Use of proteomic analysis to determine the protein constituents of synovial fluid samples from the stifle joints of dogs with and without osteoarthritis secondary to cranial cruciate ligament rupture

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Received March 16, 2017.
Accepted July 11, 2017.

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OBJECTIVE
To use proteomic analysis to determine the protein constituents of synovial fluid samples from the stifle joints of dogs with and without osteoarthritis secondary to cranial cruciate ligament rupture (CCLR).

ANIMALS
12 dogs with clinically normal stifle joints (controls) and 16 dogs with osteoarthritis secondary to CCLR.

PROCEDURES
A synovial fluid sample was obtained from all dogs. Synovial fluid total protein concentration was determined by the Bradford assay. Proteins were separated by use of a 1-D SDS-PAGE to detect protein bands that differed between dogs with and without osteoarthritis. Those protein bands then underwent trypsin digestion and were analyzed by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry, the results of which were compared with a curated protein sequence database for protein identification. One of the most frequently identified proteins, apoprotein (apo) A-I, was then quantified in all synovial fluid samples by use of a competitive-inhibition ELISA. Results were compared between dogs with and without osteoarthritis.

RESULTS
Median synovial fluid total protein and apo A-I concentrations for dogs with osteoarthritis were significantly greater than those for control dogs. The most abundant proteins identified in the synovial fluid were albumin and apo A-I.

CONCLUSIONS AND CLINICAL RELEVANCE
Results indicated that quantification of synovial fluid total protein and apo A-I concentrations might facilitate diagnosis of osteoarthritis secondary to CCLR in dogs. Further research and validation of synovial fluid apo A-I concentration as a biomarker for osteoarthritis in dogs are necessary before it can be recommended for clinical use. (Am J Vet Res 2018;79:397–403)

Osteoarthritis is a degenerative orthopedic disease that results in progressive destruction of articular cartilage, synovial membrane, extracellular matrix, and chondrocytes. The pathogenesis of osteoarthritis is not fully understood, but an imbalance of various enzymes, cytokines, and growth factors secreted by multiple cell types is thought to accentuate degradation of the extracellular matrix and thus cartilage breakdown. Several risk factors have been associated with the development of osteoarthritis in dogs. Dogs with various orthopedic diseases, such as CCLR, elbow dysplasia, hip dysplasia, and idiopathic polyarthritis, are predisposed to the secondary development of osteoarthritis, a painful condition that is difficult to diagnose during its early stages. Cranial cruciate ligament rupture, with or without a meniscal tear, is a common disorder of dogs that affects the stifle joint and often causes lameness and secondary osteoarthritis. Osteoarthritis requires intensive long-term treatment, which can place considerable financial strain on the owners of affected dogs. In a 2003 survey of American College of Veterinary Surgeons diplomates, who indicated that their area of emphasis was small animal orthopedic surgery or small animal general and orthopedic surgery and AVMA members who indicated that their professional area was at least 80% small animal practice it was estimated that the annual cost of medical management for CCLR and osteoarthritis of the stifle joint alone was $1.32 billion.

Diagnosis of osteoarthritis is challenging owing to its complex pathogenesis and disease progression.
Osteoarthritis is generally diagnosed on the basis of patient history, clinical signs, and results of diagnostic imaging, and that diagnosis can be supported by serologic assay results. Although modern imaging modalities such as MRI can improve diagnostic sensitivity for the detection of osteoarthritis, those modalities are quite expensive and not readily available in all veterinary practices. Therefore, there is a need for the development of less expensive and easily accessible methods for the diagnosis of osteoarthritis in dogs.

Because synovial fluid is closely associated with articular cartilage, the primary site for osteoarthritis progression, identification of synovial fluid components is of central importance in proteomic research. Proteomic analysis is gaining popularity for the analysis of synovial fluid, serum, and cartilage for changes associated with osteoarthritis and may be useful for elucidation of the pathophysiology of the disease as well as the discovery of new biomarkers for the diagnosis and monitoring of orthopedic diseases in animals. Proteins can be analyzed by means of 1-D or 2-D PAGE, in which a polyacrylamide gel matrix is used for protein separation, and the resulting protein bands are identified through in-gel digestion followed by mass spectrometry analyses. Polyacrylamide gel electrophoresis has been used to accurately identify synovial fluid protein patterns for human patients with rheumatoid arthritis and horses and dogs with osteoarthritis, and those protein patterns have advanced understanding of the pathophysiology of such diseases.

