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### Permalink

<https://escholarship.org/uc/item/6ft653q1>

### Journal

Analytical Chemistry, 85(20)

### ISSN

0003-2700

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### Publication Date

2013-10-15

### DOI

10.1021/ac4023082

Peer reviewed



Published in final edited form as:

*Anal Chem.* 2013 October 15; 85(20): . doi:10.1021/ac4023082.

## Development of a Capillary Electrophoresis Platform for Identifying Inhibitors of Protein-Protein Interactions

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### Abstract

Methods for identifying chemical inhibitors of protein-protein interactions (PPIs) are often prone to discovery of false positives, particularly those caused by molecules that induce protein aggregation. Thus, there is interest in developing new platforms that might allow earlier identification of these problematic compounds. Capillary electrophoresis (CE) has been evaluated as a method to screen for PPI inhibitors using the challenging system of Hsp70 interacting with its co-chaperone Bag3. In the method, Hsp70 is labeled with a fluorophore, mixed with Bag3, and the resulting bound and free Hsp70 separated and detected by CE with laser-induced fluorescence detection. The method used a chemically modified CE capillary to prevent protein adsorption. Inhibitors of the Hsp70-Bag3 interaction were detected by observing a reduction in the bound to free ratio. The method was used to screen a library of 3,443 compounds and results compared to those from a flow cytometry protein interaction assay. CE was found to produce a lower hit rate with more compounds that reconfirmed in subsequent testing suggesting greater specificity. This finding was attributed to use of electropherograms to detect artifacts such as aggregators and to differences in protein modifications required to perform the different assays. Increases in throughput are required to make the CE method suitable for primary screens but at the current stage of development it is attractive as a secondary screen to test hits found by higher throughput methods.

### Introduction

Protein-protein interactions (PPIs) are involved in key cellular processes<sup>1-3</sup> and enthusiasm is growing for developing chemical inhibitors of these contacts<sup>4-5</sup>. PPIs were previously considered to be intractable drug targets, but recent successes have demonstrated that potent and selective inhibitors can indeed be found<sup>6</sup>. More than 100 inhibitors of PPIs have now been reported in the literature and some of these molecules have low nanomolar potency<sup>7</sup>. Interestingly, a recent analysis of known PPI inhibitors suggests that the most tractable PPI targets feature a relatively small contact area, with clear energetic “hotspots”<sup>6</sup>. Conversely, other PPIs have been more difficult to target, likely because they involve large, relatively

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#### Supporting Information

Supporting information for this article includes methods and results for demonstrating effect of fluorescent labeling in Hsp70 ATPase activity, isothermal calorimetry measurements of binding affinity between Hsp70 and Bag3, dose response curves for Hsp70-Bag3 inhibitors and cross-testing between inhibitors discovered by CE and FCPIA, and comparison of IC50s for inhibitors by CE and FCPIA. This material is available free of charge via the Internet at <http://pubs.acs.org>.

flat surface areas and/or because the interactions are relatively weak<sup>6-9</sup>. Despite these challenges, a handful of inhibitors of difficult PPIs have also been reported and it seems likely that emerging discovery methods, such as fragment-based screening, will continue to expand the categories of PPIs that are considered “druggable”<sup>9</sup>.

Often, high throughput screening (HTS)<sup>10</sup> plays a critical role in the discovery of chemical PPI inhibitors. The assay platforms used for PPI targets can be divided into two general classes: those that measure binding of test molecules to one of the proteins and those that directly measure disruption of the protein-protein contact. The first class of methods relies on the idea that binding of a small molecule might potentially disrupt PPIs involving the protein target. The techniques used in this type of search include NMR, surface plasmon resonance (SPR), differential scanning fluorimetry (DSF), and *in silico* approaches. These strategies have been successful in yielding PPI inhibitors<sup>9</sup>. The alternative approach is to measure the PPI itself and then screen for compounds that block the contact. Methods such as fluorescence resonance energy transfer (FRET), AlphaLisa, fluorescence polarization (FP) and flow cytometry protein interaction assay (FCPIA) are commonly used in this type of paradigm. While these technologies are powerful, they suffer from high false positive rates, often from the presence of “aggregator” molecules<sup>11</sup>. Such compounds bind and denature a protein target causing it to aggregate. These artifacts are particularly problematic in screens of difficult PPIs because flat, poorly soluble molecules tend to interact with the relatively shallow topologies of protein-protein contacts. Likewise, intrinsically fluorescent compounds are widespread in most compound libraries and often need to be removed in secondary screens because they produce artifacts in fluorescence-based assays. However, indiscriminate removal of all fluorescent compounds necessarily removes potential inhibitors (*e.g.* false negatives).

