Characterizing the Kekkon Family as Adhesion Molecules

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ABSTRACT

The Kekkon family, represents a subfamily of LIGs in *Drosophila melanogaster*. Previous experiments suggested that Kek2 and Kek6 exhibit preferential homo- and heterophilic binding and could represent novel cell adhesion molecules. To further investigate this, these and possibly interactions among other Kek family members were pursued in cell aggregation and extracellular protein-protein (ePPI) interaction assays. While the cell aggregation assay was unable to be completed, no interactions between any Kek family members were detected in the the ePPI assay. Suggestions to overcome experimental pitfalls include: for cell adhesion - generating stable cell lines or adopt to a cell surface binding assay; for ePPI – confirm presence of protein tag/size via Western blot and generating monomeric protein tags.

ACKNOWLEDGEMENTS

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INTRODUCTION

Cell to cell interactions are a critical part of normal development. Cell-to-cell adhesion, for example, allows for juxtracrine signaling, interactions with the extracellular matrix, the epithelial mesenchymal transition, and morphogenesis (Barresi, 2019). During embryonic development, morphogenesis involves selective cell affinity caused, in part, by **C**ell **A**dhesion **M**olecules (CAMs), which underlies cell sorting in the embryo to form the germ layers and organs (Barresi, 2019). The strength of interactions between CAMs drives the creation of the three layers of the gastrula: the ectoderm, endoderm, and mesoderm. As adhesion proteins contribute critically to the formation of the tissues and organs during embryogenesis, understanding their structure and function is necessary to gain deeper insight into the mechanisms underlying embryonic development. Model organisms, such as *Drosophila melanogaster*, have contributed greatly to our understanding of cell adhesion and the molecular mechanisms through which they contribute to morphogenesis and development. CAMs represent a diverse set of molecules but are most typically single pass transmembrane proteins. On the extracellular side they include a wide array of domains and motifs, including EGF repeats, Immunoglobulin domains (Ig), Fibronectin III domains (FN3), Cadherin Repeats, and Leucine Rich Repeats (LRRs), that mediate their ability to interact with CAMs on neighboring cells. In contrast, on the intracellular side CAMs typically link to either the cytoskeleton or specific signaling pathways. The **LRR** and **Ig** domain containing, or **LIG**, family of molecules represents a set of proteins with extracellular structures indicative of CAMs, that are present in metazoans, but whose function relative to cell adhesion has been underexplored (MacLaren et al., 2004). The goal of this work was to investigate this by testing if a subset of LIGs, specifically the Kekkon (Kek) family in *Drosophila*, can act as CAMs *in vivo* and interact with each other through homo- or heterophilic interactions.

Kekkon Family of LIGs – Putative CAMs?

The Kek family proteins consists of six LIG family members, Kek1 to Kek6, exhibiting similar structures

(Figure 1). To date, the Kek family has only been found in arthropods and has no known orthologues in vertebrates (MacLaren et al., 2004). Consistent with a CAM-like structure and function, members of the family are extensively expressed during development, including the *Drosophila* nervous system (Musacchio and Perrimon, 1996;
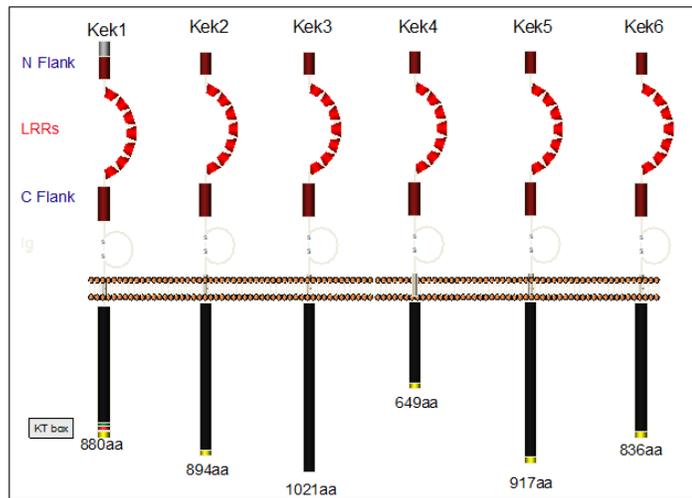
Figure 1: The Kekkon Protein Family in Drosophila melanogaster.

Evans et al., 2009). In addition to the common organization of seven LRRs followed by one Ig domain for all Keks, there are also conserved intracellular sequences and PDZ domain binding sites shared among different family members, consistent with both redundant and unique functions (MacLaren et al., 2004).

Current expression and functional data, also supports the notion of both redundant and unique functions for the Keks. Kek1 and Kek2 were first discovered based on their expression in the central nervous system (CNS) of developing Drosophila (Musacchio and Perrimon, 1996). Kek1 was then later linked to signaling by the Epidermal Growth Factor Receptor (EGFR) through its expression in oogenesis and shown to act as an inhibitor of the EGFR (Ghiglione et al., 1999). Kek5 is expressed in many *Drosophila* tissues and appears to also modulate BMP signaling (Evans et al., 2009). While the role of Kek1 and Kek5 as EGFR and BMP signaling regulators, respectively, supports interesting functions, expression data coupled with *in vitro* evidence supports a possibility that the Keks may directly interact or function redundantly (Arata, 2011). Using a co-immunoprecipitation approach, distinct homophilic and heterophilic interactions were observed between Kek family members. For example, in the co-IPs, strong homophilic and heterophilic

interactions were observed between Kek2 and Kek6, supporting, along with their structure, the possibility they are CAMs.