The purpose of the study reported here was to use proteomic analysis to determine the protein constituents of synovial fluid samples from the stifle joints of dogs with and without osteoarthritis secondary to CCLR. We hypothesized that the synovial fluid protein profiles would differ between dogs with and without osteoarthritis. Those differences might help identify biomarkers that can be used to diagnose osteoarthritis.

Materials and Methods

Animals and synovial fluid sample collection

All dogs enrolled in the study reported here were examined at the Small Animal Clinic at the Freie Universität Berlin, Germany, between April 2013 and October 2014. Because the dogs were clinical patients and all samples were collected as part of a routine diagnostic workup or during necropsy, approval of the study by the university’s animal care committee was not required. However, consent was obtained from the owners of all dogs for use of leftover synovial fluid samples in the study. Age, breed, weight, and sex were recorded for each dog. The osteoarthritis group consisted of 16 adult medium- to large-breed dogs that were examined because of CCLR with (n = 15) or without (1) medial meniscus damage. All 16 dogs had advanced osteoarthritis as diagnosed on the basis of the presence of stifle joint effusion (edges of the patellar tendon were palpably indistinct), fibrotic thickening on the medial aspect (medial buttress) of the stifle joint, and a positive cranial drawer or tibial compression test result as determined by a veterinary orthopedic surgeon and presence of radiographic abnormalities consistent with the disease in the affected stifle joint as determined by a board-certified veterinary radiologist. An orthopedic surgeon collected at least 1 mL of synovial fluid from the affected joint of each dog immediately prior to initiation of surgery to correct the CCLR. Arthrocentesis was performed before surgery to avoid contamination of the synovial fluid samples with blood.

The control group consisted of 12 client-owned dogs that did not have a clinical history of osteoarthritis. All 12 dogs were euthanized for reasons other than orthopedic or neoplastic disorders with the owners’ consent and in accordance with AVMA Guidelines for the Euthanasia of Animals. A minimum of 0.5 mL of synovial fluid was aseptically collected from 1 stifle joint of each dog within 1 hour after euthanasia.

For each synovial fluid sample immediately after collection, the color and viscosity were recorded, as was the total number of cells as determined with an automated cell counter and the protein concentration as determined by use of refractometry. A smear of the sample was prepared on a glass slide for cytologic evaluation. The remaining sample was centrifuged at 2,300 × g for 4 minutes to remove any cells or particulate matter from the fluid. The supernatant was decanted and separated into 0.5-mL aliquots. Ten microliters of protease inhibitor cocktail was added to each 0.5-mL aliquot of synovial fluid, and the samples were stored frozen at -80°C until further analysis.

Protein quantification and separation by SDS-PAGE

For each synovial fluid sample, the total protein concentration was measured by the Bradford protein assay as described. Briefly, each sample was diluted with an equal amount of reducing sample buffer and loaded into a lane of a polyacrylamide mini gel. The gel was then incubated at 95°C for 5 minutes to allow protein denaturation. The samples and low-molecular-weight range standard proteins were separated by the use of 12% SDS and PAGE. Then, the gel was stained with Coomassie brilliant blue G-250 solution and scanned with an imager to assess the molecular weights of the synovial proteins as described.