In principle capillary electrophoresis (CE) could be used to screen for modulators of PPI. A variety of CE methods have been successfully used to probe non-covalent interactions *in vitro*<sup>12-19</sup>. In this work we have explored affinity probe CE (APCE)<sup>20-21</sup> for this purpose. In this method, binding partners are combined and the mixture separated fast enough by CE that the non-covalent complex and free partners can be detected as separate peaks. Typically one of the binding partners is fluorescently labeled to allow sensitive detection by laser-induced fluorescence (LIF). This approach has been used in immunoassays<sup>12, 20</sup>, aptamer assays<sup>15, 22-23</sup>, protein aggregation assays<sup>21</sup>, and to detect protein-DNA<sup>24</sup>, protein-saccharide<sup>25-26</sup>, protein-protein<sup>27</sup>, protein-peptide interactions<sup>19, 28-29</sup>. Binding inhibitors added to mixtures can be detected by observing the shift in bound to free peak areas that result in electropherograms. This method has been used for competitive immunoassays<sup>12</sup> and to detect inhibitors in small scale screens of protein-peptide interactions, *e.g.* SH2 domains binding to short phosphorylated peptides<sup>28</sup>. Extending this approach to interactions of full proteins is of interest because many proteins do not have well-defined, linear peptide binding targets and allosteric modulation may be an important mode of interaction.

As a screening tool for discovery of PPI inhibitors, CE has a number of potential advantages over currently used screening methods. Namely, this method might allow detection of aggregators, based on the appearance of peaks different from the complex and free affinity probe. Moreover, CE would allow separation of fluorescent test compounds from the complex to prevent interference in binding detection. This feature has the potential to identify and possibly “rescue” false negatives. CE, especially in microchip format, is also compatible with scale up to HTS based on its low sample consumption, fast separation speed, high degree of automation and straight-forward quantification<sup>30-33</sup>. Indeed, commercial systems are available that use microchip electrophoresis for screening enzymatic reactions, *e.g.* Caliper LabChip EZ reader.

Realizing the potential of CE for screening PPI requires addressing a number of specific technical challenges, such as the tendency of some proteins to adsorb to CE columns and difficulties with obtaining adequate resolution of bound and free protein under non-denaturing conditions. Moreover, this method has yet to be tested head-to-head with existing HTS platforms to rigorously uncover whether it has any demonstrable advantages. Finally, we consider it important to attempt pilot studies on a challenging and physiologically meaningful PPI to meet the goal of adding CE to the armamentarium of methods for meeting the specific and emerging challenges of difficult PPIs.

Here, we report the first use of CE as a PPI screening platform. As a target, we selected the PPI between heat shock protein 70 (Hsp70) and Bcl2-associated anthanogene 3 (Bag3). Hsp70 is a molecular chaperone that cooperates with Bag3 to regulate protein quality control<sup>34</sup>. The Bag3-Hsp70 complex stabilizes a number of key oncogenes, making it a promising anti-cancer target.<sup>35–40</sup> However, this complex has a large predicted contact interface ( $>1800 \text{ \AA}^2$ ) with moderate affinity, placing it in the category of a challenging PPI. Previously, CE has been used to study the interaction between heat shock proteins and small molecule immunosuppressants.<sup>41–42</sup> In this research, we used APCE to detect formation of the Hsp70-Bag3 complex by labeling the Hsp70 with a fluorophore. Further, by modifying the CE column we limited undesirable protein adsorption which enabled screening of a pilot collection of 3,443 small molecules against this target. Concurrently, we screened the same library using FCPIA. A comparison between the results of these parallel screens revealed strengths and weaknesses of the methods. From these studies, we conclude that CE is a promising method for finding inhibitors of PPIs and a significant advantage is that it enables early detection of aggregators.

## Experimental

### Protein Purification and Labeling

Human Hsp70 (HSPA1) was purified as previously described<sup>43–44</sup> using a N-terminal 6xHis tag and Ni-NTA column, followed by overnight TEV Protease cleavage of the His tag and lastly an ATP-agarose affinity column. Human N-terminal 6xHis-tagged Bag3 was purified based on previous reports<sup>45</sup>. Briefly, Bag3 was purified by ammonium sulfate (0–30% of saturation) followed by a Ni-NTA column and overnight TEV cleavage of the His tag. Bag3 was dialyzed overnight into Buffer A (25mM HEPES, 10mM NaCl, 15mM - mercaptoethanol, 0.1mM EDTA, pH 7.6) and subjected to ion-exchange chromatography on a Mono-Q HR 16/10 column (GE Healthcare). Finally, Bag3 was subjected to size exclusion chromatography on a Superdex 200 gel filtration column (GE Healthcare). Where indicated, Hsp70 and Bag3 were labeled with Alexa Fluor® 488 5-SDP ester (Life Technologies) according to the suppliers instructions. For FCPIA, Hsp70 was biotinylated using EZ-link NHS-Biotin (Thermo Scientific) according to the supplier instructions. After labeling, the proteins were subjected to gel filtration to remove any unreacted label.