There is further evidence that the Kek proteins are involved in adhesion and cell-cell junctions (Arata, 2011). Specifically, Arata found that when misexpressed in the ovary, Kek6 is expressed in bicellular regions and excluded from tricellular junctions (TCJs) in the follicle cells in developing egg chambers (Figure 2; Arata, 2011). One model to explain this localization pattern was that Kek6 interacts in a homophilic fashion, causing it to
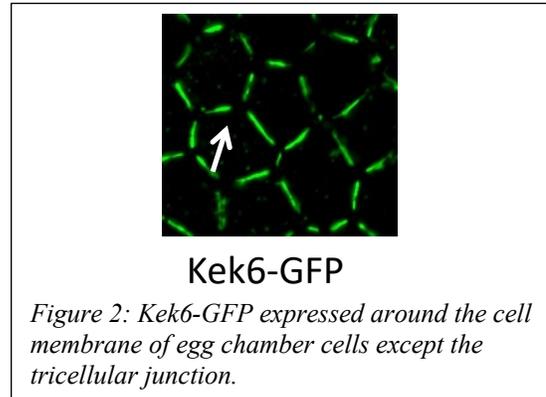
Kek6-GFP

Figure 2: Kek6-GFP expressed around the cell membrane of egg chamber cells except the tricellular junction.

stabilize at bicellular junctions rather than TCJs, where it would be unable to bind a partner due to physical space in the tricellular region. The absence of cross cellular binding would consequently lead to Kek6 turnover thereby causing loss of expression in the TCJ. To test this model, Arata generated mosaics with
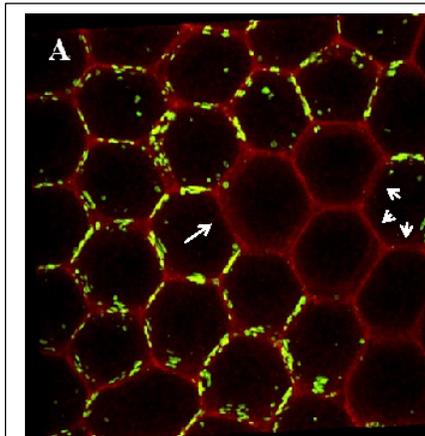
Figure 3: Mosaic expression of Kek6-GFP in egg chamber cells. White arrows indicate loss of bicellular expression in adjoining cells exhibiting mosaic expression.

Kek6 and showed that Kek6 must indeed be expressed on two adjacent cells to stabilize its expression, presumably through homophilic interactions (Figure 3, Arata 2011). The mechanism on how Kek6 putatively binds to itself and the functional relevance *in vivo* of any such binding is currently unknown. There is evidence that Kek6 is involved in neuromuscular junction plasticity, further suggesting its importance in junction biology and development (Ulian-Benitez et al., 2017). Taken together, the expression of the Kek family in the developing nervous system, the *in vitro* evidence of homophilic and heterophilic interactions, and Kek6's bicellular localization, are suggestive of CAM like activities and highlights the need for further investigating the Keks as CAMs.

Cell Adhesion, Junctions and Neural Development

As mentioned above morphogenesis involves extensive cell-cell interactions, including adhesion. Critical to this intercellular communication and adhesion are cell junctions. Junctions refer to "points of cell-cell or cell-matrix contact" in tissues and can be classified as occluding junctions, anchoring junctions, and communicating junctions (Alberts, 2002). Cellular junctions are important for organism development functioning in cell signaling, adhesion, and maintenance of trans-epithelial barriers (Arata, 2011). In invertebrates, such as *Drosophila*, the main types of junctions include sub-apical region, adherens junctions, septate junctions (Figure 4A), and the more specific type called TCJs (Figure 4B; Bosveld et al., 2018). The junctions in invertebrates are made of tricellular channel diaphragms and include
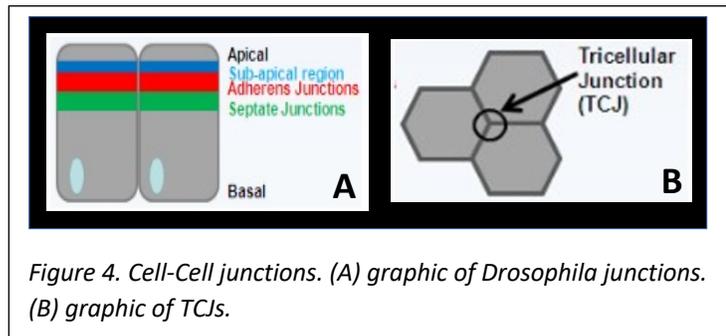
Figure 4. Cell-Cell junctions. (A) graphic of Drosophila junctions. (B) graphic of TCJs.

additional parts such as SJ strands, lateral limiting strand, and additional proteins such as Gliotactin (Furuse et al., 2014; Auld et al., 1995).

Perhaps the most elegant use of cell-cell interactions, CAMs, and adhesion is in the construction of metazoan nervous systems. For example, the adult CNS of *Drosophila melanogaster* contains approximately 150,000 neurons and 15,700 glial cells, all derived from the embryonic and larval nervous systems (Jenett et al., 2012; Crews, 2019). Neural development involves numerous steps, including the formation of neuroblasts and neurons, involving stem cell niches, as well neuronal wiring and synapse formation. All of these steps involve cell-cell interactions, often involving CAMs, with errors during development or ultimately in junction structure, synapses, or signaling underlie various diseases of the nervous system (Sun and Xie, 2012). Given their expression in the developing nervous system, CAM like structure, reported homo- and heterophilic interactions, and Kek6's bicellular localization, investigating the Kek family of LIGs could lead to the identification of a new family of CAMs. This could ultimately provide a better understanding of how cellular adhesion is modulated during development.

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METHODS

Cell Culture and Transfection

In order to test for interactions between secreted versions of the Kek proteins, pIB constructs with an alkaline phosphatase (AP) tag (Bait) and a construct with an Fc tag (Prey) for each secreted Kek version were transfected and supernatants collected 10-12 days post transfections. To prepare for transfection, S2* cells were seeded and grown to confluency in a T-75 flask. Cells were kept at 25°C with no CO2 for incubation. When at confluency, the media in T-75 was removed and cell were resuspended with 6mL of Ultra Low Schneider's Media. The cell concentration was calculated with a hemocytometer. One ml of cells at 5×10^6 cells/mL were then plated per well of a 6 well plate. Before transfection, cells were rinsed with 2mL of sterile 1X PBS, then 1.6mL of Ultra Low Schneider's Media was added to each well. Next, transfection complexes were added. The transfection complexes were prepared using the Qiagen transfection kit and the construct. 800ng of the pIB-AP or pIB-Fc constructs were added to EC buffer until 100uL was reached. When starting the transfection, 6.4uL of enhancer was added to complexes, they were then incubated at RT for 5 minutes. 8uL of Effectene transfection reagent was then added and the complexes were incubated for 10 minutes at RT. 600uL of Ultra Low Schneider's Media was added to each complex tube. In the cell culture hood, each transfection complex was then added to a designated well and incubated for twelve days at 25°C. After incubation period, the supernatant/media was removed from each well, centrifuged at 1000 rpm, and sterile filtered using a 0.22um filter. Samples were then aliquoted and stored at 4°C.