MALDI-TOF-MS and protein identification

For each sample, protein bands of interest were excised from the polyacrylamide gel, destained, reduced with a reducing agent, alkylated with...
iodoacetamide, and digested with trypsin at 37°C overnight (approx 14 hours). Then, the peptides were analyzed with MALDI-TOF-MS as described. The mass spectrometry data were transferred to software for alignment with a curated protein sequence database by use of a database search engine. Search parameters were as follows: taxonomy was restricted to canine, mass tolerance was 200 ppm, the enzyme used was trypsin, missing cleavages ≤ 1 were accepted, and variable modifications were defined as carbamidomethylation of cysteine. For each protein identified, the database search engine generated a protein score and associated P value, which was the probability that the observed match between the experimental (synovial fluid) protein and database protein sequence was a random event. A protein score > 57 was required to achieve a value of P < 0.05, which was indicative of reliable protein identification. Identified proteins with a protein score > 57, as well as those with a protein score ≤ 57 and a sequence coverage > 20%, were considered potential candidates for further investigation as diagnostic biomarkers for osteoarthritis.

Quantification of apo A-I

Apolipoprotein A-I was identified by in-gel digestion and subsequent MALDI-TOF-MS analyses as a potential marker for osteoarthritis. Therefore, apo A-I concentrations were quantified in synovial fluid samples from all dogs in duplicate by use of a commercially available competitive-inhibition ELISA in accordance with the manufacturer’s instructions. Briefly, the ELISA was performed by use of a microtiter plate, the wells of which were precoated with apo A-I. Competitive inhibition was mediated between the apo A-I in the wells of the microtiter plate and the apo A-I in the synovial fluid samples or a standard protein (control). After the samples in the wells were incubated with a horseradish peroxidase-conjugated antibody specific for apo A-I, color was developed by the addition of 3,3′,5,5′-tetramethylbenzidin. The reaction was terminated by the addition of a stop solution, and the light absorbance of each well was measured at 450 nm by use of a spectrophotometer. The minimum detectable apo A-I concentration was 7 ng/mL. Per the ELISA user manual, the intra-assay and interassay coefficients of variation were < 8% and < 10%, respectively.

Statistical analysis

Descriptive data were generated. The respective data distributions for synovial fluid total protein and apo A-I concentrations were assessed for normality by means of the Kolmogorov-Smirnov test. Neither variable was normally distributed; therefore, the Mann-Whitney U test was used as described to compare those variables between control dogs and dogs with osteoarthritis. The constituent proteins within the synovial fluid were categorized on the basis of molecular weight, and the relative percentage of constituent proteins within each molecular-weight category was compared between the control and osteoarthritis groups by means of a t test. All analyses were performed with a software program, and values of P < 0.05 were considered significant.

Results

Dogs

The 16 dogs that comprised the osteoarthritis group included 3 sexually intact females, 8 sexually intact males, and 5 neutered males. The group had a median age of 7.3 years (range, 0.6 to 13 years) and weight of 26 kg (range, 7 to 40 kg). The most frequently represented breeds were German Shepherd Dog (n = 2), Jack Russell Terrier (2), and American Staffordshire Terrier (2); there were also 2 mixed-breed dogs and 8 dogs of other breeds.

The 12 dogs of the control group included 2 sexually intact females and 10 sexually intact males. The group had a median age of 7.5 years (range, 2 to 11 years) and weight of 37.5 kg (range, 22 to 43 kg). Breeds represented included Rottweiler (n = 4), Great Dane (2), Dalmatian (2), and Cocker Spaniel (2); there were also 2 mixed-breed dogs in the control group.

Quantitative and qualitative protein analysis

The median synovial fluid total protein concentration for the osteoarthritis group (31.4 mg/mL; range, 18.3 to 41.6 mg/mL) was significantly (P < 0.001) greater than that for the control group (19.0 mg/mL; range, 5.49 to 30.0 mg/mL; Figure 1). One-dimensional SDS-PAGE was used to separate the proteins within each synovial fluid sample on the basis of molecular-weight (Figure 2). The proteins were then categorized into 18 molecular weight categories, and the relative percentage of the synovial fluid total protein concentration constituted by proteins of each molecular weight category was summarized (Table 1). Qualitative analysis revealed that the predominant synovial fluid proteins in both the control and osteoarthritis groups had molecular weights of 21 to 30 kDa and 51 to 60 kDa. In fact, all study dogs had synovial fluid proteins with molecular weights that fell within those 2 ranges. However, when the relative percentage of the constituent proteins within each molecular-weight category was compared between the control and osteoarthritis groups, the synovial fluid of dogs in the osteoarthritis group had significantly higher proportions of proteins with molecular weights of 21 to 30 kDa (P = 0.005), 41 to 50 kDa (P = 0.011), 71 to 89 kDa (P = 0.012), 81 to 90 kDa (P = 0.021), 111 to 120 kDa (P = 0.010), 131 to 140 kDa (P = 0.001), and 151 to 160 kDa (P = 0.032) and a significantly (P = 0.002) lower proportion of proteins with molecular weights of 51 to 60 kDa than the synovial fluid of dogs in the control group.