### Capillary Surface Modification

The inner surface of a 30 cm long fused silica capillary (Polymicro Technologies; Phoenix, AZ) with 50  $\mu\text{m}$  inner diameter and 360  $\mu\text{m}$  outer diameter was activated for derivatization by pumping the following through the capillary at 30 psi for 1 h each: 1) methanol at room temperature; 2) RCA solution ( $\text{NH}_4\text{OH}:\text{H}_2\text{O}_2:\text{H}_2\text{O}$  v:v:v = 1:1:5) at 120 °C; 3) 0.1 M HCl at 90°C. Temperature was maintained by an oil bath. After activation, the residual liquid was purged out of the capillary and the capillary inner surface was dried with dry nitrogen flow at 165 °C overnight. To derivatize the capillary, 10% (volume percent) 1H, 1H, 2H, 2H-perfluorooctyltrichlorosilane (Sigma, St. Louis, MO) in toluene was continuously infused

for 3 h at 120 °C. The derivatized capillary was rinsed with toluene and methanol before purging with nitrogen and drying in an oven at 80 °C overnight.

### Capillary Electrophoresis

A Beckman Coulter MDQ/PACE was used for CE experiments unless otherwise noted. The capillary was mounted to have a separation length of 10 cm. Capillary temperature was kept at 25 °C for all experiments. Separation buffer was 10 mM sodium phosphate with 0.01% Tween 20 (w:v) adjusted to pH 7.5. All separation buffers were made fresh every day from 5x stock solution. All buffer solutions were made using water purified and deionized to 18 M resistivity using a Series 1090 E-pure system (Barnstead Thermolyne Cooperation, Dubuque, IA) and then filtered through a 0.2 µm pore size membrane (Whatman, GE). The separation method consisted of three steps: 1) 1 min rinsing using with separation buffer; 2) pressure injection at 0.3 psi for 5 s; 3) separation at 500 V/cm with normal polarity resulting in 14 µA current. (During screening, this procedure was repeated without interruption or regenerating the column until the column failed, usually by breaking and typically after over 500 injections.) 0.5 psi pressure was applied during separation to generate flow in the same direction of EOF to decrease separation time to 3 min. LIF detection was accomplished using a 20 mW optically pumped semiconductor Sapphire laser (Coherent, Santa Clara, CA) coupled to the Beckman Coulter MDQ/PACE LIF detection module through an optical fiber. The LIF was equipped with  $\lambda_{ex}/\lambda_{em}$  filters of 488 nm/520 nm. All data were collected by 32 Karat software and further processed using software written in-house<sup>46</sup>.

### Small Molecule Libraries

The chemical library of 3,443 distinct compounds was assembled at the University of Michigan's Center for Chemical Genetics (CCG) from several small libraries. The MicroSource MS2000 library contains 2000 bioactive compounds with a minimum of 95% purity. The collection contains 958 known therapeutic drugs, 629 natural products and natural product derivatives, 343 compounds with reported experimental biological activities and 70 compounds approved for agricultural use. The CCG Focused collection includes ~1000 small molecules that target specific activities (e.g., Wnt Pathway) and natural products. The CCG Biofocus NCC library is an NIH Clinical collection that contains ~450 small molecules that have a history of use in human clinical trials including some FDA approved drugs. The activity of promising compounds was confirmed with repurchased samples from commercial sources including Sigma-Aldrich, Enzo Life Sciences, Cayman Chemical, Acros Organics, Alfa Aesar, and MP Biomedicals.

### Screening by Capillary Electrophoresis

Binding reactions were performed in 96-well conical bottom PCR plates (ISC BioExpress). A stock solution of Alexa Fluor® 488 labeled Hsp70 (Hsp70-488) and unlabeled Bag3 was prepared fresh daily in assay buffer (25 mM HEPES, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 0.3% Tween-20 pH 7.5) so that the final concentration of both proteins was 500 nM in the assay. Compounds and DMSO were dry spotted in plates prior to protein addition using a Mosquito liquid handler (TTP LabTech). Assay buffer (5 µL) was added to each well except for positive control wells that received 5 µL of unlabeled Hsp70. Hsp70-Bag3 solution (10 µL) was then added to each well. All additions were made using a Matrix Electronic Multichannel pipette (Thermo Scientific). Plates were incubated for at least 15 min at ambient temperature and then analyzed on the CE system. Dose response curves were obtained the same way except varying the inhibitor concentration.

## High-throughput Flow Cytometry Protein Interaction Assay

The assay procedure was adopted from previous reports<sup>47</sup>. In brief, biotinylated Hsp70 was incubated with streptavidin coated polystyrene beads (Spherotech) for 1 h prior to assay for immobilization. A stock solution of Alexa Fluor® 488 labeled Bag3 was prepared in assay buffer (25 mM HEPES, 10 mM KCl, 5 mM MgCl<sub>2</sub>, 0.3% Tween-20 pH 7.5) so that the final concentration of Bag3 was 30 nM in the assay. Assay buffer (5 µL) was added to each well of a black 384 well plate (Thermo Scientific), followed by compound or DMSO addition (0.2 µL) using a Biomek HDR (Beckman). Positive control wells received 5 µL unlabeled Hsp70 instead of assay buffer. Bag3 solution (10µL) was then added to each well, followed by Hsp70-bead addition (5 µL). All components other than compounds were added using a Multidrop dispenser (Thermo Fisher Scientific). Plates were incubated for 15 min then analyzed using a Hypercyt liquid sampling unit in line with an Accuri® C6 Flow Cytometer. Median bead associated fluorescence was calculated using Hyperview software for each well and data was uploaded to the Mscreen database.