Cell Aggregation Transfection and Assay

S2* cells in a T25 flask were grown to 80% confluency in Normal Schneider's media + 10% FBS. Next, the cells were resuspended in 3mL of Schneider's Media and counted. Cells were diluted to 3×10^5 cells/mL and 0.8mL of dilution was added to a 24 well plate. Cells were then incubated for 24 hours at 25°C. For the transfection, the Qiagen transfection kit was used. The next day, transfection complexes were created by mixing together 1.5uL of the Driver: Arm-GAL4 plasmid (100ng/uL), 1.5uL of the Responder: Kek6-GFP

pUAST plasmid (100ng/uL), and 72uL of the EC buffer. For the negative control, the same reagents minus the pUAST plasmid were mixed together. Transfection complexes were stored at 4ºC until needed. To begin the transfection, 2.4uL of the Enhancer solution, 6uL of Effectene Transfection Reagent, and 0.4mL of media were added to the transfection complexes and then immediately added to the wells of the plate. The cells were then incubated for 4 to 7 days at 25ºC. After the incubation period, cells were observed under a fluorescent microscope to observe GFP expression. For the cell aggregation assay, protocols from Islam et al, Brittle et al, and Johnson et al were compared to create the protocol. Cells were resuspended and 1mL of culture (concentration of approximately 2.5×10^5 cells/mL) was transferred to a new well in a 24 well plate. Plate was placed at room temperature on a shaking platform at varying speeds (both 100 and 250 rpm were tested). The cells were observed under the fluorescent microscope after 2, 24, and 48 hours on the shaker.

AP Protein Quantification

Kek family Bait proteins from previous transfections were quantified using their AP enzyme tags. Calf intestinal alkaline phosphatase (CIP) was used to create a standard curve and to determine Bait concentrations in each sample. CIP dilutions were 200U/L, 150U/L, 100U/L, 50U/L, 20U/L, were triplicates, and were made in Normal media supernatant (NMS) from untransfected cells processed the same way as samples. Dilutions were also made of each Kek sample with NMS in triplicates. 60uL of the Kek samples were used. Both standards and samples were added to designated wells in a 96 well plate. Blanks were also added and were made of NMS only. Samples were read on the Victor Nivo plate reader and the plate reader was warmed to 37ºC prior to reading. 100uL of PNPP substrate was added to each well and the absorbance was then read at wavelength 405nm starting at time "0" and then every minute for 10 minutes with each well being read for 0.1 seconds. CIP standard absorbance data was plotted against time and a trendline was created. The equation of the trendline was used for calculating the concentration of the Kek protein sample based on the absorbance value (if these values appeared on the standard curve). Once

the protein concentrations were collected, each AP tagged protein was normalized to 0.24pmol for the ELEXIS assay.

Fc Quantification

The quantification for Kek Prey samples was different from the Bait/AP samples and was an ELISA-based assay to detect the Fc tag. The standard used for this assay was ChromPure Human IgG Fc Fragment. Wells of a 96 well plate were incubated in the coating antibody (rabbit anti-human Fc antibody, Jackson Labs #309-005-008) at 4°C overnight. For each sample, the dilution factors were 50 and samples were made with Syd labs assay diluent. 50uL of the standards and each sample were added to designated wells. The plate was incubated at 25°C for 1 hour on a rocking platform. Wells were then washed three times with 200uL Syd labs wash buffer. Next, the detection antibody (dAB) (Peroxidase-conjugated F(ab')2 Goat anti-Human IgG Fc, Jackson Labs #109-036-170, stock [.8mg/mL]) was diluted 1/10,000 (1ul in 10mLs Syd labs conjugate buffer). 50uL of the dAB was added to each well, then the plate was incubated for 30 minutes at 25°C. Wells were next washed three times with 200uL wash buffer. Samples were read on the Victor Nivo plate reader and the plate reader was warmed to 25°C prior to reading. Once the plate reader was at temperature, 50uL of TMB Ultra (Thermo) substrate was added to wells before immediately putting plate in the plate reader. Absorbance was then read at wavelength 650nm starting at time "0" and then every minute for 10 minutes with each well being read for 0.1 seconds. Samples were compared to a standard curve of the IgG Fc fragment (absorbance data for each concentration was plotted against time and a trendline was created). The equation of the trendline was used to calculate the concentration of the Kek protein sample based on the absorbance value (if these values appeared on the standard curve). Once the protein concentrations were collected, each Fc tagged protein was normalized to 2.75mU for the ELEXIS assay.

EPPI Assay

To detect interactions between secreted and tagged versions of the Kek family, the ELEXIS assay was used (Figure 5; Wojtowicz et al., 2007; Putnam, 2021). Briefly, wells of a Microfluor 2 Nunc white 96 well

plate were coated with 50uL of 2ug/mL of anti-AP antibody (Novus Bio). The plate was then incubated

