INTRODUCTION

We have evaluated the signaling of the SOMAscan™ Assay by investigating mouse plasma, rat plasma and cerebrospinal fluid (CSF), and cat and dog serum and plasma by the methods described within this Technical Note. Many individual SOMAmer® reagents used in the SOMAscan™ Assay generated to pure human proteins cross-react with non-human orthologs and therefore can be used in the SOMAscan™ Assay to identify differential expression of these analytes in non-human samples. SOMAscan™ AssayFirst, the samples were assayed by dilution linearity assessment. Second, the signaling analytes were assessed by calculating the F-statistic (F-stat) of the ratio of population variance to assay variance for each measurement. The population variance for "real" measurements must be greater than assay noise; non-signaling analytes are expected to have population variances similar to assay variance since both are measuring noise. Our results suggest that the SOMAscan™ Assay can measure hundreds of proteins reproducibly and reliably in small sample volumes from these species. Due to the high degree of homology between primates (1) we have noted that the majority of SOMAscan™ reagents will signal in samples from non-human primates. Estimates of differential protein regulation in the SOMAscan™ Assay in non-human species have already led to novel discoveries (2) indicating the SOMAscan™ Assay can offer powerful, hypothesis-free biomarker discovery for many applications from preclinical models in drug discovery to animal health in veterinary sciences.

DATA/RESULTS

SOMAscan™ Assay cross-reactivity to mouse proteins

The ability of the SOMAscan™ Assay to measure non-human species has been evaluated by different methods. The mouse is the best studied non-primate species at this time because of its high utility in life sciences research and the corresponding availability of purified proteins. Two hundred and thirty-nine (239) purified mouse proteins were tested for cross- reactivity to SOMAmer® reagents derived to human proteins. The recombinant murine proteins were pooled, serially titrated from 10 nM to 30 fM, and profiled in the SOMAscan™ Assay in buffer. Eighty (80) of the SOMAmer® reagents passed evaluation using criteria of RFU values > 35,000 RFU at 10 nM and apparent K_D values < 1 nM, for a 33% (80/240) pass rate. Representative plots for SOMAmer® reagents that passed are shown in Figure 1. The apparent dissociation constants (K_D-apparent) ranged from 0.8 to 500 pM for SOMAmer reagents that passed.

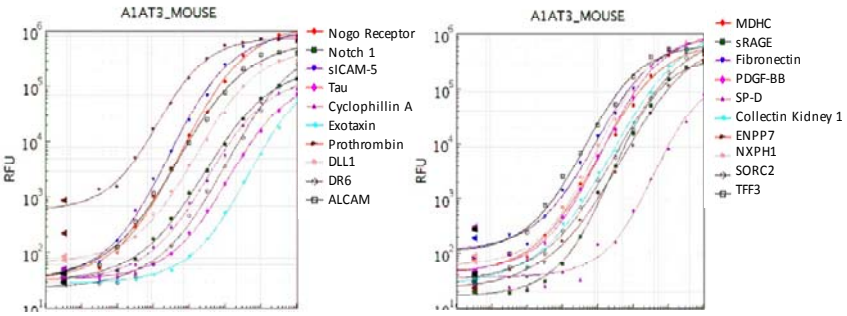

Figure 1. Buffer dose-response curves of human-derived SOMAmer reagents binding to murine orthologs.
Representative plots of the 80 mouse proteins that passed cross-reactivity criteria are shown.
Plotted are SOMAscan™ Assay measurements in relative fluorescent units (RFU) of mouse proteins titrated from 10 nM to 30 fM in buffer. All 239 mouse proteins were pooled and run against the 1129 SOMAmer® reagents in the SOMAscan™ Assay 1.3k.

The degree of homology was compared between these 239 mouse and human orthologs to see if there was a relationship between pass rate and homology. The homology of these 239 orthologs was calculated applying the Smith- Waterman algorithm to the mouse sequence that gave the highest percent identity to the human sequence, even though sometimes this meant that the annotated names were not the same (i.e., the names were given before the genomes were sequenced). The 80 mouse proteins that cross-reacted to the human-derived SOMAmer® reagents had a higher average percent identity to human sequence than the 160 mouse proteins that did not pass, 84% versus 75% average identity (p-value < 1.6e-11) (Figure 2A). Although this

means that the likelihood of cross-reactivity increases with increasing percent amino acid identity, it is important to keep in mind that some SOMAmer® reagents cross-reacted to murine proteins with less than 60% amino acid identity to the human ortholog (Figure 2A), perhaps suggesting that binding epitopes may be present with higher similarity than the overall protein. To ascertain if the extrapolation to the entire SOMAscan™ menu is valid we tested whether the 239 proteins selected for analysis were skewed towards greater similarity to the human ortholog than the remaining proteins. The analysis showed that, in fact, the opposite was true, the median
percent identity for the untested 889 proteins was 86.1% versus 78.1% for the 239 tested proteins (p-value = 2.6e-8)(Figure 2B). In addition, 70 of the untested mouse proteins were shown to have >99% identity to human proteins on the menu. Together these data suggest that, if one extrapolated to the entire menu, it is not unreasonable to expect that one-third (33% of the 1129 SOMAmer® reagents) of the SOMAmer® reagents in the SOMAscan™ Assay could signal reliably.