Protein identification

The proteins most frequently identified by MALDI-
Figure 1—Box-and-whisker plots of stifle joint synovial fluid total protein (A) and apo A-I (B) concentrations for 12 adult dogs that were euthanized for reasons other than orthopedic or neoplastic disorders (control dogs) and 16 adult medium- to large-breed dogs with advanced osteoarthritis (OA dogs) secondary to CCLR. Advanced osteoarthritis was diagnosed on the basis of the presence of stifle joint effusion (edges of the patellar tendon were palpably indistinct), fibrotic thickening on the medial aspect (medial buttress) of the stifle joint, a positive cranial drawer or tibial compression test result, and radiographic abnormalities consistent with the disease in the affected stifle joint. For each plot, the upper and lower edges of the box delimit the interquartile (25th to 75th percentile) range, the horizontal line within each box represents the median, and the whiskers delimit the range. The median synovial fluid total protein and apo A-I concentrations for the OA dogs were significantly ($P < 0.001$) greater than the corresponding concentrations for the control dogs.

Figure 2—Representative photographs of polyacrylamide mini gels following SDS-PAGE to identify patterns for stifle joint synovial fluid proteins from 3 control dogs (C1, C2, and C3) and 3 dogs with osteoarthritis (OA1, OA2, and OA3) described in Figure 1. The gels subsequently underwent MALDI-TOF-MS, and the resulting data were transferred to software for alignment with a curated protein sequence database by use of a database search engine. Search parameters were as follows: taxonomy was restricted to canine, mass tolerance was 200 ppm, the enzyme used was trypsin, missing cleavages $\leq 1$ were accepted, and variable modifications were defined as carbamidomethylation of cysteine. For each protein identified, the database search engine generated a protein score and associated $P$ value, which was the probability that the observed match between the experimental (synovial fluid) protein and database protein sequence was a random event. A protein score $> 57$ was required to achieve a value of $P < 0.05$, which was indicative of reliable protein identification. Identified proteins with a protein score $> 57$, as well as those with a protein score $\leq 57$ and a sequence coverage $> 20\%$, were considered potential candidates for further investigation as diagnostic biomarkers for osteoarthritis. Encircled bands represent proteins of interest identified in the study. See Figure 1 for remainder of key.
Relative percentage of synovial fluid constituent proteins within each molecular-weight category as determined by 1-D SDS-PAGE for 16 dogs with osteoarthritis of a stifle joint secondary to CCLIR (osteoarthritic group) and 12 dogs with clinically normal stifle joints (control group). 

<table>
<thead>
<tr>
<th>Molecular weight category (kDa)</th>
<th>Osteoarthritis group</th>
<th>Control group</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 20</td>
<td>6.03 ± 2.81</td>
<td>6.56 ± 6.57</td>
<td>0.84</td>
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<td>21–30</td>
<td>14.1 ± 2.59</td>
<td>8.68 ± 3.89</td>
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<td>31–40</td>
<td>4.64 ± 2.68</td>
<td>2.93 ± 1.45</td>
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<td>41–50</td>
<td>3.66 ± 2.79</td>
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<td>51–60</td>
<td>42.2 ± 4.48</td>
<td>69.3 ± 20.1</td>
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<td>61–70</td>
<td>5.39 ± 5.85</td>
<td>4.68 ± 6.51</td>
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<tr>
<td>71–80</td>
<td>4.60 ± 3.91</td>
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<td>81–90</td>
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<tr>
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<td>0.032</td>
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<td>171–180</td>
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<td>—</td>
<td>0.15</td>
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<tr>
<td>&gt; 180</td>
<td>2.56 ± 2.10</td>
<td>1.10 ± 2.34</td>
<td>0.228</td>
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</tbody>
</table>

Values of P < 0.05 were considered significant. 
* = No proteins were detected within this molecular weight range.