overnight at 4°C on a rocking platform. The next day, each well was washed four times with 300uL PBST (1X PBS and 0.05% tween20). A blocking solution of 2.25% casein (300uL) was added to the wells and incubated at 25°C for 2.5 hours on a rocking platform. During the blocking step, the respective Bait,
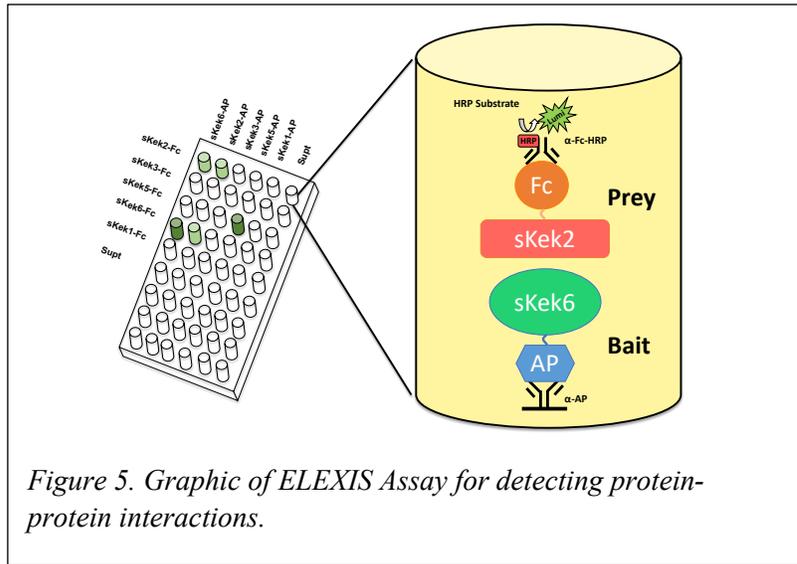
Figure 5. Graphic of ELEXIS Assay for detecting protein-protein interactions.

and Prey samples were mixed with the detection antibody and kept on ice until ready to be added to the wells after the blocking step. For the detection antibody, the antibody was diluted with Ultralow Ig FBS media supernatant from untransfected cells to 1.5ug/mL. For each of the interactions, final volumes were brought to 50uL with Ultralow Ig FBS supernatant. After the blocking incubation, the samples were added to their wells. The plate was incubated at 25°C for 2 hours on a rocking platform. After the incubation, wells were washed four times with 300uL of PBST then eight times with 1X PBS. Next 100uL of Femto substrate (Thermo) was added to the plate and was immediately moved to the Victor Nivo plate reader, which was prewarmed to 25°C. Once the plate reader was at temperature, substrate was added to wells before immediately putting plate in the plate reader to detect luminescence starting at time "0" and then every minute for 5-10 minutes with each well being read at 1000ms.

RESULTS

Can Kek family members act as CAMs in vivo?

The goal of this work was to determine if a subset of LIGs, specifically the Kekkon (Kek) family in *Drosophila*, can act as CAMs *in vivo*, possibly through homo- or heterophilic interactions. Cell aggregation assays in *Drosophila* have been used previously by different investigators to demonstrate the ability of various transmembrane proteins to act as cell adhesion molecules, although published methodologies vary widely. However, given the *in vivo* evidence from Kek6's bicellular localization that it may bind across cells in a homophilic fashion, I chose to focus on first test the ability of Kek6 to act as a CAM in a cell aggregation assay. To generate S2 cells expressing Kek6, the GAL4/UAS system was used (Klueg et al., 2002). Cells were transiently co-transfected a GAL4 driver (Arm•GAL4) and a UAS responder (UAS-full-length Kek6 with a C-terminal GFP fusion) and expression monitored
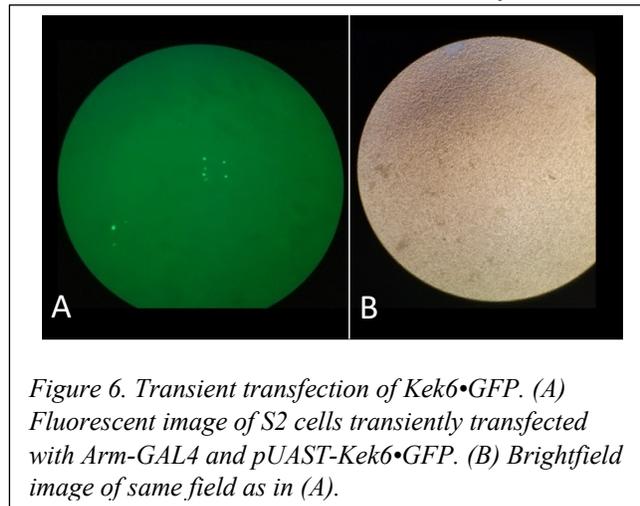
Figure 6. Transient transfection of Kek6•GFP. (A) Fluorescent image of S2 cells transiently transfected with Arm-GAL4 and pUAST-Kek6•GFP. (B) Brightfield image of same field as in (A).

using a fluorescent dissecting microscope. Although, expression was detected as seen in Figure 6, transfection efficiency was extremely low. One possibility for the low efficiency was because two different plasmids needed to be present in the same cell for expression.

Simultaneously with assessing expression, due to the lack of a consistent published method, I also tried a varying different parameters, including different plate surfaces, well sizes, rotating plates at different RPMs, and how to provide a simple metric for quantifying aggregation. Under the various conditions and parameters tested, the negative control cells still formed aggregates. This, coupled with the low transfection efficiency and transient nature of expression using the GAL4/UAS system, led me to shift focus to testing the ability of Kek family members to interact via the ELEXIS assay.

Do Kek family members bind in a homo- or heterophilic fashion?

Expression/Quantification of Kek Family Baits and Preys

Homo- and heterophilic interactions between the extracellular domains of transmembrane proteins have been detected using a variety of approaches. In *Drosophila,* extensive analyses of the ability of the

extracellular region of the transmembrane molecule Down Syndrome Cell Adhesion Molecule (DSCAM) to interact were performed using an ELISA based sandwich assay, developed by Wojtowicz, 2007 and here termed the **e**nzyme **l**inked **ex**tracellular **i**nteraction
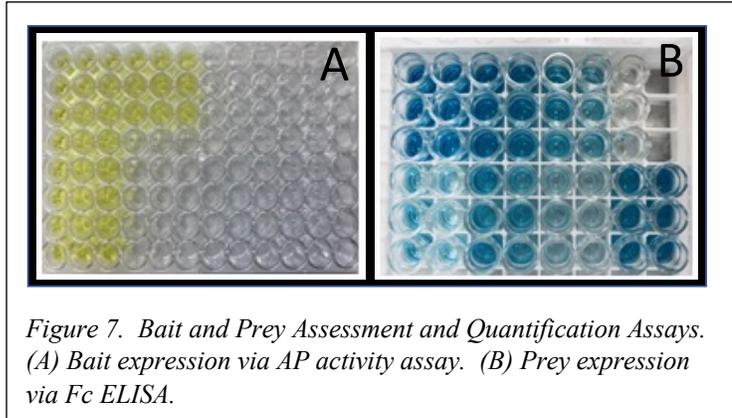
Figure 7. Bait and Prey Assessment and Quantification Assays. (A) Bait expression via AP activity assay. (B) Prey expression via Fc ELISA.

screen or ELEXIS assay (Wojtowicz et al., 2007; Putnam, 2021). To test for homo- or heterophilic interactions between the Kek family members using the ELEXIS assay, Kek Baits (Alkaline Phosphatase tag) and Preys (Fc tag) needed to be produced. Because I wanted to test the interactions in both orientations, Baits and Preys for each respective Kek were transfected (all Kek Bait/Prey plasmids had been previously generated in the lab). After transfection, Bait (Kek2,3,5 & 6) and Prey (Kek1,2,3,5 & 6) supernatants were recovered and processed (see Materials and Methods, Kek1-AP had been previously generated).

