Supplemental Methods

Animals and animal models
Eight-week old CD-1 mice, strain 022, were purchased from Charles River and housed in the NIDDK animal facility. We followed National Institutes of Health guidelines for the use and treatment of laboratory animals and the institute animal care and use committee approved all procedures. All animals had free access to water and chow, except during the 12-hour period of the furosemide stress test, when chow was removed.

Cecal ligation and puncture was performed to induce sepsis, largely as previously described (1). Briefly, under isoflurane anesthesia a midline incision was made, and the cecum was located and removed from the peritoneal cavity. The cecal contents were palpated to the tip and the distal 15 mm, filled with contents, were ligated with 4-0 silk suture. A 21 g needle was then passed through the ligated cecum allowing the contents to leak out through two holes. The cecum was returned to the peritoneal cavity and the incision closed. All mice received a slow release formulation of buprenorphine (SR Veterinary Technologies, Windsor, CO) subcutaneously for analgesia during CLP surgery and every 72 hours thereafter. Fluids (40 mL/kg) were given intraperitoneally at the time of surgery (0.9% saline) and then subcutaneously at 6 hours, and every 12 hours thereafter (0.6% saline). Antibiotics were given with fluids starting at 6 hours (14 mg/kg Primaxin [imipenem and cilastatin, Merck & Co, Whitehouse Station, NJ] to start and then 7 mg/kg subsequently).

Monitoring & Euthanasia
In survival studies all mice were monitored at least every 6 hours from 18 to 72 hours, and then every 8 hours until 7 days, when the study was concluded. Mice were scored on their respiratory pattern, activity and general appearance and euthanized if they exceeded a threshold value.

In acute studies, mice were euthanized immediately after the completion of the FST at 54 hours. Serum was collected by cardiac puncture and kidney tissue stored in formalin or RNAlater.
**FST & FEST**

At 42 hours mice were given 1 mg/kg furosemide with their scheduled fluids administered via subcutaneous injection and moved to individual metabolic cages (Hattaras Instruments, Cary, NC) for a 12 hour urine collection. Mice had free access to water, but chow was withheld during this period.

**Vasopressin Treatment**

The performance of FST and FEST was assessed in a separate experiment with vasopressin administered to mice. At the time of CLP surgery, a micro-osmotic pump (Durect, Cupertino, CA) was implanted in the peritoneal space loaded with either water or vasopressin (Sigma Aldrich, St. Louis, MO) scaled from a dose equivalent to 0.08 U/min in humans (2). The dose assumed a 70 kg human and a 35 g mouse for 0.0024 U/hr. A 1003D pump was used with a nominal infusion rate of 1 ul/hr. Using the manufacturer supplied formula this was adjusted to 0.68 ul/hr based on a predicted body temperature of 30 °C due to sepsis-induced hypothermia.

**Furosemide quantification**

Urinary furosemide concentration was determined using a custom HPLC assay. An 8 µl sample of urine was mixed with 160 µl acetonitrile to precipitate proteins. After centrifugation (13,000 x g for 15 min at 4 °C) the supernatant was removed, dried by Speedvac (Thermo Savant, Holbrook, NY), and redissolved in 192 µl mobile phase. Measurement was performed in duplicate with a 25 µl injection volume. The mobile phase was 70% 20 mM potassium phosphate pH 4.5 / 30% acetonitrile. The HPLC (1100 system, Agilent Technologies, Palo Alto, CA) assay was optimized for a 50 mm PRP-C18 column (Hamilton, Reno, NV) with a 10 minute isocratic elution and detection by absorbance at 335 nm, with a retention time of approximately 2.6 minutes.

**Blood chemistries**

Serum BUN, phosphorus, glucose, lactate dehydrogenase, creatine kinase, amylase, alkaline phosphatase, aspartate transaminase, and alanine transaminase were measured using an autoanalyzer (Hitachi 917, Boehringer Mannheim, Indianapolis, IN).
Gene expression

At euthanasia, mouse kidney tissue was collected and preserved in RNAlater overnight and then stored at -80 °C until processing. RNA was extracted using the Qiagen RNeasy kit following the manufacturer’s instructions. Taqman reverse transcription reagents (Thermo Fisher, Livermore, CA) were used to prepare cDNA and then gene expression was quantified using the following Taqman assays; OAT1 Mm00456258_m1, OAT3 Mm00459534_m1, NKCC2 Mm01275821_m1, IL10 Mm00439614_m1, IL6 Mm00439614_m1, IL1β Mm00434228_m1, and TNFα Mm00443258_m1. All gene expression was normalized against 18S rRNA expression.

Statistics and data analysis

The diagnostic ability of FST and FEST was assessed by receiver operating characteristic curves and levels in the mice that survived compared to those that did not were compared by t-test. Associations between measured values were analyzed by linear regression. Gene expression was visualized as log 2 fold change from the average expression. Fisher’s exact test was used to investigate the validity of applying FST and FEST cut-off values to the vasopressin treatment experiment. Prism 7 (GraphPad Software, La Jolla, CA) was used for all statistical tests.

References

Supplementary Figure 1: Consistent recovery in a mouse model requires a 12 hour collection.
Supplementary Figure 2: Correlation of FST and FEST with serum organ injury markers
Supplementary Figure 3: FST and FEST performance in female mice following CLP surgery

FST

A

Urine volume (ml)

Survived
Died

0
1
2
3

B

Sensitivity%

100% - Specificity%

AUC = 0.948
p = 0.0004

C

Time of death (hr)

FST (ml)

R^2 = 0.152
p = 0.3001

FEST

D

Furosemide excretion %

Survived
Died

0
20
40
60
80
100

E

Sensitivity%

100% - Specificity%

AUC = 1.0
p = 0.0001

F

Time of death (hr)

Furosemide excretion %

R^2 = 0.233
p = 0.2259
Supplementary Figure 4: Correlation between FST and FEST

- Survived female
- Died female
- Survived male
- Died male

$R^2 = 0.662$

$p < 0.0001$
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**Supplementary Table 1**: Comparison of predicted outcomes from applying the optimal cut-offs from the male and female combined group to the vasopressin experiment. TN: True Negatives, survival correctly predicted. TP: True Positives, mortality correctly predicted. FN: False Negatives, survival incorrectly predicted. FP: False Positives, mortality incorrectly predicted.
**Supplementary Figure Legends**

**Supplementary Figure 1** Furosemide excretion in the 12 hours following bolus administration in individual healthy (non-CLP) mice. Urine was collected in the periods 0-6 hours and 6-12 hours following administration and the fraction of furosemide recovered in each period expressed as a percentage of the total recovered.

**Supplementary Figure 2** Performance of FST and FEST during sepsis in female mice. FST: A) Comparison of urine volume following furosemide bolus in mice that survived and died. B) Receiver operator characteristic curve for FST. C) Plot of urine volume against time of death. FEST: D) Comparison of furosemide excretion following furosemide bolus in mice that survived and died. E) Receiver operator characteristic curve for FEST. F) Plot of furosemide excretion against time of death.

**Supplementary Figure 3** Comparison of furosemide-induced urine volume with furosemide excretion in both male and female septic mice.