TITLE: Validation of APF as a Urinary Biomarker for Interstitial Cystitis

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Validation of APF as a Urinary Biomarker for Interstitial Cystitis

The purpose of this study is to develop and characterize a surface plasmon resonance (SPR)-based assay that can specifically detect binding of APF to its cellular receptor, cytoskeleton associated protein 4 (CKAP4), immobilized on a sensor chip surface and to test the ability of this SPR-based assay to discriminate and measure the concentration of APF in urine from well-defined IC patients vs. age-matched, asymptomatic controls. Our results demonstrate that we have successfully developed an SPR assay using a CKAP4 127-360 biosensor with sufficient binding efficiency to detect as-APF in urine with detection limits in the high nM to uM range. Urine specimens from 14 (47%) of 30 women diagnosed with IC/PBS demonstrated as-APF binding activity to the CKAP4127-360 biosensor compared with 22 (73%) of 30 asymptomatic control women. Thus, urine specimens from women with IC were less likely to demonstrate significant APF/CKAP4 binding activity (47%) than specimens from asymptomatic control women (73%). When compared to cellular proliferation assay results, agreement with clinical diagnosis is decreased by the SPR method vs. cellular proliferation method (47% vs. 77%). Importantly, the correlativity between both assays was 58% (35 of 60 samples), suggesting that the SPR method has potential utility as a non-invasive means for diagnosing IC in women. The observation that APF activity is detectable in urine specimens from 47% of asymptomatic control women by the cellular proliferation assay and 73% of asymptomatic control women by the SPR binding assay, suggests that other urinary constituents may be contributing to the measured response. Using a targeted mass spectroscopy method, we determined that APF/as-APF were present in the pM to nM range in all urine samples regardless of patient diagnosis (control vs. IC) suggesting that both peptides may exist at basal levels in normal bladder physiology.
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1. INTRODUCTION:

Interstitial cystitis (IC) is a chronic, debilitating bladder disease that affects mostly women and is frequently misdiagnosed due to lack of a non-invasive test to detect the disease. While the cause remains unknown, biomarkers for IC have been described, including antiproliferative factor (APF), a glycopeptide that is detectable in the urine of 95-97% of IC patients vs. normal controls. Validation of APF as a biomarker and etiologic agent for IC has been hindered by the absence of robust assays to detect and measure its concentration in patient urine. The purpose of this study is 1) to develop and characterize a surface plasmon resonance (SPR)-based assay that can specifically detect binding of APF to its cellular receptor, cytoskeleton associated protein 4 (CKAP4), immobilized on a sensor chip surface and 2) to test the ability of this SPR-based assay to discriminate and measure the concentration of APF in urine from well-defined IC patients vs. age-matched, asymptomatic controls. Approximately 90 patients will participate in this study. Urine specimens from 30 IC patients will be collected by Dr. Phillip Hanno and his staff during routine office visits to the Penn Urology IC Clinic at the University of Pennsylvania Hospital for the management of IC. Urine specimens from asymptomatic controls will be collected at TCMC by Betsy Mead, the clinical research coordinator. All processed urine samples will be shipped on dry ice to Dr. Susan Keay, who will blindly test the fresh urine specimens for APF activity by 3H-thymidine incorporation in addition to 30 banked frozen specimens (from another 15 IC female patients and 15 age-matched, asymptomatic controls) for comparison to results obtained from the SPR assay. We expect that the SPR-assay will overcome current barriers associated with validation of APF as a diagnostic biomarker for IC by being able to specifically detect the presence of APF in urine and accurately quantitate its levels for the first time. This would meet a critical need for an affirmative, diagnostic test for IC with the advantages of being rapid, specific, and non-invasive; further, it would present major learning opportunities for advancing our knowledge about the contribution of APF to IC.

2. KEYWORDS:

APF: antiproliferative factor
CKAP4: cytoskeleton associated protein 4
ED: extracellular domain
FL-CKAP4: full-length cytoskeleton associated protein 4
HT-CKAP4: histidine (x6) tagged-cytoskeleton associated protein 4
IC: interstitial cystitis
kDa kilodalton
KLH: keyhole limpet hemocyanin
mAb: monoclonal antibody
NTA: nitrilotriacetic acid
PAGE: polyacrylamide gel electrophoresis
PBST: phosphate buffered saline containing Tween-20
SDS: sodium dodecyl sulfate
SPR: surface plasmon resonance

3. ACCOMPLISHMENTS:

Major Project Goals

The original Statement of Work indicated two specific aims or major goals to be accomplished during the 3-year funding period, with emphasis on the first aim in the first two years and the second aim in the final year: 1) Develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF (1-24 months) 2) Determine the ability of the SPR-based assay to detect APF in urine from patients with IC (1-36 months)

The status of milestones for tasks related to each aim of the project is summarized below.

Specific Aim 1:
Task 1a. Optimization of rCKAP4 activity and immobilization on sensor chip surface (months 1-12)

**Milestone:** Improved CKAP4 binding efficiency to APF in a purified and non-purified system (ie, urine)

Completion Date: Completed July 2016

Task 1b. Optimization of conditions for sensor chip surface regeneration and binding reproducibility (months 1-12):

**Milestone:** Full regeneration and CKAP4/APF binding reproducibility following 3 repeated cycles of analyte and regeneration injections.

Completion Date: Completed January 2015

Task 1c. Characterization of the SPR-based assay using synthetic APF (months 12-18):

**Milestone:** Characterization of 20 samples containing various levels of synthetic APF spiked in urine from healthy donors as a first test of the system’s diagnostic ability.

Completion Date: Completed August 2016

Specific Aim 2:

Task 2a. Regulatory review and approval by Institutional Review Boards and DoD Human Research Protection Office (months 1-3):

Completion Date: Completed August 2013

Task 2b. Recruitment of human subjects for urine sample acquisition (months 4-24):

**Milestone:** Acquisition of 60 urine specimens from 30 IC/PBS patients and 30 age-matched, asymptomatic controls.

Completion Date: Completed April 2016

Task 2c. Testing of biological urine specimens by the cellular proliferation assay (months 4-30)

**Milestone:** To have tested 90 urine specimens for APF activity using the cellular proliferation assay.

Completion Date: Completed June 2016

Task 2d. Testing of biological urine specimens by the SPR-based assay and comparison of results with cellular proliferation assay results (months 24-36)

**Milestone:** To have tested 90 urine specimens for APF using the SPR-assay.

Completion Date: Completed September 2016

Task 2e. Statistical analysis (months 24-36)

**Milestone:** Assessment of APF's utility as a diagnostic biomarker for IC and the first direct measurement of APF in human urine.