Table 1: Bait and Prey Concentrations	
Protein	**Conc.**
Kek2-AP	200 (Uu/uL)
Kek3-AP	88.9 (Uu/uL)
Kek5-AP	252.6 (Uu/uL)
Kek6-AP	165.9 (Uu/uL)
Kek1-Fc	0.029 (pmol/uL)
Kek2-Fc	0.039 (pmol/uL)
Kek3-Fc	0.047 (pmol/uL)
Kek5-Fc	0.103 (pmol/uL)

To confirm expression all Bait and Preys were first assessed and then quantified. An Fc ELISA was performed for the Preys (Fc tagged proteins) and an AP enzymatic activity assay was performed for the Baits (AP tagged proteins) (Figure 7). All transfected Kek family Baits and Preys were successfully

expressed and quantified (Table 1). Although individual Bait and Prey protein concentrations varied, all proteins were expressed at concentrations allowing for each sample to be used in the ELEXIS assay.

The ELEXIS Assay Does Not Detect Interactions b/w Kek Family Members

With the Kek family Bait and Prey proteins in hand, I carried out a complete set of Bait/Prey pairwise combinations in both orientations, in the ELEXIS assay with all relevant negative controls (Bait/Supt only, Prey/Supt only, and Supt only) and Kek1 Bait/dEGFR Prey as a positive control. An interaction between proteins was defined using an extracellular protein-protein interaction (ePPI) value, or the luminescence signal of the Bait/Prey signal minus the Prey/Supt only signal which was then divided by the Bait/Supt only signal (see methods). Confirming the validity of the assay and experiment, the Kek1/dEGFR positive

Figure 8. Assessment of Kek family Interactions via the ELEXIS Assay.
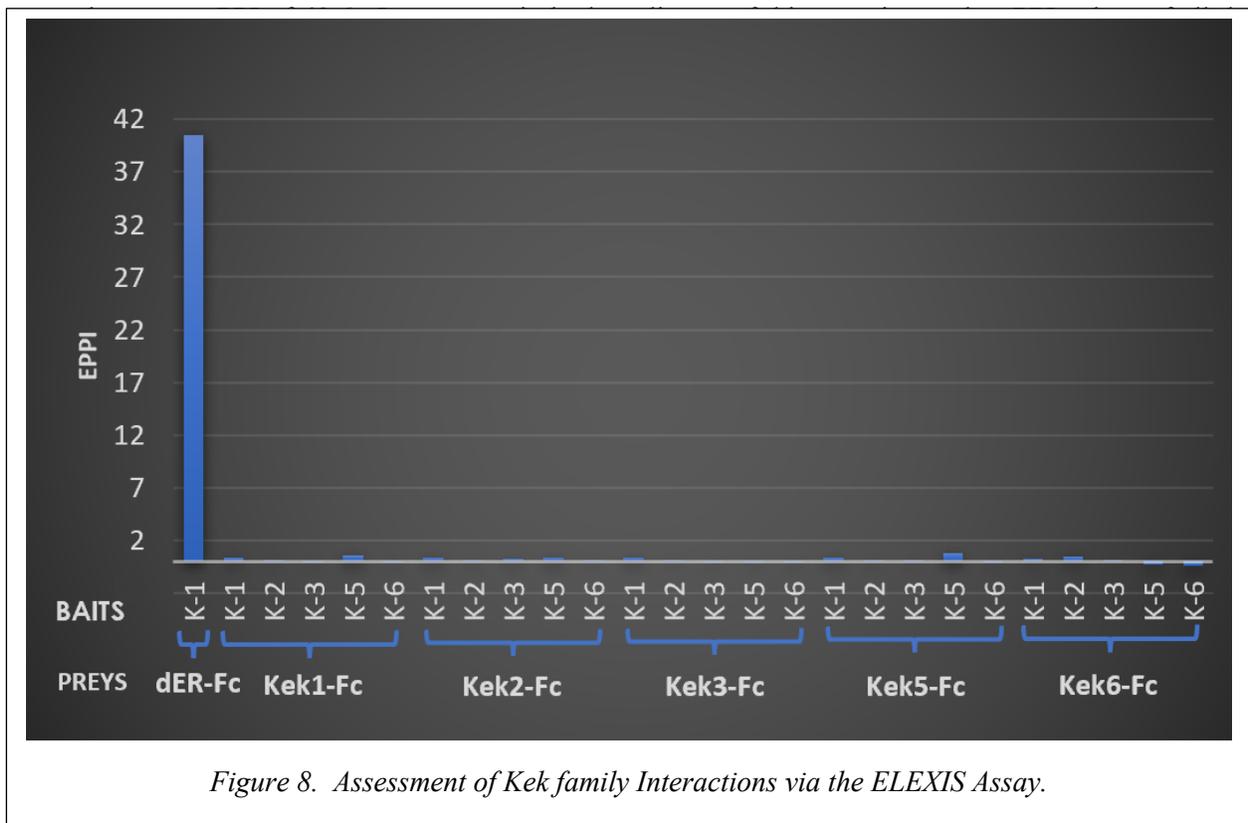
DISCUSSION

The goal of this work was to investigate if a subset of LIGs, specifically the Kek family in *Drosophila*, act as CAMs *in vivo* and display homo- or heterophilic interactions *in vitro*. To address this, I pursued two distinct lines of experimentation: first, could the Keks drive aggregation in a cell-based assay; second, did the extracellular regions of Keks exhibit homo- or heterophilic interactions in an *in vitro* (ELEXIS) assay.

