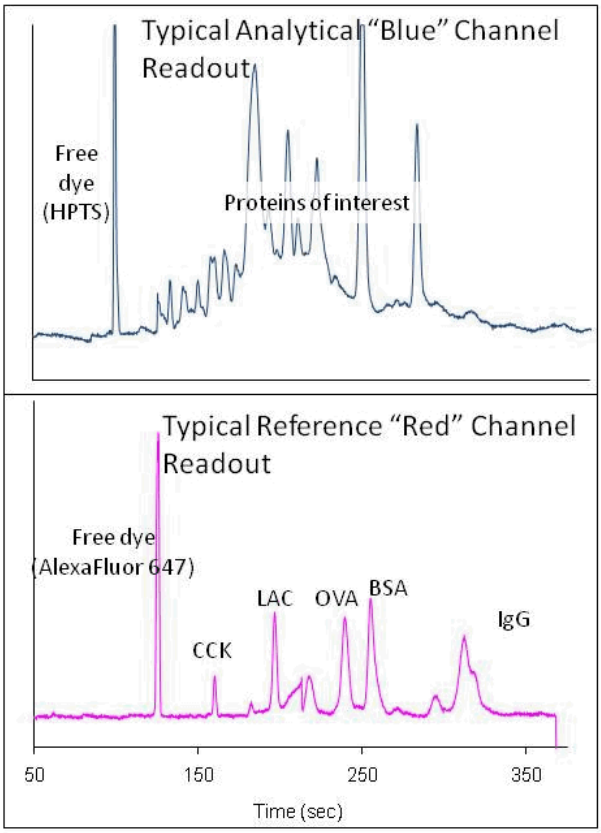

Figure 3: Typical overlaid traces ("Blue" channel only) comparing the related T2 and T4 bacteriophages with the corresponding separation via SDS-PAGE.