## Results and Discussion

### Development of a CE-based assay for Hsp70 binding to Bag3

In APCE, one of the binding partners is normally labeled to allow sensitive detection of the complex by LIF. For these experiments we labeled recombinant, human Hsp70 (HSP1A1) with Alexa-488, such that there was an average of 0.5 fluorophore per protein. This modification did not affect the activity of Hsp70 as measured by comparing the ATPase activity of unmodified and labeled samples (Supporting Information Figure S-1).

Initial experiments were directed at identifying separation conditions that would allow detection of Hsp70–488 and its complex with Bag3. Samples containing 0.5 µM Hsp70–488 or Hsp70–488 and Bag3 mixed at a 1:1 ratio (0.5 µM each) were analyzed by APCE. In this stage of the study, we found that the biggest challenge was Bag3 adsorption. For example, when using unmodified fused silica for the CE capillary, the Hsp70–488 peak was detected and it decreased upon addition of Bag3; however, no complex peak was detected (Figure 1A). To prevent adsorption, the capillary surface was modified using a perfluorinated silane and 0.01% (w/v) Tween-20 was added to the electrophoresis buffer<sup>48</sup>. Using these modifications, the mixture of labeled Hsp70 and unlabeled Bag3 was readily observed as two individual bands with migration times of 96 and 122 s, corresponding to the complex and the free Hsp70 respectively (Figure 1B). A “bridge” is observed between the complex and free peak which is attributed to Hsp70–488 that dissociated during separation (Figure 1B).

To evaluate whether this method could faithfully recapitulate the known affinity of the Hsp70-Bag3 complex, we titrated Bag3 into Hsp70–488 and measured formation of the complex by CE. The peak area of complex plus “bridge” increased as a function of Bag3 concentration (Figure 2A). (Peak area of complex was considered to be the beginning of the complex peak to the first rise of the free peak.) Plotting this peak area against Bag3 concentration and using non-linear regression to fit a 1:1 binding model yielded a  $K_D$  of  $23 \pm 8$  nM (Figure 2B), which is in good agreement with the 15 nM  $K_D$  obtained from isothermal titration calorimetry (ITC, Supporting Information Figure S-2) and values determined using SPR<sup>49</sup>. Because ITC was performed with unlabeled Hsp70-Bag3, these results suggest that the binding affinity was not affected by the label and that CE can determine comparable measures of affinity.

To confirm that the peak attributed to complex was in fact Hsp70-Bag3 and demonstrate that we could detect inhibition of this complex, we performed competition experiments in which unlabeled Hsp70 was added at different concentrations to the Hsp70–488 and Bag3 mixture.



These experiments showed that the complex peak decreased with added Hsp70 to yield an  $IC_{50}$  of 0.24  $\mu\text{M}$  (Figure 2D). These results confirm that the peak is a specific complex between Hsp70–488 and Bag3 and that bound to free ratios can be used to detect an inhibitor.

### Adapting CE to a Screening Format

After validating the CE method as an effective platform for monitoring the Hsp70-Bag3 interaction, we evaluated its potential for screening using automated analysis from 96-well microtiter plates. Each CE assay required ~ 6.5 min to complete, including 1 min rinsing, 5 s injection and 3 min separation. This protocol allowed us to screen 220 samples per day. Thus, while the method is not yet practical for screening large (*e.g.* 100,000) chemical libraries, it is suitable for proof-of-principle pilot screens.

The robustness of the CE platform was tested by performing a sequence of control assays from a 96-well plate. Each sample contained Hsp70–488 (0.5  $\mu\text{M}$ ) mixed with Bag3 (0.5  $\mu\text{M}$ ). Half of the samples were positive controls (2  $\mu\text{M}$  unlabeled Hsp70 added) and half were negative controls (only 1% DMSO added). The calculated Z-factor for this experiment was 0.78, well above the suggested minimum for HTS (~0.50)<sup>50</sup>. We also tested the robustness of FCPIA for measuring this PPI. Briefly, Hsp70 was biotinylated and immobilized on streptavidin coated polystyrene beads, while Bag3 was fluorescently labeled with Alexa-Fluor 488 (see Experimental). The two partners were then incubated together and analyzed using an Accuri flow cytometer to measure bead-associated fluorescence<sup>51</sup>. This platform yielded a similar affinity for the Hsp70-Bag3 complex (15  $\pm$  4 nM) and a Z-factor of 0.86. Together, these experiments established two platforms for screening of the Hsp70-Bag3 complex.