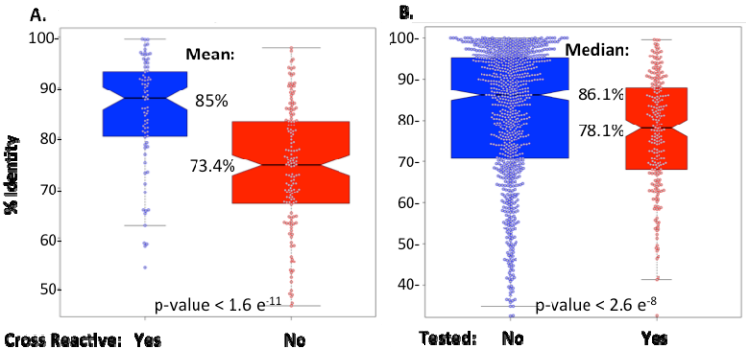

Figure 2. Percent identity of mouse and human orthologs as a function of cross-reactivity to human- derived SOMAmer® reagents or testing. (A) The % identity of the 239 mouse proteins that were tested for ability to cross-react to human-derived SOMAmer® reagents described in Fig. 1 was calculated aligning to the most closely related human ortholog using the Smith-Waterman algorithm. The mean % identity between those that cross-reacted (85%) to those that did not (73.4%) was significantly different (p-value < $1.6e^{-11}$). (B) The median % identity between mouse and human orthologs that were tested (78.1%) or not tested (86.1%) was significantly different (p-value <$2.6e^{-8}$).

Mouse Models

Two notable mouse-only models have been run in the SOMAscan™ Assay with excellent results. In one study profiling young and old mice, 13 analytes were found which reliably distinguished young from old mice, p-value < 1.8e-5 (2). In total, 122 analytes were different between young and old mice with false discovery rate (FDR) cutoff of 0.2. Many analytes had biological plausibility such as GDF-11 (2); for some the relevance to aging is novel at this time. In another mouse study, plasma from mice with genetic mutations linked to dysfunctional socialization was profiled in the SOMAscan™ Assay. Seventeen proteins were found to be significantly different among all 6 groups with an FDR cutoff of 5% (q-value < 0.05). Out of all pair-wise comparisons 62 analytes were statistically significant, many of which were biologically relevant. In both studies, analytes reflective of sample handling were evident which permitted the exclusion of poorly handled samples, an important component of biomarker discovery efforts (3).

The SOMAscan™ Assay has also been used to detect differential expression in drug-treated, preclinical xenograft models. In one study plasma was obtained from mice implanted with either a pancreatic or breast human xenograft before and after monoclonal antibody therapeutic treatment (unpublished data). Two hundred and fifty (250) proteins changed in response to xenograft alone, likely a combination of proteins that come from the human xenograft and the mouse response to xenograft. Many of these proteins decreased upon drug treatment correlating with decreasing tumor size. In another example, tumor tissue from mouse xenografts were profiled in the SOMAscan™ Assay, and while over 250 analytes changed significantly, these were human grafts and therefore the analytes are presumed to be human (4). These examples demonstrate the power of applying the human-derived SOMAmer reagents in the SOMAscan™ Assay to other species, exposing deeper biological knowledge in preclinical models.

Profiling Plasma from non-Human samples in the SOMAscan™ Assay

Rat: To simulate an outbred population, a small sample set of EDTA-plasma from rats of different strains were

evaluated using SOMAscan™ to estimate the number of signaling analytes. For the rat assay, 14 rat samples were obtained from 14 different rats of different strains, ages 6 weeks to 4 months, from equal gender (7 males, 7 females) distributions. The samples were assayed as a single pool of all samples. The number of analytes found to be signaling in rat plasma was determined to be 495 based on F-Stat >106.5, a FDR-corrected 95% confidence cutoff for 1129 measurements.

Mouse: Mouse EDTA-plasma was collected in 19 different pools of plasma collected from 15 different strains of mice ranging in age from 5 weeks to 15 weeks including 15 females and 4 males. The samples were assayed as a single pool of all samples. The number of analytes found to be signaling in mouse plasma was found to be 643 based on F-Stat >15.55, a FDR-corrected 95% confidence cutoff for 1129 measurements.