Completion Date: 90% complete

Accomplishments

The original Statement of Work indicated two specific aims to be accomplished over the course of the 3-year contract period. In the first two years of the study, we predominantly focused our effort on the first specific aim: developing and characterizing the SPR-based assay. In year 3, we shifted our focus to the second aim, which included testing of the acquired biological urine specimen by the cellular proliferation assay and the SPR assay. Below we present the original SOWs (in italics) and our major accomplishments (underlined) over the three-year contract period.

**SOW – Specific Aim 1/Task 1:** Develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF. This has been accomplished as described under each task below.

1a: Optimization of rCKAP4 activity and immobilization on sensor chip surface (months 1-12). In order to develop a robust method to immobilize CKAP4 onto a sensor chip surface to measure APF binding, optimization of recombinant CKAP4 (rCKAP4) activity was pursued and achieved. Various strategies
were employed to promote CKAP4’s most physiologically relevant conformation in order to obtain improved APF binding efficiency. Early results indicated that a truncated form of CKAP4 consisting of its extracellular domain (ED) achieved stronger binding to APF than full-length forms tagged with Histidine at the N- or C-terminus. Therefore, we focused our efforts in years 2 and 3 on characterization of four CKAP4 deletion mutants of the extracellular region in an effort to identify the primary APF binding domain and thus establish a robust binding assay with much higher sensitivity.

Using structural prediction software, we analyzed the CKAP4 ED and determined the optimal deletion strategy for generating mutants of this region while preserving structural and functional domains. Four CKAP4 deletion mutant constructs consisting of amino acids 106-602, 127-360, 361-524, and 525-602 were generated by PCR and subcloned into the pET15b bacterial expression vector. All of the mutants (tagged at N-terminus with His6) were expressed successfully in bacteria. Each mutant was successfully purified, dialyzed, and immobilized to a CM5 chip via amine-coupling to measure its APF binding response. As shown in Figure 1, the 127-360, 361-524, and 525-602 mutants exhibited specific binding to active APF, with mutant 127-306 exhibiting a slightly higher binding affinity and intensity. Table 1 lists the parameters of the binding kinetics for the APF interaction with the CKAP4 extracellular domain mutants using a 1:1 binding kinetics model. It is interesting to point out that both CKAP4127-360 and CKAP4361-524 exhibited a fast association constant \( k_{on} \) and a slow dissociation constant \( k_{off} \), therefore yielding high binding affinity \( K_D = k_{off}/k_{on} \); \( K_D \) for CKAP4 127-360: 1.34\( \mu \)M, and \( K_D \) for CKAP4 361-524: 2.27\( \mu \)M), which fits the general model of strong interaction and suggests that the primary APF binding site is located within the Aa 127-524 region of the CKAP4 extracellular domain. In contrast, interaction of APF with the CKAP4 525-602 ligand (Fig. 1C) displayed different binding kinetics with a slower association phase and a rapid dissociation phase, conveying a much lower affinity with a \( K_D \) value of 29.64 \( \mu \)M. Although the 106-602 mutant also bound APF efficiently (data not shown), it did not show reproducible binding, suggesting that the presence of the receptor transmembrane domain requires an alternative immobilization strategy and sensor chip surface to preserve the structure of CKAP4 (ie, L1 chip). Thus, we determined that the CKAP4127-360 and CKAP4361-524 mutants exhibit improved binding to APF with normal proportional response to increasing dose of ligand and the maximum binding response (Rmax), making a robust SPR-based assay feasible in a purified system.

Table 1. Kinetic parameters of the interaction between APF and CKAP4 ED deletion mutants

<table>
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<tr>
<th>Ligand</th>
<th>( k_{on} ) ( M^{-1}s^{-1} \times 10^2 )</th>
<th>( k_{off} ) ( s^{-1} \times 10^{-4} )</th>
<th>( K_D ) ( \mu M )</th>
<th>( R_{max} )</th>
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<td>1.77</td>
<td>52.5</td>
<td>29.64</td>
<td>27.36</td>
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</table>
Figure 1. Binding kinetics of APF interaction with CKAP4 extracellular domain deletion mutants. Three CKAP4 ED deletion mutants were immobilized onto the Fc2 channel of CM5 chip as the ligand; the Fc1 channel was treated similarly but without the protein as the control. Multi-cycle kinetics assay were performed by the injection of various concentrations of as-APF (2.5 – 40 µM, colored lines) over CM5 chip surfaces on which CKAP4_{127-360} (A), CKAP4_{361-524} (B), and CKAP4_{525-602} (C) were immobilized via amine-coupling. The adjusted sensorgrams (Fc2-Fc1) were overlaid to calculate the binding kinetics. The 1:1 binding model was used to fit the data and fitting curves (black lines) are also shown. The parameters of binding kinetics are shown in Table 1.
Based our findings using CKAP4 variants, we sought to test an alternative, parallel approach that could significantly increase the sensitivity of our SPR assay. This methodology utilized a monoclonal anti-APF antibody as the biosensor in the SPR assay to detect APF as the analyte. Five purified monoclonal antibodies that displayed specific activity against APF-KLH were tested for APF specificity by SPR. We used two different immobilization strategies—an amine-coupling approach and an indirect capture approach. In both instances, the APF monoclonal antibody (mAb), HL-2411, displayed specific binding against APF, as-APF, KLH-APF, but not APF lacking its sugar moieties; further, this binding was more enhanced with indirect capture, suggesting that this immobilization approach presents the antibody in a better conformation that can be recognized by APF. Next, we compared the sensitivity of the HL-2411 antibody biosensor to our CKAP4 biosensor (both CKAP4_{127-360} and CKAP4_{361-524}) in the SPR assay using spiked control urine. Importantly, we determined that the CKAP4 biosensor(s) has the greater dynamic range and the most likelihood of success for detecting APF in urine and therefore discontinued pursuit of the HL-2411 antibody as an alternate method to enhance SPR sensitivity.