### Screening and Selection of PPI inhibitors

Using these conditions, we screened a pilot library of 3,443 compounds by CE using the workflow shown in Figure 3. A comparable workflow was used for a FPCIA screen. In the CE screen, each well produced an electropherogram, which was then integrated and the peak area ratios (bound Hsp70–488 to free Hsp70–488) were used to identify putative inhibitors.<sup>52</sup> Ratios were used instead of single peak areas to minimize artifacts from variations in injection volume, light source instability or sample evaporation effects. This approach also allowed the results from each individual microtiter well to be submitted to the screening database as a convenient single point data; however, the full electropherograms were stored and could also be retrieved. Test compounds in both the CE and FCPIA screens were screened at a single concentration (20  $\mu\text{M}$ ) and those that blocked the PPI with a percentage of inhibition that was  $\geq 3$  standard deviations (SD) from the negative controls were considered “hits”. Using this criterion, CE identified 79 primary hits (2.3%), while FCPIA identified 117 (3.4%) as shown in Figure 4. Active molecules from the CE screen were further triaged based on visual inspection of the raw electropherograms. This analysis readily identified aggregator molecules and compounds with high intrinsic fluorescence because of irregular electropherograms (see Figure 5). Removing these artifacts reduced the number of putative inhibitors to 48 (1.4%). Interestingly, only 6 primary hits were common between the two assays, suggesting a large number of false positives and/or negatives.

To explore the quality of the screening, we performed confirmation and dose response curve (DRC) analyses (Figure 4C). DRC analyses confirmed that 69% (33 of 48) of compounds identified by CE had  $IC_{50}$  values of at least 400  $\mu\text{M}$  whereas 50% (59 of 117) of compounds identified by FCPIA had similar potency. CE had a lower hit rate and a greater fraction of hits reconfirmed suggesting fewer false positives in screening.

Select compounds from both lists of confirmed inhibitors were then repurchased from commercial sources: 14 compounds from the CE screen and 18 from the FCPIA screen including 2 shared inhibitors. Only hits that were available from different commercial sources and were deemed to have potential for further development as drugs or chemical probes were selected for retesting. Repurchased compounds were tested by DRC in the original screening platforms and 8 were reconfirmed from each list. These results suggest that some of the hits are due to degraded compounds or other artifacts from the library, a common observation in screening.

The 8 confirmed hits from each assay were tested by DRC on the other assay. Interestingly, all (8/8) repurchased compounds that reconfirmed in CE were also confirmed in FCPIA; however, only 50% (4/8) of the compounds that showed activity in FCPIA were considered inhibitors in the CE platform. (DRC from cross testing are shown in Supporting Information Figure S-3).

Of the 4 compounds found by FCPIA that reconfirmed by CE, two were detected and two were missed in the original CE screen. The two inhibitors overlooked by CE were confirmed at a concentration 5 times higher than the concentration used for the screen. The use of lower concentrations during the screen combined with lower Z-factor of the CE assay led to their being missed in the original screen. Of the 8 compounds found by CE that reconfirmed by FCPIA, 6 were missed in the original FCPIA screen. Two of these, celastrol<sup>53</sup> and myricetin<sup>54</sup>, are known modulators of Hsp70. These results suggest that CE gave fewer false negatives. Nevertheless, it is apparent that the screens complement each other.

### Evaluation of the CE Platform and Opportunities for Further Optimization

Compared to the standard screen by FCPIA, CE produced lower hit rates with a higher percentage of compounds that were eventually confirmed by retesting using multiple techniques. We suspect that the enhanced reproducibility and specificity of CE might derive from the use of the electropherogram to triage molecules, which allowed streamlining of the hit selection process at multiple stages. For example, molecules that induce Hsp70–488 aggregation were readily identified by the appearance of sharp spikes corresponding to insoluble particulates (Figure 5B). Removing these aggregators from future consideration greatly streamlined the subsequent confirmation steps. CE also allowed direct detection of intrinsically fluorescent compounds, which might otherwise give false positives in a fluorescent screening assay and require secondary screens for their removal (Figure 5C). In principle, these compounds could be further evaluated in screens by CE, but we did not utilize this possibility in this pilot work.

Another difference between the assays is that the CE assay used labeled Hsp70 and allowed interaction in free solution. In contrast, the FCPIA used labeled Bag3 and immobilized Hsp70. This difference may have also yielded some differences in the results such as the 4 hits confirmed by FCPIA but not detected by CE and the rank order of IC<sub>50</sub>s (see Supporting Information Figure S-4). A label or surface immobilization may yield subtle changes in protein conformation, affect access to the binding site, or inhibit allosteric mechanisms. Thus, in principle the lower degree of protein modification required by CE would be advantageous.

Despite these advantages, the application of CE to screening PPI is still in its infancy and several issues need to be addressed. We observed that peak area ratios began to drift after about 500 injections. This was attributed to deterioration of the surface coating because switching to a new capillary restored the original bound to free ratio. This effect may have contributed to the lower Z-factor of the CE method and it might have caused us to overlook some active compounds. More stable coatings are likely to help this effect. Of course, not all



proteins will require coatings. Another limitation is the current requirement for covalent labeling with fluorophores, which has the potential to interfere with the PPI itself depending on the system. Use of post-column derivatization, native protein fluorescence, or label-free detection methods are alternatives that may eliminate this requirement.

At the throughput enabled by a commercial CE instrument, this assay is best suited as a secondary screen to test hits found by higher throughput methods. Use of novel microchip systems<sup>55</sup> or adoption to commercial microchip electrophoresis, e.g. CaliperLabChip EZ reader, will be required to make this a HTS tool suitable for screens of larger chemical collections.