Dog: EDTA-plasma samples were harvested from dogs and evaluated in the SOMAscan™ Assay to estimate the number of signaling analytes. In this experiment, EDTA-plasma was obtained from 8 purebred beagles ranging from 3 – 5 years of age. The samples were evaluated for signaling analytes and reproducibility by running 9 replicates over 3 different assay runs. The number of analytes found to be signaling in this limited population of animals was determined to be 294 based on F-Stat > 29, a FDR corrected 95% confidence cutoff for 1129 measurements. The %CV for the SOMAmer reagents binding to analytes in dog plasma was excellent with median total CV of 3.9%.

Cat: EDTA-plasma was harvested from 8 different domestic short hair cats ranging between 8 – 22 months, with equal gender representation. The samples were evaluated for signaling analytes and reproducibility by running 9 replicates over 3 different assay runs. The number of analytes found to be signaling in this population of animals was 687 based on F-Stat > 29, a FDR corrected 95% confidence cutoff for 1129 measurements. The %CV for the SOMAmer reagents binding to analytes in cat plasma was excellent with median total CV of 4.4%.

These results suggest the human-derived SOMAscan™ Assay may have powerful utility in preclinical development and veterinary sciences.

Profiling rat CSF in the SOMAscan™ Assay

Cerebrospinal fluid was obtained from 6 rats and assayed individually to determine population variance. Six replicates of a calibrator sample (pooled sample of rat CSF) were also assayed to determine intra-assay variance. Signaling analytes were assessed as described above. The number of analytes found to be signaling in this limited population of rats was based on F-Stat $\geq$ 5.05, a FDR corrected 95%confidence cutoff for 1128 measurements and determined to be 402 for CSF.

Sample Volumes Requirements

Sample volume requirements for EDTA-plasma and serum from tested species are listed in Table 2. Due to the high degree of protein homology between primates we assume the majority of SOMAmer reagents derived to human proteins will exhibit high cross-reactivity to non-human primates and this has borne out in preliminary experiments with samples from these animals. Experience shows that the concentrations of proteins are different in different matrices and species and this is reflected in the smaller sample volume requirements from monkeys as compared to humans (Table 2). We are continually investigating new sample types and are open to discuss developing additional sample types with collaborators. Specific protocols for various sample preparations are available (5).

Table 2. Species Tested and Sample Volumes Required

Species	Requested Volume* (uL)
Human	150
Mouse (serum/plasma)	70
Rat (serum/plasma)	50
Non-human primate (serum/plasma)	120
Dog (serum/plasma)	90

Cat (serum/plasma)	90
Rat CSF	150

**Lower volumes are feasible when availability is limited.*

Verification

Once the SOMAscan™ Assay has been used to analyze non-primate samples, it is recommended to verify the identity of analytes of interest in an identified protein signature. The two methods suggested here depend on the availability of the orthologous protein and related detection reagents. The easiest method is to purchase or generate the purified protein and run it in a single analyte buffer titration SOMAscan™ Assay. If an ELISA is available one can compare ELISA results to the SOMAscan™ Assay, keeping in mind that the epitope recognized in an ELISA may not be the same as that recognized by the SOMAmer reagent. If an ELISA is not available, the SOMAmer reagent can be used to capture purified protein, if available, or if protein is at high endogenous levels, affinity capture may be achieved directly from matrix. After affinity capture from matrix the identity of the protein can be verified by peptide mass fingerprinting. The most definitive method would be to perform a pull down using sds-page gels followed by identification of the protein via mass spectrometry.

CONCLUSION

In conclusion, the SOMAscan™ Assay can reproducibly and reliably measure hundreds of proteins in small sample volumes from mice, rats, cats and dogs better than any available alternative; genetically similar non-human primates appear to cross-react even more completely. Using the SOMAscan™ Assay to measure the proteome of non-human species has already led to novel discoveries (2) indicating the SOMAscan™ Assay can offer powerful, hypothesis-free protein biomarker discoveries leading to deeper biological understanding of these species. Utilizing the same reagents in multiple species and in *in vitro* studies, creates an opportunity for a single translational platform, minimizing the technical risk of moving between different platforms.

REFERENCES

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(3) Williams S, *et al.* (2012). Exposing the criminal record of every blood sample: use of SOMAmer technology and sample mapping vectors to mitigate false biomarker discoveries. Poster presentation at Tri-Con 2012 in San Francisco.

(4) Ayers D, *et al.* (2012) Differential protein signatures in erlotinib-sensitive and resistant lung cancer cell lines in vitro and in vivo. Poster presentation at ADAPT 2012 in Washington D.C.

(5) SSM-001 *Recommended Sample Handling and Processing*, obtained from SomaLogic, Inc.