1b: Optimization of conditions for sensor chip surface regeneration and binding reproducibility (months 1-12). Task 1b under Aim 1 was accomplished as amine-coupling was determined to be the optimal method for CKAP4 biosensor immobilization and CKAP4/APF binding reproducibility in the SPR assay.

1c: Characterization of the SPR-based assay using synthetic APF (months 12-18). We characterized the APF binding potential of four CKAP4 deletion mutant constructs and determined that CKAP4_{127-360} had the highest potential to detect and quantitate APF in urine samples. Since urine is a non-purified system, we tested several methods of urine peptide extraction to enrich samples for the APF peptide while minimizing background interference from other urinary constituents. These methods included urine protein concentration through proprietary ion exchange resins and peptide enrichment. We tested five different combined chromatographic methods—hydrophilic interaction chromatography (HILIC), ion-pairing hydrophilic interaction chromatography (HILIC-IP), solid phase extraction (SPE), and ion exchange chromatography (IEC)—for enrichment of APF peptide and for removal of urinary constituents that might potentially interfere with the SPR assay sensitivity. As shown in Figure 2, we established that the SPE resin is most efficient to enrich as-APF peptide from urine.

**Figure 2.** as-APF can be successfully detected by LC-MS after purification by a combined method. Control urine sample was charged with 5uM of as-APF, followed by the partial enrichment by ultrafiltration and then purified by five different methods, including HILIC, HILIC+IP, SPE+IEC, SPE, and IEC, among which the SPE purification turned out to be the best as shown by the MS spectrum above.
1) HILIC: Hydrophillic Interaction Chromatography (PolyHydroxyEthyl A TopTips from PolyLC Inc.) Binding condition: 2% FA-Formic Acid
2) HILIC-IP: Ion-Pairing Hydrophilic Interaction Chromatography (same resin as above, different binding conditions done with 0.1% TFA)
3) IEC: Ion Exchange Chromatography. (proteaspin urine protein concentration kit from Norgen Biotech)
4) SPE: Solid Phase Extraction. (Oasis HLB SPE cartridges from Waters)

Using this urine enrichment strategy, we tested the dynamic range and sensitivity of the SPR-based assay employing the CKAP4_{127-360} mutant as the immobilized biosensor in a non-purified system. For this experiment, we used urine from twenty healthy donors spiked with as-APF as the mimetic for IC patient urine and CKAP4_{127-360} as the ligand, since it yielded the highest binding affinity as observed earlier. Various dilutions of a control urine sample spiked with APF were prepared and injected over a CM5 chip surface pre-treated with the CKAP4_{127-360} ligand (Fc2 channel). To account for background interference from urinary constituents, corresponding dilutions of unspiked control urine were also run to serve as individual controls. The representative sensograms (Fig. 3A-D) demonstrated that it was possible to detect low concentrations of as-APF in urine (a non-purified system) using a CKAP4 biosensor in an SPR-based assay. APF/as-APF concentrations as low as 1.25 µM were easily detectable (Fig. 3D). Characterization of 20 samples containing various levels of synthetic APF spiked in urine from healthy donors demonstrated that detection limits for this assay are in the high nanomolar to micromolar range.

![Figure 3. Detection of as-APF in spiked urine.](image-url)

Control urine was mixed 1:1 with as-APF stock (200 µM) solution. Various dilutions of this mixture in SPR running buffer were prepared and injected over a CM5 chip surface immobilized with CKAP4_{127-360} by amine-coupling on the Fc2 channel. Fc1 channel was similarly treated but without CKAP4, as the reference. Final concentrations of as-APF were 40 µM (A), 20 µM (B), 5 µM (C), and 1.25 µM (D). Resulting sensograms were corrected for background interference from urinary constituents, and re-plotted after subtracting the RU trace from the reference (Fc1) channel.
**SOW – Specific Aim 2/Task 2:** *Determine the ability of the SPR-based assay to detect APF in urine from patients with IC (months 1-36).* This has been accomplished as described under each task below.

**2a:** *Regulatory review and approval by Institutional Review Boards and DoD Human Research Protection Office (months 1-3):* This has been accomplished and approval has been received by all regulatory agencies.

**2b:** *Recruitment of human subjects for urine sample acquisition (months 4-24):* The recruitment of human subjects for urine sample acquisition has been accomplished. Fresh urine specimens were acquired from 30 IC/PBS patients at the University of Pennsylvania and from 30 age-matched, asymptomatic controls at The Commonwealth Medical College.

**2c:** *Testing of biological urine specimens by the cellular proliferation assay (months 4-30):* Testing of 60 urine specimens for APF activity using the cellular proliferation assay has been accomplished. The goal of testing 90 specimens was not met due to an insufficient volume of banked, frozen urine that was determined to be required for testing each specimen by both the cellular proliferation assay and the SPR-based assay. Thus, for the purpose of comparing assay results, only the 60 fresh urine specimens were tested as described below.

De-identified urine specimens from 30 IC/PBS patients and 30 age-matched, asymptomatic normal controls were shipped to Dr. Susan Keay’s laboratory and tested for APF activity by $^3$H-thymidine incorporation. For the APF activity assays, primary bladder epithelial cell explants (from a normal donor without evidence for urinary tract disease) were plated at a density of $1 \times 10^4$ cells per well in MEM medium containing 10% heat-inactivated fetal calf serum, 1% glutamine, and 1% antibiotic/antimycotic solution onto Corning 96 well tissue culture plates (VWR Scientific Products, Bridgewater, NJ) and incubated at 37°C overnight. The medium was changed to serum-free MEM containing 1% glutamine and 1% antibiotic/antimycotic solution, and the cells were incubated at 37°C overnight. On the third day, urine specimens from IC/PBS patients or controls were corrected to pH 7.2 and 300 mOsm, filtered through a 0.2-mm pore filter (Gelman Sciences, Ann Arbor, MI), diluted 1:2 in MEM ("serum-free MEM" containing only glutamine and antibiotics/antimycotics) and applied to the cells. Cell controls received serum-free MEM alone. After another 48 hours of incubation at 37°C, the cells were pulsed with 1 μCi per well $^3$H-thymidine (NEN DuPont, Wilmington, DE) and incubated for another 48 hours at 37°C. Cells were then trypsinized, and insoluble cell contents harvested and methanol-fixed onto glass fiber filter paper. The amount of radioactivity incorporated was determined as counts per minute using a Beckman LS 3801 scintillation counter (Beckman Instruments, Los Angeles, CA). A significant inhibition of $^3$H-thymidine incorporation was defined as a mean decrease in counts per minute of 2 standard deviations from the mean of control cells for each plate and considered positive for APF activity.