Additional work needed to establish robust cell aggregation assay

Cell-based aggregation assays in *Drosophila* have been used previously to demonstrate the capacity of transmembrane proteins to act as CAMs, but published methodologies vary widely. Although numerous parameters were tested (plate surfaces, well size, RPMs, duration of shaking, aggregation size metric), I was unable to establish negative control conditions exhibiting minimal aggregation. Coupled with the low efficiency observed in transient co-transfections using the Arm-GAL4 Driver and the UAS-Kek6•GFP Responder plasmids, my focus shifted to testing for interactions among the Keks in the *in vitro* ELEXIS assay.

ELEXIS assay does not detect interactions between Kek family members

Kek family members in both Bait and Prey configurations were successfully expressed and quantified, allowing me to test for homo- and heterophilic interactions in all pairwise (25) combinations. No detectable interaction was observed for any Kek family in pairwise combinations, while the positive control, Kek1/dEGFR, had an ePPI consistent with its known interaction (Figure 8). As discussed in the Introduction, previous co-IP data from the lab supported homo- and heterophilic interactions between family members. While it is possible that the co-IP results are misleading, the ELEXIS also presents experimental complexities that could result in flawed outcomes.

First, although the positive control indicated that the quantification and interaction steps were carried out properly, it is possible that proteolysis of the Baits and/or Preys during expression led to loss of the Kek

portion of the fusion. Since the protein quantifications depend solely on the AP and Fc tags, any loss of the Kek portion through proteolysis would not be detected. Therefore, Western Blots would need to be done to confirm the presence of complete protein fusions and rule out proteolysis. Alternatively, family member interactions could require the transmembrane and intracellular domains, which are lacking from the secreted versions used in the ELEXIS assay. Use of *in vivo* experiments, such as the cell aggregation assay or even a cell binding assay, provide options in which full-length versions of family members could be used.

One important possibility for the lack of any detectable interactions in the ELEXIS assay could involve steric hindrance. The tags could be too large and blocking sites on the Keks that interact. While both homo-and heterophilic interactions can be detected in this assay, false negatives have also been observed in this and similar ePPI assays that require tagged or multimerized versions of the molecules of interest (Wojtowicz et al., 2007). Particularly relevant for the Preys used in this study is the Fc tag. It has been discussed in previous studies that the Fc tag can dimerize with itself (Suzuki et al., 2018). There is a cysteine in the Fc portion of the heavy chain that drives disulfide bond formation, thereby allowing the heavy chains of the antibody to form a dimer (Lippold et al., 2019). If dimerization among the Prey molecules is occurring prior to their secretion, as in the case of the native heavy chains, then the Preys would be dimeric. A dimeric form of the Kek prey molecules could hide binding interfaces, thus resulting in a steric block to any interactions with a prospective binding partner. To address this issue, using distinct monomeric tags or at least a monomeric Fc tag fused to the Kek Preys provide a potential solution to this issue.

Future Directions

While this project did not reveal Kek family interactions and provide evidence of a role as CAMs, valuable insight into future alterations to experimental approaches was obtained. With respect to the cell-based aggregation assay, low transfection efficiency can be overcome by both changing from a binary to a single vector expression system and generating stable cell lines expressing the full-length Kek family members fused to GFP and another fluorescent tags. With this in mind, steps to design and generate a new base

expression vector with a selectable marker for *Drosophila* cells were initiated to be able to generate stable cell lines (Figure 9). This would overcome the low transfection efficiency issues and allow for a robust approach to the cell aggregation assay. In addition, the stable cell lines could be used with the secreted tags

Figure 9. Generating Fluorescent Protein Tagged Expression Plasmid for Stable Cell Lines.
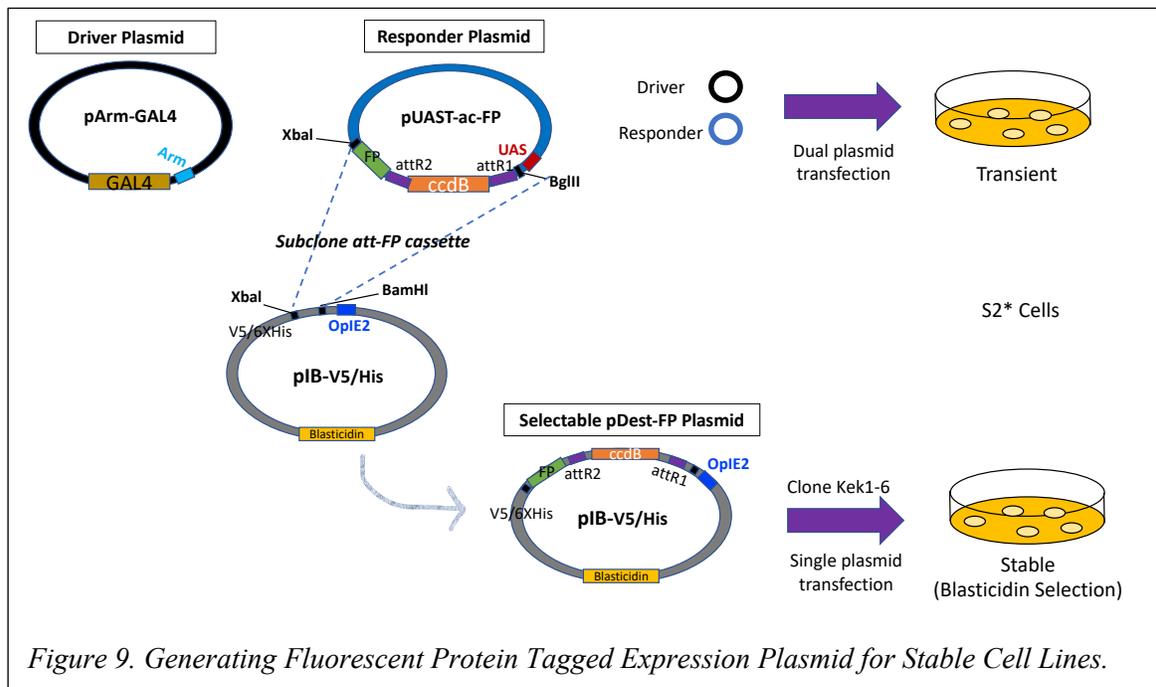
from the ELEXIS assay in cell surface binding assays to also address homo- and heterophilic interactions in a more *in vivo* approach. Briefly, a stable cell line expressing a given full-length Kek family member fused to a fluorescent tag are incubated with media containing a secreted version of a Kek with a different tag. Cells are then washed and the presence of any surface bound secreted protein assessed by through the presence of its tag.