Table 1—Relative percentage of synovial fluid constituent proteins within each molecular-weight category as determined by 1-D SDS-PAGE for 16 dogs with osteoarthritis of a stifle joint secondary to CCLIR (osteoarthritic group) and 12 dogs with clinically normal stifle joints (control group).

Quantification of apo A-I

Because the MALDI-TOF-MS results suggested that apo A-I might be an important biomarker for osteoarthritis in the synovial fluid of dogs, the apo A-I concentration was quantified in the synovial fluid samples of all dogs. The apo A-I concentration was below the lower limit of quantification (7 ng/mL) for 7 of the 12 control dogs, but was quantifiable in all 16 dogs of the osteoarthritis group. The median synovial fluid apo A-I concentration for the osteoarthritis group (191 µg/mL; range, 38 to 390 µg/mL) was significantly (P < 0.001) greater than that for the control group (0 µg/mL; range, 0 to 155 µg/mL; Figure 1). The synovial fluid apo A-I concentration ranged from 39 to 155 µg/mL (median, 120 µg/mL) for the 5 control dogs with quantifiable concentrations.

Discussion

Results of the present study indicated that the synovial fluid total protein concentration in osteoarthritic stifle joints of dogs was significantly greater than that in clinically normal (control) stifle joints. That finding was consistent with results of studies of synovial fluid protein concentration in dogs with degenerative joint disease. Results of another study suggest that synovial fluid total protein concentration has greater accuracy for the diagnosis of idiopathic arthritis in dogs (cutoff concentration, > 2.54 g/dL; sensitivity, 58.3%; specificity, 95.5%) than plasma C-reactive protein concentration. Synovial fluid total protein concentration may be a useful biomarker for the diagnosis of osteoarthritis in the stifle joints of dogs because it can be easily assessed in veterinary laboratories, but further research is necessary to verify that supposition and establish reference limits and diagnostic sensitivity and specificity.

Results of proteomic studies indicate that alterations in synovial fluid protein expression are associated with the development of osteoarthritis in dogs, and those proteins may be useful as biomarkers for detection of disease. In the present study, the constituent proteins of synovial fluid were qualitatively and quantitatively compared between dogs with and without osteoarthritis. Although 3 proteins (serum albumin, apo A-I, and haptoglobin) were frequently identified in the synovial fluid of both osteoarthritic and control joints, only serum albumin and apo A-I had protein scores that suggested those proteins might be useful as diagnostic biomarkers for osteoarthritis.

Albumin is an important constituent in plasma and is classified as a negative acute-phase protein, which indicates that its concentration decreases in response to inflammation. In humans, albumin is highly concentrated in synovial fluid relative to other proteins, and different isoforms of albumin have been detected in plasma and synovial fluid of patients with juvenile idiopathic arthritis. Studies regarding albumin in the synovial fluid of dogs are scarce. In 1 study, the albumin ratio for synovial fluid and serum did not differ significantly between healthy dogs and dogs with degenerative joint disease. However, we could not definitively rule out that the synovial fluid samples evaluated in the present study were not contaminated with blood, which could have affected both the protein concentrations and patterns for those samples. When evaluating synovial fluid protein content, it is absolutely necessary to avoid contamination of samples with blood during arthrocentesis. Further research is necessary to evaluate synovial fluid albumin as a diagnostic biomarker for osteoarthritis in dogs.