Cellular proliferation assay results are summarized in Table 2. Urine specimens from 23 (77%) of 30 women diagnosed with IC/PBS significantly inhibited human bladder epithelial cell proliferation compared with 14 (47%) of 30 asymptomatic control women. Thus, osmolality- and pH-corrected urine specimens from women with IC were significantly more likely to inhibit human bladder epithelial cell proliferation (77%) than specimens from asymptomatic control women (47%).

**2d:** *Testing of biological urine specimens by the SPR-based assay and comparison of results with cellular proliferation assay results (months 24-36):* Testing of 60 urine specimens for APF binding to the CKAP4 biosensor by the SPR assay and comparison of results with the cellular proliferation assay results has been accomplished. As mentioned above, the goal of testing 90 specimens was not met due to limited volume of banked, frozen urine required for testing by both assays. Thus, for the purpose of comparing assay results, only the 60 fresh urine specimens were tested as described below.

SPR assays were performed on a Biacore X-100 instrument (GE Healthcare) with the chip surface at 25°C using PBS containing 0.005% Tween-20 as running buffer. De-identified urine specimens that had been stored at -80°C prior to testing, were thawed on ice and clarified by
centrifugation at 500 x g for 5 min at 4°C. Supernatants were subjected to ultrafiltration with a 10 kD MWCO centrifugal filter (Amicon Ultra, Millipore) until 95% sample volume could be recovered as filtrate. 2 CM5 sensor chips were utilized for the assay, and prepared by immobilizing ~1400 RU of CKAP4<sub>127-360</sub> on Fc2 by amine-coupling. The Fc1 channel was treated similarly but without CKAP4 and served as the reference. The processed urine samples were diluted 5-fold in SPR running buffer and injected over the sensor surface at 30 µl/min flowrate for 120 s, to screen for APF binding, and reference-subtracted SPR binding responses were plotted. The sensor surface activity for APF-binding was checked at the beginning, middle, and the end of each run with an as-APF standard series (5 µM – 40 µM). Figure 4A and C, show average reference-subtracted SPR binding responses for the as-APF standard series from 3 run checkpoints, for Chip 1 and Chip 2, respectively. Figure 4B and D, indicate reference-subtracted SPR binding responses for samples #5 - #46 (Chip 1), and samples #47 - #78 (Chip 2), respectively.

Figure 4. SPR-based detection of APF binding using CKAP4<sub>127-360</sub> as a biosensor. (A & C) show average reference-subtracted SPR binding responses for the as-APF standard series from 3 run checkpoints, for Chip 1
and Chip 2, respectively. (B & D) indicate reference-subtracted SPR binding responses for samples #5 - #46 (Chip 1), and samples #47 - #78 (Chip 2), respectively.

SPR-binding assay results are summarized in Table 2. Urine specimens from 14 (47%) of 30 women diagnosed with IC/PBS demonstrated as-APF binding activity to the CKAP4 127-360 biosensor compared with 22 (73%) of 30 asymptomatic control women. Thus, urine specimens from women with IC were less likely to demonstrate significant APF/CKAP4 binding activity (47%) than specimens from asymptomatic control women (73%). When compared to results obtained from the cellular proliferation assay, these data suggest that agreement with clinical diagnosis is decreased by the SPR method vs. cellular proliferation method (47% vs. 77%), and that measurement of urine antiproliferative factor activity is more sensitive. Importantly, the correlativity between both assays was 58% (35 of 60 samples), suggesting that the SPR method has potential utility as a non-invasive means for diagnosing IC in women.

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<th>Sample ID</th>
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<th>APF Binding to CKAP4 Detected by SPR Assay</th>
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**Table 2. Comparison of SPR-based assay results with cellular proliferation assay results.** APF activity was assessed using the cellular proliferation assay established in Dr. Keay's lab. APF binding to the CKAP4 biosensor was assessed by SPR. Similar results are highlighted in yellow, while different results are highlighted in blue. Similar results were obtained for 35 (58%) of 60 samples, while 25 (42%) of 60 samples had different results. The asterisk (*) indicates that the assay result is in agreement with the patient diagnosis.

**Similar Results:** 35/60 = 58.3%

**Different Results:** 25/60 = 41.6%

Because the cellular proliferation assay and the SPR-based assay depend on APF biological activity for measurement, both methods lack some degree of specificity. The observation that APF activity is detectable in urine specimens from 47% of asymptomatic control women by the cellular proliferation assay and 73% of asymptomatic control women by the SPR binding assay, suggests that other urinary constituents may be contributing to the measured response. To clarify the specific contribution of APF and/or as-APF for the biological activities measured in urine specimens by these two methods, a targeted LC-MS method was used. This chemical method can definitively detect APF and as-APF in a urine specimen by generating specific signature peaks for each peptide, while separating the interference from other urinary biomolecules (Fig. 5). Thus, we used this method to determine if APF and/or as-APF co-exist in IC and/or control urines and to measure their quantity.