Likewise, to better define if any of the experimental pitfalls described above for the ELEXIS assay could explain the lack of detectable Kek family interactions, it will be important to address these potential issues. Most directly, is confirmation of protein fusions. While there has been no significant evidence to date of proteolysis for Kek Bait (AP-tagged) family members, this has not been directly tested here (Putnam, 2021). Therefore, to first determine if any proteolysis is occurring, all Bait and Preys proteins need to be tested by Western blotting to assess their sizes. The presence of primarily full-length molecules and lack of

proteolysis would rule out a lack of interactions due simply to separation of the Keks and tags. However, if proteolysis is occurring, size exclusion columns could be used to recover only the full-length/uncleaved Bait/Prey forms of the Keks. Then, protein quantification can be repeated and the ELEXIS assay run. Assuming minimal proteolysis, to address the possibility of Prey Fc dimerization causing steric hindrance, a new set of constructs with a monomeric Fc tag could be generated. In the Prey base vector, the cysteine causing dimerization could be mutated to create a monomeric Fc and a new set of Prey constructs could be transfected, quantified, then tested again in the ePPI assay. If a monomeric Fc tag results in a higher ePPI signal for a given Kek family interaction, then steric hindrance due to Fc dimerization was likely preventing the detection of Kek family interactions via the ELEXIS assay.

Using these experimental suggestions, if it is revealed that the Keks can act as CAMs and that there are interactions between family members, the next steps would be to define how they are interacting. Which motifs/domains or sequences of the Keks are contributing to these functions or interactions; is it the LRR, the Ig domain, or both? To test this, structural and functional studies could be performed on the Keks. Deletion constructs lacking either the LRR or Ig domains could be created, and then tested in the revised assays to elucidate the mechanism of any Kek CAM function or family interaction. Ultimately, a better understanding of the function of these proteins and how they contribute to cell-cell interactions will provide insight to their roles in development.

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APPENDIX:

AP Quantification Raw Values

AP Quantification Data

Fc Raw Values

Fc Quantification Data

ELEXIS Data

AP Quantification Raw Values

Plate Layout

	7	8	9	10	11	12
A						
B						
C				1-Kek-1 AP	2-Kek-1 AP	3-Kek-1 AP
D				1-Kek-2 AP	2-Kek-2 AP	3-Kek-2 AP
E				1-Kek-6 AP	2-Kek-6 AP	3-Kek-6 AP
F	Blank	Blank	Blank			
G						
H						

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 1 Time(s) 0
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							0.401	0.378	0.365	0.27	0.286	0.263
B							0.362	0.347	0.336	0.3	0.319	0.274
C							0.324	0.322	0.328	0.298	0.399	0.278
D							0.282	0.346	0.327	0.284	0.501	0.265
E							0.266	0.336	0.295	0.276	0.275	0.269
F							0.247	0.257	0.254			
G							0.305	0.302	0.281			
H							0.262	0.26	0.241			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 2 Time(s) 59.9
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							0.54	0.521	0.5	0.322	0.34	0.315
B							0.461	0.444	0.432	0.396	0.421	0.365
C							0.393	0.392	0.396	0.365	0.405	0.345
D							0.323	0.386	0.363	0.335	0.547	0.314
E							0.285	0.354	0.309	0.322	0.317	0.316
F							0.252	0.259	0.257			
G							0.347	0.346	0.324			
H							0.266	0.261	0.252			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 3 Time(s) 119.1
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							0.682	0.67	0.637	0.378	0.398	0.367
B							0.561	0.548	0.53	0.496	0.529	0.463
C							0.462	0.466	0.464	0.438	0.488	0.415
D							0.36	0.423	0.401	0.39	0.603	0.364
E							0.302	0.373	0.329	0.364	0.363	0.36
F							0.252	0.262	0.258			
G							0.393	0.392	0.369			
H							0.269	0.266	0.248			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 4 Time(s) 178.3
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12

	1	2	3	4	5	6	7	8	9	10	11	12
A							0.834	0.819	0.778	0.43	0.457	0.418
B							0.666	0.652	0.633	0.596	0.636	0.563
C							0.529	0.537	0.534	0.508	0.567	0.486
D							0.397	0.46	0.433	0.444	0.667	0.417
E							0.317	0.383	0.344	0.411	0.408	0.405
F							0.252	0.263	0.261			
G							0.441	0.44	0.416			
H							0.271	0.268	0.25			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 5 Time(s) 237.4
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							0.986	0.971	0.924	0.488	0.518	0.471
B							0.774	0.759	0.737	0.699	0.769	0.664
C							0.603	0.611	0.605	0.582	0.652	0.561
D							0.432	0.501	0.472	0.496	0.724	0.466
E							0.333	0.403	0.36	0.458	0.454	0.455
F							0.253	0.263	0.26			
G							0.49	0.486	0.463			
H							0.274	0.269	0.25			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 6 Time(s) 296.6
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.14	1.129	1.073	0.547	0.581	0.531
B							0.884	0.87	0.844	0.806	0.865	0.77
C							0.677	0.691	0.678	0.66	0.738	0.639
D							0.468	0.543	0.512	0.554	0.785	0.522
E							0.351	0.425	0.378	0.506	0.505	0.503
F							0.255	0.266	0.26			
G							0.54	0.536	0.516			
H							0.276	0.272	0.253			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 7 Time(s) 355.8
Plate 0
Barcode 0
Wavelengt 405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.3	1.29	1.227	0.611	0.648	0.582
B							0.997	0.984	0.955	0.916	1.007	0.881
C							0.753	0.772	0.754	0.737	0.826	0.719
D							0.514	0.586	0.552	0.616	0.859	0.579
E							0.371	0.445	0.397	0.555	0.557	0.555
F							0.256	0.266	0.263			
G							0.596	0.588	0.566			
H							0.28	0.275	0.255			