Recent interest in PPI inhibitors has driven a search for new methodologies that are suitable for HTS. In particular, HTS methods that permit screening of relatively weak or transient interactions are becoming an area of need. The experiments with CE described here provide a framework for development of this method as a robust tool for the discovery of new inhibitors of PPIs that complements existing techniques.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

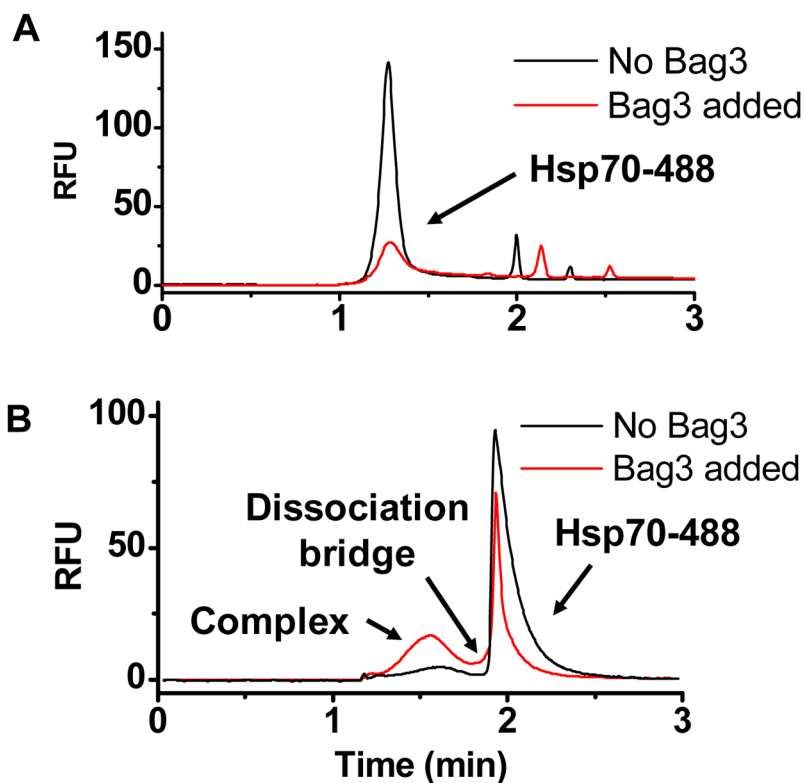
This work was supported by NIH GM102236 to R.T.K. and NIH NS095690 to J.E.G. The authors thank Martha Larsen and the University of Michigan CCG for technical assistance.