In order to chemically detect and analyze APF and as-APF by MS, urine samples were acidified to pH 3 with 10% formic acid. To determine the quantity of APF and/or as-APF in a sample, APF/as-APF standard curves were generated (Fig. 6). Pure compound solutions (200µM each of APF, as-APF and Glu-1-Fibrinopeptide B (Glu-Fib, GFP, control peptide) were mixed at 1:1:1 ratio and diluted with matrix (control urine) to 1000fmol/ul, 100fmol/ul, 10fmol/ul with 10-fold increment to 0.01fmol/ul. 100ul of urine samples or standard curve samples were desalted using with SPEcpTC18 (Agilent) and vacuum dried. After re-solubilized with 100ul 0.1% TFA, 1ul was analyzed by nano LC-MS/MS with a Dionex RSLC and QExactive HF (ThermoFisher). Samples were loaded onto a self-packed 100um x 2cm trap packed with Magic C18AQ, 5um 200 A (Michrom Bioresources Inc, Auburn, CA) and washed with Buffer A (0.2% formic acid) for 5 min with flow rate of 10ul/min. The trap was brought in-line with the homemade analytical column (Magic C18AQ, 3um 200 A, 75 µm x 50cm) and peptides fractionated at 300 nL/min with a multi-step gradient (4 to 15% Buffer B—0.16% formic acid 80% acetonitrile) in 10 min, and 15% to 50% B in 40 min. Mass spectrometry data was set for PRM on target list of 596.3217
(as-APF), 741.87 (APF) and 785.842 (Glu-Fib) throughout the run. MS parameters: resolution: 30,000, isolation width: 2.0 dalton, AGC target 5E5, Max ion time: 100ms. HCD collision energy: 20% relative collision energy for as-APF and APF, 25% for Glu-Fib. The PRM data were analyzed using automatic processing in Xcalibur. Two transitions each were used for quantitation of each compound: as-APF: m/z: 596.3217-620.3796, 596.3217-719.4487; APF: m/z: 620.3740-620.3796, 620.3740-719.4487; Glu-Fib: m/z: 785.842-684.3463, 785.842-813.3887. The peaks were isolated with 10ppm window within 2min retention time and integrated using ICIS algorism with smoothing point set to 5. Peak recognition and integration were inspected manually in Xcalibur Quan Browser. The area under the curve of the two transitions was summed to represent quantitation of the peptide(s). Fig. 7 demonstrates the quantitation of peptides in sample 15 and sample 26 as examples.

As-APF:  
m/z: 620.3796  
m/z: 719.4487

APF:  
m/z: 620.3740  
m/z: 719.4487

Glu-Fib:  
m/z: 813.3887  
m/z: 684.3463

Figure 5. MS spectra of two signature peaks of APF and as-APF (100nM) along with control peptide Glu-Fib (20nM). These peaks were used for quantitation based on standard curves.
Figure 6. Standard curves for APF and as-APF
Figure 7. Quantitation of endogenous APF and as-APF peptide in samples 15 and 26.
Using the targeted MS method described above, we identified both APF and as-APF in all urine samples regardless of patient diagnosis (ie, control vs. IC), suggesting that APF and as-APF may exist at basal levels in normal bladder physiology. While the quantities of both peptides were very low and varied among patient samples, as-APF was detected more sensitively. Further, based on normalization against the standard curve, as-APF concentration levels were consistently higher than APF in all urine samples tested (Fig. 8 & 9). Based on the standard curve, 60% of samples were calculated to have as-APF from 0 nM (sample 14) to 0.2 nM (sample 42). To calculate concentrations for other samples, standard curves with even lower concentrations (down to 0.001nM) need to be repeated. Overall, our data demonstrate that APF and as-APF exist in pM to nM range in original urine samples. The concentrations of sample 14 to 45 are consistent with their APF levels, the rest of samples are not consistent. Correlativity between the MS data and the SPR and cellular proliferation assays is problematic as both depend on APF/as-APF biological activity and have much higher interference from other urinary constituents (ie, biomolecules).

**Figure 8. Measurement of APF in urine samples.** Original urine samples were centrifuged and ultra-filtered to remove all particles, and then processed and analyzed by nano-LC- MS/MS as described in “Method”. The sum of area of two representative MS/MS peaks was used to indicate the relative concentration of each sample. Based on the standard curve, the concentrations of all samples are lower than 0.01nM, but the identity of APF was completely confirmed by targeted MS analysis. Note: sample 5, 8 and 11 were used for pre-test, only as-APF was detected. After optimization of detecting both APF and as-APF, these samples were not enough, so no consistent data were obtained for these samples.
Figure 9. Measurement of as-APF in urine samples. This experiment was performed as described in Figure 8. However, as-APF was detected more sensitively and at higher concentrations in the urine samples. Based on the standard curve, 60% of samples were calculated to have as-APF from 0 nM (sample 14) to 0.2 nM (sample 42). To calculate concentrations for other samples, standard curves with even lower concentrations (down to 0.001 nM) will be repeated.

2e: Statistical analysis (months 24–36). Assessment of APF’s utility as a diagnostic biomarker for IC and the first direct measurement of APF in human urine has been determined preliminarily as described above, but not yet fully accomplished (90% complete). This is due in part to the very low concentrations of each peptide present in urine. While SPR was able to detect APF/as-APF binding to the CKAP4 127-360 biosensor, binding kinetics for the interaction(s) could not be established; thus, APF/as-APF concentration could not be determined by this method (with detection limits in the high nanomolar to micromolar range). With further refinement of APF enrichment techniques, the sensitivity of the assay could be increased and this obstacle overcome.

The observation that APF activity is detectable in urine specimens from 47% of asymptomatic control women by the cellular proliferation assay and 73% of asymptomatic control women by the SPR binding assay, suggests that other urinary constituents may be contributing to the measured response. To clarify the specific contribution of APF and/or as-APF for the biological activities measured in urine specimens by these two methods, a targeted mass spectrometry (MS) method was used. This chemical method was able to detect and quantitate APF and as-APF in a urine specimen by generating specific signature peaks for each peptide, while separating the interference from other urinary biomolecules (Fig. 5). We confirmed that both APF and as-APF were present in all urine samples regardless of patient diagnosis (ie, control vs. IC), suggesting that both peptides may exist at basal levels in normal bladder physiology. While the quantities of both peptides were very low and varied among patient samples, as-APF was detected more sensitively. Further, based on normalization against the standard curve, as-APF concentration levels were consistently higher than APF in all urine samples tested (Fig. 8 & 9). Based on the standard curve, 60% of samples were calculated to have as-APF from 0 nM (sample 14) to 0.2 nM (sample 42).
To calculate concentrations for other samples, standard curves with even lower concentrations (down to 0.001 nM) need to be repeated and will be accomplished within the next few months. At that point in time, a full statistical analysis of the data can be completed. Overall, our data demonstrate that APF and as-APF exist in pM to nM range in original urine samples. The concentrations of sample 14 to 45 are consistent with their APF levels, the rest of samples are not consistent. Correlativity between the MS data and the SPR and cellular proliferation assays is problematic, as both depend on APF/as-APF biological activity and have much higher interference from other urinary constituents (ie, biomolecules).