In the present study, apo A-I was detected in synovial fluid samples from all 16 dogs in the osteoarthritis group but only 5 of the 12 dogs in the control group, and the median synovial fluid apo A-I concentration for dogs in the osteoarthritis group was significantly greater than that for dogs in the control group. Thus, it appeared that synovial fluid apo A-I might be a useful diagnostic biomarker for osteoarthritis in dogs. To our knowledge, the role of apo A-I in the pathogenesis of osteoarthritis in dogs has not been elucidated, but apo A-I has a role in the pathogenesis of various orthopedic diseases in humans. Similar to albumin, apo A-I is a negative acute-phase protein in blood,
and it is also an essential constituent of high-density lipoproteins. In humans, the role of apo A-I in the pathogenesis of osteoarthritis is somewhat equivocal. It has an inhibitory effect on the synthesis of tumor necrosis factor-α and interleukin-1β and therefore has an anti-inflammatory effect in the pathogenesis of several orthopedic diseases. Apolipoprotein A-I also impaired collagen-induced inflammation in a rodent model for rheumatoid arthritis in humans. However, in human patients with rheumatoid arthritis, the primary extravascular inflammatory process enhances high-density lipoprotein and apo A-I modulation both qualitatively and quantitatively. Pro-inflammatory properties of apo A-I include the induction of matrix metalloproteinase-1 and -3 expression by chondrocytes and fibroblast-like synoviocytes, a pathophysiologic mechanism associated with the development of osteoarthritis. High concentrations of apo A-I have been detected in synovial fluid of human patients with rheumatoid arthritis and juvenile idiopathic arthritis, and high synovial fluid apo A-I concentrations may modulate the inflammatory process and lead to the development of osteoarthritis. Human patients with rheumatoid arthritis have an abnormally increased concentration of apo A-I in inflamed synovial tissues, particularly in perivascular areas that are infiltrated by macrophages and T cells. In a study of horses with osteoarthritis, synovial fluid apo A-I concentrations were abnormally increased before treatment but decreased following treatment with autologous-conditioned serum, which was accompanied by an improvement in joint function.

Other studies have used a proteomic approach to identify various apolipoprotein isoforms in the serum and synovial fluid of dogs with osteoarthritis. However, neither those studies nor another study of synovial fluid biomarkers has evaluated apo A-I in synovial fluid as a diagnostic biomarker for osteoarthritis in dogs. Although the results of the present study suggested that synovial fluid apo A-I may be a useful biomarker for osteoarthritis in the stifle joints of dogs, further research involving a larger population of dogs with and without osteoarthritis is necessary to validate our findings. It remains unknown whether the depletion of abundant proteins, such as albumin and immunoglobulins, in synovial fluid might be beneficial for elucidation of the pathogenesis of orthopedic diseases in dogs or useful as diagnostic biomarkers in clinical patients because their identification and quantification require sensitive procedures.

In the present study, synovial fluid total protein and apo A-I concentrations for dogs with osteoarthritis of a stifle joint secondary to CCLR were significantly greater than those for control dogs without osteoarthritis. Thus, quantification of synovial fluid total protein and apo A-I concentrations may be useful for the diagnosis of osteoarthritis in dogs, but the use of synovial fluid apo A-I concentration as a diagnostic biomarker for osteoarthritis in dogs requires further research and validation before it can be recommended for use in clinical settings.

Acknowledgments

Dr. Shahid received funding from the German Academic Exchange Service (DAAD) and Higher Education Commission of Pakistan. The authors declare that there were no conflicts of interest. The authors thank L. Häussler for technical assistance.

Footnotes

a. Plaxan, Biosystems Switzerland AG, Muttenz, Switzerland.
b. Sigma-Aldrich Corp, Munich, Germany.
c. Bio-Rad Laboratories GmbH, Munich, Germany.
d. ChemiDoc XRS scanner system, Bio-Rad Laboratories GmbH, Munich, Germany.
e. Quantity One, Bio-Rad Laboratories GmbH, Munich, Germany.
f. Tris-(2-carboxyethyl)-phosphine hydrochloride, Carl Roth GmbH & Co, Karlsruhe, Germany.
g. Sigma-Aldrich, Deisenhofen, Germany.
h. Serva Electrophoresis GmbH, Heidelberg, Germany.
i. AutoflexSpeed, Bruker Daltonik GmbH, Karlsruhe, Germany.
m. Cusabio, College Park, Md.

References