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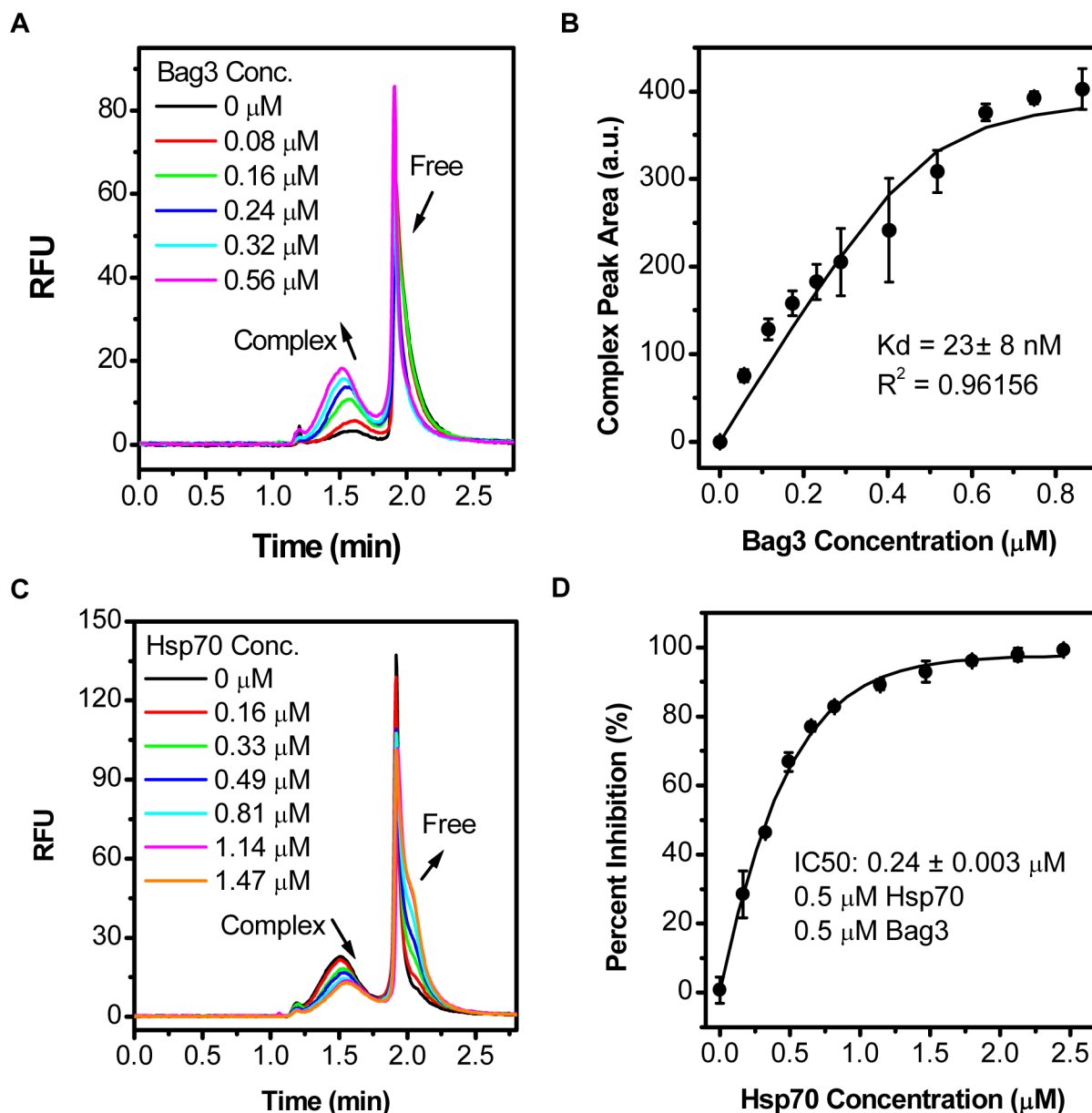
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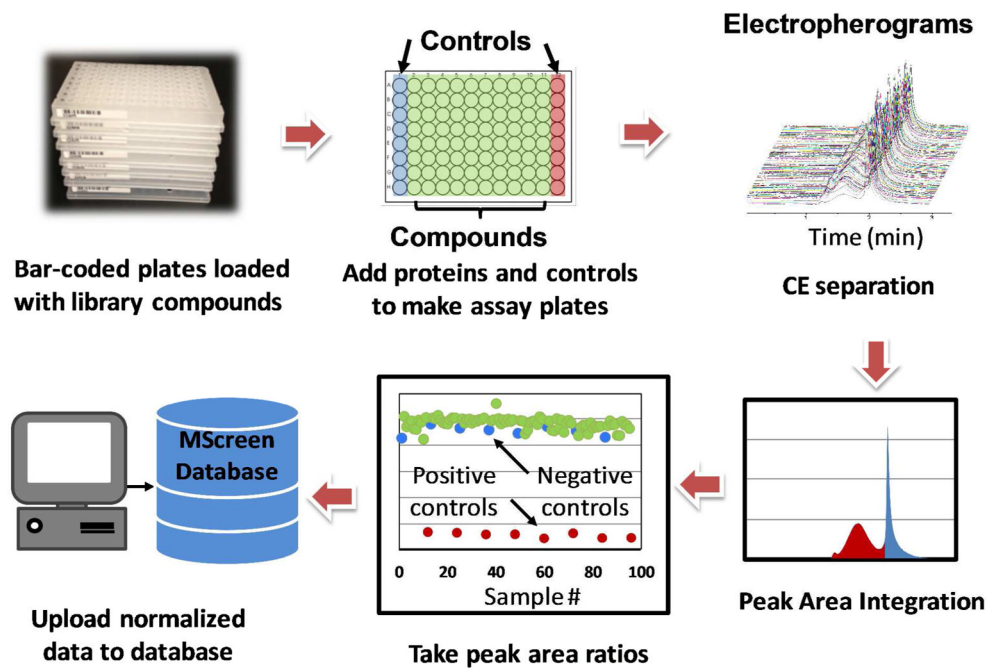
**Figure 1.**

Electropherograms of Hsp70-488 with and without Bag3 added using (A) bare silica capillary; (B) PFOTCS modified capillary. Free Hsp70-488 decreases with added Bag3 in (A), but no complex peak is detected suggesting adsorption induced by Bag3. A complex peak with dissociation, forming a bridge to the free Hsp70-488, is observed with the modified capillary. Separations were performed at 500 V/cm through 10 cm effective length. 0.5 psi was applied to drive flow and decrease analysis time in (B).