**Provided Opportunities for Training and Professional Development**

Several abstracts were presented throughout the 3-year contract period (see 6b under products). Two, in particular, resulted in professional development opportunities for the post-doctoral fellow supported by this project, Burzin Chavda, PhD, who presented our findings at two national conferences (American Society for Molecular Biology and Biochemistry Annual Meetings). We also submitted an abstract, which was accepted as a poster for the 2015 Military Health System Research Symposium (MHSRS) on 01-Jun-2015. Dr. Planey attended the MHSRS conference on August 17-20 and presented the team’s findings.

An important training activity that stemmed from this project was the development of Dr. Chavda’s skills with surface plasmon resonance technology and the Biacore platform. The expertise he developed in this technology as a direct result of this project was used to assist others in the department of basic sciences to attain proficiency in this technology, holding multiple one-on-one training sessions with faculty and students. Further, his acquired proficiency has enabled him to secure a position at the California Institute for Biomedical Research in San Diego, CA where he will be further utilizing his expertise in this area.

**Dissemination of Results to Communities of Interest**

In year one of the contract period, I organized the Interstitial Cystitis Symposium at the Commonwealth Medical College which included the following speakers: Robert J. Echenberg, MD, FACOG; Diana Pope-Albright, PT, DPT, MS; Sonia Lobo Planey, PhD and three IC patients who shared their experience. One of those patients was a local high school student who was intimately involved in the planning. We organized the event in an effort to raise awareness of this disease in Northeastern, Pennsylvania, which resulted in a regional outreach/support group for patients with IC. I presented my research to an audience of patients, medical students, and health care providers at the event, which was held on 22-Feb-2014.

Results of our work have also been disseminated at TCMC’s annual Spring Research Symposium, Faculty Research Symposium, Summer Research Immersion Program, and Pennsylvania Junior Academy of Science/TCMC Fellowship Program, through medical student, undergraduate student, and high school student participation in specific aspects of this research project over the course of the three-year contract period.

**Plan for Accomplishing Goals Next Reporting Period**

Nothing to Report

4. **IMPACT:**

**Impact on the Development of the Principal Discipline(s) of the Project**

The biophysical interaction of APF with its cellular receptor, CKAP4, has not been previously characterized. The results of our current study demonstrate direct binding of CKAP4 to APF and/or as-APF with real time kinetics, providing an important tool to characterize how the two interact specifically to elicit APF’s pathological effects. As shown in Figure 1 and Table 1 of this report, regions of the CKAP4 C-terminal, extracellular domain are required for binding APF and as-APF. Table 1 lists the parameters of the binding kinetics for the APF interaction with the CKAP4 extracellular domain mutants using a 1:1 binding kinetics model. We determined that the CKAP4_{127-360} and CKAP4_{361-524} mutants exhibit improved binding activity to APF as compared to the full-length extracellular domain, making it
possible to detect low concentrations of as-APF in urine, thereby establishing a foundation for a non-invasive diagnostic assay for IC. Further, these data have revealed novel APF binding site(s) suggesting that targeting this region of CKAP4 to inhibit APF binding may be a useful strategy for treating IC-related bladder pathology.

Since we were able to obtain real-time binding kinetics for APF binding to CKAP4 by SPR, we sought to determine the suitability of CKAP4_{127-360} to quantitate APF in urine. Our results show that immobilized CKAP4_{127-360} can detect APF charged in normal urine in a dose-dependent manner in the high nanomolar to micromolar range. However, the concentrations of APF and as-APF peptide in patient urine specimens as determined by targeted MS, revealed that both peptides exist at levels in the pM to nM range, well below the established detection limit of our current SPR assay. Thus, we could not determine APF and/or as-APF concentrations from our SPR binding response sensorgrams. With further refinement of the CKAP4 biosensor (making it smaller to magnify the binding response of APF analyte) or improvement of the APF enrichment strategy to enhance sensitivity, this label-free technique employing CKAP4 as a biosensor to detect and measure APF in urine has potential as a suitable approach for IC diagnosis.

One very unique aspect of our findings from this project was that as-APF, the desialylated APF analogue, showed greater CKAP4 biosensor binding activity than APF in all our assays, across various technology platforms. While the quantities of both peptides were very low and varied among patient urine samples, as-APF was detected more sensitively. Further, based on targeted MS with normalization against the standard curve, as-APF concentration levels were consistently higher than APF in all urine samples tested. APF is a nonapeptide containing a 2,3-sialylated core 1 \( \alpha \)-linked disaccharide linked to the N-terminal threonine residue (Neu5Ac \( \alpha \) 2–3Gal \( \beta \) 1–3GalNAc \( \alpha \) –O– TVPAAVVVA). Earlier APF studies, supported in part by our data, have shown that the APF saccharide moiety is an absolute requirement for biological activity, and that such activity does not depend on the presence of the terminal sialic acid. Thus, it is important to note that such structural constraints required for APF’s biological activity are also required for its interaction with CKAP4, which provides further implication of CKAP4’s role as a cellular receptor for APF.

Indications that as-APF has greater binding affinity than APF for CKAP4, and that most structural and biochemical studies to date have focused solely on as-APF give credence to an earlier suggestion that APF may be a precursor of as-APF, perhaps resulting by the action of an intracellular neuraminidase.\(^{11,12}\) A recently published structural study has revealed the active APF conformation using as-APF.\(^{13}\) It has shown that hydrophobic folding within the nine amino acids (TVPAAVVVA), further stabilized by interaction with the saccharide moiety, provides a collapsed conformation for optimal biological function. Thus, addition of the terminal sialic acid moiety can destabilize the hydrophobic core or disturb the conformation to linear form direction, thereby resulting in lower molecular binding activity to CKAP4. Whether or not as-APF is a naturally occurring, active form of APF in pathological conditions such as IC requires further verification. Based on data generated by targeted MS in this study, all urine specimens contained both full length APF and as-APF in pM to nM range regardless of patient diagnosis (ie, control vs. IC). Importantly, this finding suggests that APF and as-APF may exist at basal levels in normal bladder physiology, but contribute to IC pathology/symptomology once a certain threshold level is reached or rather when a specific form predominates. Alternatively, pathogenic variants of CKAP4 may exist and contribute to the pathologic effects of APF and/or as-APF. Future studies will be necessary to determine the significance of this unexpected finding.

What was the Impact on other Discipline(s)
We expect that with additional refinements, the SPR-based assay will overcome current barriers associated with validation of APF as a diagnostic biomarker for IC by being able to specifically detect and measure APF levels in urine. Thus, this assay represents a tangible product that could be developed into an affirmative diagnostic test for IC with the advantages of being rapid, repeatable, and non-invasive. This would potentially impact clinical practice by obviating the need for more costly invasive procedures such as cystoscopy and hydrodistension and curtail rates of misdiagnosis or
improper treatment of patients who have symptomological overlap with IC. Accurate and earlier diagnosis would improve patient care and quality of life leading to better management of comorbidities and reduce healthcare costs.

Further, the findings of this study represent a substantive departure from the status quo by shifting focus to a mechanistic understanding of how CKAP4 regulates the biological activity of APF and/or as-APF using surface plasmon resonance to define their biophysical interactions and to identify the specific binding region that constitutes a potential target for APF inhibition. Future studies aimed at determining how CKAP4 manifests APF’s pathological effect in IC, may open new therapeutic horizons that have previously been unattainable with other biological markers and could lead to the development of therapeutic agents for IC that specifically bind to APF or CKAP4 to block its effects on the bladder epithelium.

**What was the Impact on Technology Transfer**
Throughout the three-year contract period, two non-provisional patents were issued (see Inventions, Patent Applications, and/or Licenses); however, to date there has been no institutional effort to identify corporate partners to further develop and/or commercialize and license this technology.

**What was the Impact on Society Beyond Science and Technology**
Nothing to Report

5. **CHANGES/PROBLEMS:**

**Changes in Approach and Reasons for Change**
One problem that we encountered with development of the SPR assay was interference from other urinary constituents in urine. Because the SPR-based assay (like the cellular proliferation assay) depends on the biological activity of APF, other biomolecules present in urine can presumably interfere with the measured response. In an attempt to minimize this problem, we established a combined chromatography method that allowed us to enrich for APF peptide while minimizing background interference from other urinary constituents. However, we observed multiple forms of APF in these charged, control urine samples (intact, as-APF and further deglycosylated APF). This raised a question about which APF forms are predominant in IC patient urine. Our SPR method can detect both APF and as-APF in charged urine samples; however, it may not detect APF containing only on sugar moiety. To address this issue, we used a targeted mass spectrometry (MS) method to identify and quantitate the predominant APF form in IC patient urine samples following testing by SPR. This turned out to be an important change/addition to our original protocol, because the method enabled us to clarify the specific contribution of APF and/or as-APF for the biological activities measured in urine specimens by SPR and to quantitate APF and as-APF (at levels below detection by SPR).

Another important change was that we could not meet our goal of testing 90 urine specimens due to an insufficient volume of banked, frozen urine that was determined to be required for testing each specimen by both the cellular proliferation assay and the SPR-based assay. Thus, for the purpose of comparing assay results, only the 60 fresh urine specimens were tested.

**Actual or Anticipated Problems or Delays and Actions or Plans to Resolve Them**
During the three-year contract period, we encountered several problems that were mostly resolved. The first problem we encountered was with our supply of synthetic APF peptide, which we used for characterization of the SPR-based assay. We used a new vendor to synthesize the peptide; however, we observed differences in its binding characteristics by SPR. To resolve the issue, we contacted our original supplier to provide additional APF peptide. Despite a delay, they were able to synthesize more peptide for us. We also ordered the peptide more regularly and in larger quantities, so as to avoid running out of stock.

Another problem that we encountered with development of the SPR assay was interference from other urinary constituents in urine. Because the SPR-based assay (like the cellular proliferation
(assay) depends on the biological activity of APF, other biomolecules present in urine can presumably interfere with the measured response. In an attempt to minimize this problem, we established a combined chromatography method that allowed us to enrich for APF peptide while minimizing background interference from other urinary constituents. However, we observed multiple forms of APF in these charged, control urine samples (intact, as-APF and further deglycosylated APF). This raised a question about which APF forms are predominant in IC patient urine. Our SPR method can detect both APF and as-APF in charged urine samples; however, it may not detect APF containing only one sugar moiety. To address this issue, we used a targeted mass spectrometry (MS) method to identify and quantitate the predominant APF form in IC patient urine samples following testing by SPR. This turned out to be an important change to our original protocol, because the method enabled us to clarify the specific contribution of APF and/or as-APF for the biological activities measured in urine specimens by SPR and to quantitate APF and as-APF (at levels below detection by SPR).

Because TCMC does not have a mass spectrometry facility on-site, we had to rely on two outside core facilities at Penn State University and Rutgers to complete the targeted MS studies. We experienced significant delays working with the Penn State facility to obtain our results in year 3. Towards the end of the contract period, we engaged the Rutgers facility and worked exclusively with them to complete the MS testing, which was completed in mid September. The delays in working with the outside facilities did not leave sufficient time to interpret all of the MS results, particularly in light of the unexpected finding that both APF and as-APF peptides were present in all of the urine samples irrespective of diagnosis. Continued analysis of these data is ongoing in the context of the cellular proliferation and SPR assay results and could not be completed within the contract period.

Changes that had a significant impact on expenditures
Nothing to report

Significant Changes in Use or Care of Human Subjects
Nothing to report

6. PRODUCTS:

Publications, Conference Papers, and Presentations

a. Journal Publications:

b. Abstracts/Presentations:
   • Interstitial Cystitis Symposium – The Commonwealth Medical College; Speakers: Robert J. Echenberg, MD, FACOG; Diana Pope-Albright, PT, DPT, MS; Sonia Lobo Planey, PhD. I organized this regional event and presented my research to an audience of patients, medical students, and health care providers in an effort to raise awareness of this disease in Northeastern, Pennsylvania on 22-Feb-2014.
   • “Investigating cytoskeleton-associated protein 4 variants for optimal binding to antiproliferative factor by surface plasmon resonance” and authored by Chavda B, Ling J, Planey SL. Seventh Annual Northeastern Pennsylvania Faculty Symposium, The Academic Advisory Council of The Institute for Public Policy & Economic Development, Kings College was presented on 13-Apr-2014.
   • “Characterization of the binding of antiproliferative factor to cytoskeleton associated protein 4 by surface plasmon resonance” and authored by Chavda B, Ling J, and Planey SL was presented at the American Society for Molecular Biology and Biochemistry Annual Meeting in San Diego, CA on 26-Apr-2014.
   • “Development of a surface plasmon resonance-based assay to detect antiproliferative factor in interstitial cystitis patient urine” and authored by Chavda B, Ling J, and Planey SL was
presented at the American Society for Molecular Biology and Biochemistry Annual Meeting in Boston, MA on 29-Mar-2015.