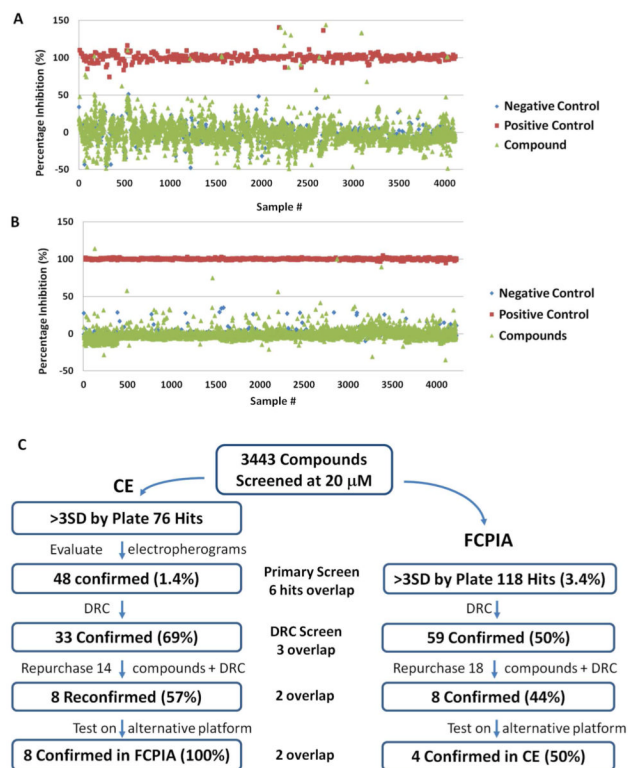
**Figure 2.**

Determination of dissociation constant ( $K_d$ ) and  $IC_{50}$  of binding by CE-LIF. (A) Electropherograms of 0.5  $\mu\text{M}$  Hsp70 with increasing concentrations of Bag3; (B) Saturation curve from the titration experiments in Figure 2A by plotting the peak area of complex and dissociation bridge against Bag3 concentration. Non-linear regression determined the  $K_d$  to be  $23 \pm 8 \text{ nM}$ . (C) Electropherograms of 0.5  $\mu\text{M}$  Hsp70 and Bag3 with increasing concentration of unlabeled Hsp70. (D) Peak area ratio (bound to free)'s response to increasing Hsp70 concentration.  $IC_{50}$  can be determined for unlabeled Hsp70 to be  $0.240 \pm 0.003 \mu\text{M}$ .

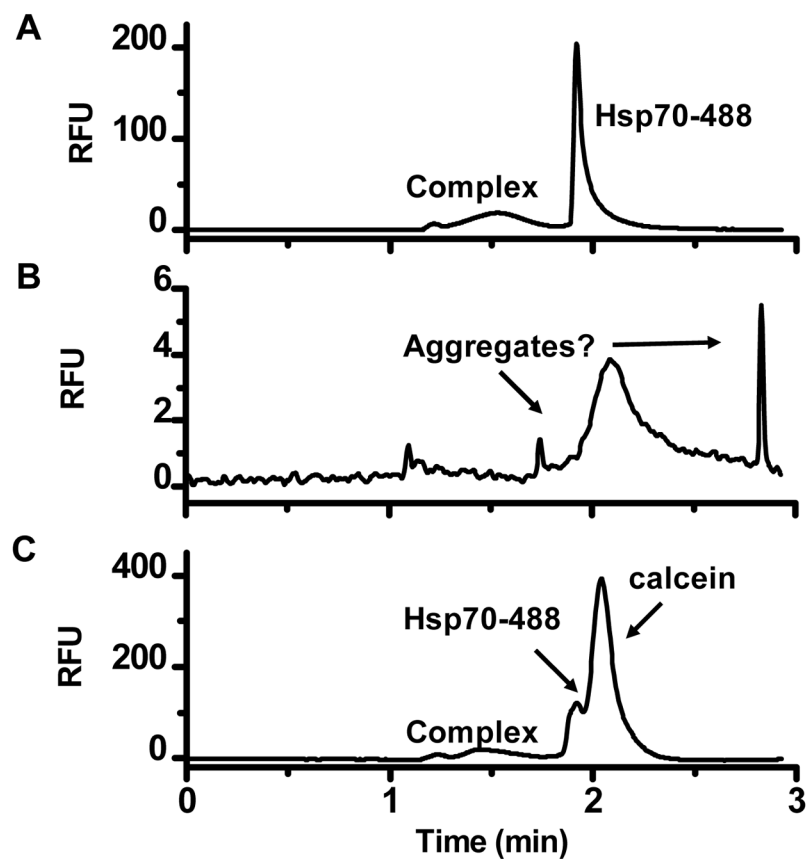


**Figure 3.** Illustration of workflow for CE screen. Library compounds were placed in 96 well plates along with controls for assay. After CE separation, peaks were integrated and bound to free ratio for Hsp70–488 measured. Resulting ratios were analyzed to determine those that varied by more than 3 standard deviations from control averages to identify hits.



**Figure 4.**

Results from primary screen of 3,443 compounds using CE and FCPIA. (A) Campaign wide results from CE screen. Negative controls (DMSO) are shown in blue, positive controls (2  $\mu$ M Hsp70) are shown in red, and compound data points are shown in green. Hits were determined by signal relative to controls on each plate. Z score for each plate was calculated and average was 0.58. (B) Campaign wide results from FCPIA screen. Negative controls (DMSO) are shown in blue, positive controls (1  $\mu$ M Hsp70) are shown in red, and compound wells are shown in green. Average plate Z score was 0.86. (C) Summary of triage and confirmation of hits for CE and FCPIA screen.



**Figure 5.** Sample electropherograms of 0.5  $\mu\text{M}$  Hsp70-488 and 0.5  $\mu\text{M}$  Bag3 with (A) 15  $\mu\text{M}$  Epigallocatechin gallate (EGCG), a confirmed PPI inhibitor; (B) 20  $\mu\text{M}$  haematoxylin, identified as an aggregator; (C) 20  $\mu\text{M}$  calcein added, identified as a fluorescent compound that interferes with detection of free Hsp70-488.