- “Development of a surface plasmon resonance-based assay to detect antiproliferative factor in interstitial cystitis patient urine” and authored by Chavda B, Majernick T, Ling J, and Planey SL was accepted as a poster for the 2015 Military Health System Research Symposium (MHSRS) in Ft. Lauderdale, Florida and presented on 18-Aug-2015.

Website(s) or other Internet site(s)
Nothing to Report

Technologies or Techniques
Nothing to Report

Inventions, Patent Applications, and/or Licenses
Non-provisional Patents:


Other Products
An alternative approach taken to increase the sensitivity of the SPR assay has resulted in the production of five purified monoclonal antibodies that display specific activity against APF-KLH in a dot blot assay and specific binding to the active form of APF by the SPR assay. HL-2411, in particular, displays specific and strong binding against KLH-APF and as-APF, but not APF lacking its sugar moieties. The four remaining monoclonal antibodies—HL-2408, HL-2409a, HL-2409b, and HL-2412—also display specific binding against APF, as-APF, and KLH-APF albeit with varying sensitivities. Using a beads based approach followed by liquid chromatography/mass spectroscopy (LC-MS) for APF identification, we determined that all of the antibodies were able to capture APF, although HL-2411 bound APF most robustly, resulting in an approximately 10-fold increase in the amount of APF captured. This antibody also generated positive Western blot bands against both APF and as-APF peptides using Simple Western technology (Protein Simple), indicating that it is specific enough to detect APF. Thus, we determined that the HL-2411 monoclonal antibody shows great promise for further development as a diagnostic tool in an antibody-based immunoassay for APF detection.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS:

Participating Individuals

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<td>Thomas Majernick, MS</td>
<td>Research Technician</td>
<td>No change since previous quarterly report</td>
<td>100</td>
</tr>
</tbody>
</table>
Changes in Active Other Support of the PD/PI(s) or Senior/Key Personnel Since the Last Reporting Period

A slight reduction in support was made for Dr. Ling and Betsy Meade in year 3 to reflect their tapering role in the project as patient recruitment neared completion.

Partnering Organizations

Organization Name: Hospital of the University of Pennsylvania
Location of Organization: Philadelphia, Pennsylvania
Partner’s contribution to the project: Facilities and collaboration.

We are collaborating with Dr. Phillip Hanno and his health care team to recruit IC patients for this study during routine office visits to the Penn Urology Clinic at HUP for the management of IC as well as with the retrieval of patient information for use in future correlation analysis.

Organization Name: University of Maryland
Location of Organization: Baltimore, Maryland
Partner’s contribution to the project: Facilities and collaboration.

We are collaborating with Dr. Keay and individuals in her laboratory to blindly test biological urine specimens by the cellular proliferation assay. Dr. Keay will send her results to us for comparison with the SPR assay results and will assist in the interpretation of the final analysis.

8. SPECIAL REPORTING REQUIREMENTS:
Quad Chart

9. APPENDICES:

References:

Validation of APF as a Urinary Biomarker for Interstitial Cystitis
PR121048 - Investigator-Initiated Research Award
W81XWH-13-1-0454

PI: Sonia Lobo Planey, Ph.D. Org: The Commonwealth Medical College
Award Amount: $945,142.00

Study Aim(s)
Aim 1: To develop and characterize a SPR-based assay employing a CKAP4 immobilized biosensor to detect APF.
Aim 2: Determine the ability of the SPR-based assay to detect APF in urine from patients with IC.

Approach
To accomplish Aim 1 we will optimize rCKAP4 activity and sensor chip immobilization to achieve maximal CKAP4/APF binding efficiency and reproducibility. We will then determine linearity and range of the assay using known concentrations of synthetic APF spiked in urine from healthy donors. In Aim 2 we will determine the ability of the SPR-based assay to detect and quantitate APF in urine samples from patients with IC (45) versus non-IC, age-matched controls (45). Sensitivity and specificity of the SPR assay to detect APF will be determined by comparison with cellular proliferation assay results obtained by Dr. Keay. Statistical analysis will be performed to assess APF’s utility as a diagnostic and/or prognostic biomarker for IC.

Goals/Milestones

<table>
<thead>
<tr>
<th>CY 13 Goal</th>
<th>CY 14 Goal</th>
<th>CY 15 Goal</th>
<th>CY 16 Goal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPR assay development</td>
<td>SPR assay characterization</td>
<td>Improved CKAP4/APF binding efficiency and reproducibility</td>
<td>Characterization of 20 samples using synthetic APF</td>
</tr>
<tr>
<td>Optimization of rCKAP4 activity and immobilization</td>
<td>Improvement of CKAP4/APF binding efficiency and reproducibility</td>
<td>Testing for APF activity by cellular proliferation assay</td>
<td>First measurement of APF and as-APF in human urine</td>
</tr>
<tr>
<td>Testing for APF by SPR-assay underway</td>
<td>Characterization of 90 biological specimens</td>
<td>Testing for APF by SPR-assay underway</td>
<td>Testing APF’s utility as a diagnostic biomarker for IC</td>
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<tr>
<td></td>
<td>Statistical analysis and comparison of assay results</td>
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</table>

Comments/Challenges/Issues/Concerns
- Statistical analysis not completed and on going

Budget Expenditure to Date
Projected Expenditure: $945,142
Actual Expenditure: $929,688

Timeline and Cost

<table>
<thead>
<tr>
<th>Activities</th>
<th>CY 13</th>
<th>CY 14</th>
<th>CY 15</th>
<th>CY 16</th>
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</thead>
<tbody>
<tr>
<td>Task 1a-b. Improved CKAP4/APF binding efficiency and reproducibility</td>
<td>$50</td>
<td>$323</td>
<td>$313</td>
<td>$259</td>
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<tr>
<td>Task 1c. Characterization of SPR-based assay using synthetic APF</td>
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<tr>
<td>Task 2a-b. Subject recruitment and acquisition of 90 urine specimens</td>
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<tr>
<td>Task 2c-e. Sample testing by SPR assay and validation of APF</td>
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Estimated Budget ($K) | $50 | $323 | $313 | $259 |

Updated: (December, 2016)