2 OPERATI ABS (S)-Kinetics
TEMPERAT 36.5
Cycle 8 Time(s) 415
Plate 0

Barcode   0
Wavelengt   405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.457	1.45	1.382	0.674	0.715	0.64
B							1.116	1.098	1.071	1.028	1.112	0.992
C							0.836	0.854	0.833	0.817	0.918	0.802
D							0.563	0.627	0.593	0.673	0.926	0.637
E							0.387	0.465	0.418	0.607	0.609	0.609
F							0.258	0.27	0.262			
G							0.651	0.641	0.623			
H							0.281	0.277	0.258			

2 OPERATI ABS (S)-Kinetics
TEMPERAT   36.5
Cycle   9 Time(s)   474.1
Plate   0
Barcode   0
Wavelengt   405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.618	1.611	1.541	0.741	0.785	0.702
B							1.237	1.218	1.188	1.142	1.262	1.11
C							0.917	0.942	0.914	0.904	1.015	0.886
D							0.608	0.673	0.639	0.737	0.993	0.7
E							0.408	0.481	0.436	0.662	0.663	0.664
F							0.261	0.273	0.269			
G							0.709	0.7	0.68			
H							0.285	0.282	0.262			

2 OPERATI ABS (S)-Kinetics
TEMPERAT   36.5
Cycle   10 Time(s)   533.3
Plate   0
Barcode   0
Wavelengt   405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.778	1.776	1.69	0.804	0.856	0.762
B							1.354	1.336	1.302	1.251	1.365	1.225
C							0.997	1.026	0.993	0.985	1.109	0.974
D							0.645	0.718	0.684	0.798	1.06	0.76
E							0.427	0.499	0.453	0.714	0.716	0.72
F							0.263	0.272	0.269			
G							0.765	0.755	0.735			
H							0.286	0.282	0.263			

2 OPERATI ABS (S)-Kinetics
TEMPERAT   36.5
Cycle   11 Time(s)   592.5
Plate   0
Barcode   0
Wavelengt   405

	1	2	3	4	5	6	7	8	9	10	11	12
A							1.939	1.941	1.854	0.874	0.929	0.826
B							1.475	1.456	1.421	1.373	1.516	1.344
C							1.084	1.113	1.077	1.071	1.205	1.062
D							0.69	0.762	0.73	0.862	1.129	0.821
E							0.447	0.526	0.475	0.768	0.773	0.776
F							0.263	0.275	0.272			
G							0.825	0.812	0.793			
H							0.29	0.285	0.266			

Plate Layout

	1	2	3	4
A	1-sK3AP (25ul)	1-sK3AP (75ul)		
B	2-sK5AP (25ul)	2-sK5AP (75ul) NM		NM
C				
D				
E				
F				
G				
H				

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE   36.7
Cycle   1 Time(s)   0
Plate   0
Barcode   0
Wavelength(nm)   405

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.322	0.318	0.362									
B	0.325	0.365	0.296	0.287								
C	0.314	0.338	0.334	0.319								
D	0.325	0.358	0.311	0.301								
E	0.295	0.3	0.29	0.285								
F	0.298	0.31										
G	0.289	0.301										
H	0.299	0.316										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE   36.7
Cycle   2 Time(s)   59.7
Plate   0
Barcode   0
Wavelength(nm)   405

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.334	0.347	0.48									
B	0.355	0.443	0.298	0.289								
C	0.33	0.374	0.417	0.404								
D	0.349	0.412	0.351	0.341								
E	0.298	0.305	0.3	0.292								
F	0.304	0.326										
G	0.293	0.308										
H	0.302	0.321										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE   36.7
Cycle   3 Time(s)   118.9
Plate   0
Barcode   0
Wavelength(nm)   405

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.35	0.379	0.6									
B	0.386	0.523	0.299	0.29								
C	0.348	0.416	0.5	0.488								
D	0.371	0.469	0.395	0.39								
E	0.301	0.31	0.309	0.303								
F	0.311	0.344										
G	0.3	0.318										
H	0.306	0.326										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE   36.7
Cycle   4 Time(s)   178
Plate   0
Barcode   0
Wavelength(nm)   405

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.364	0.412	0.727									
B	0.421	0.607	0.3	0.29								
C	0.365	0.457	0.589	0.581								
D	0.394	0.528	0.441	0.436								
E	0.308	0.316	0.32	0.314								
F	0.319	0.36										
G	0.301	0.326										
H	0.309	0.334										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE   36.7
Cycle   5 Time(s)   237.2
Plate   0
Barcode   0

Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.378	0.444	0.858									
B	0.456	0.69	0.299	0.292								
C	0.384	0.498	0.681	0.673								
D	0.42	0.584	0.487	0.485								
E	0.307	0.32	0.332	0.327								
F	0.328	0.379										
G	0.304	0.335										
H	0.312	0.343										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE	36.7											
Cycle	6 Time(s)		296.3									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.393	0.479	0.993									
B	0.493	0.78	0.297	0.294								
C	0.401	0.541	0.775	0.771								
D	0.445	0.647	0.535	0.536								
E	0.314	0.326	0.343	0.337								
F	0.336	0.399										
G	0.309	0.341										
H	0.318	0.351										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE	36.7											
Cycle	7 Time(s)		355.5									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.407	0.512	1.129									
B	0.53	0.868	0.297	0.294								
C	0.418	0.586	0.871	0.872								
D	0.474	0.709	0.583	0.587								
E	0.315	0.333	0.355	0.348								
F	0.347	0.418										
G	0.312	0.35										
H	0.32	0.356										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE	36.7											
Cycle	8 Time(s)		414.6									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.423	0.55	1.27									
B	0.569	0.959	0.298	0.295								
C	0.44	0.629	0.972	0.975								
D	0.5	0.771	0.636	0.641								
E	0.316	0.338	0.367	0.361								
F	0.353	0.438										
G	0.316	0.36										
H	0.324	0.37										

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE	36.7											
Cycle	9 Time(s)		473.8									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.437	0.588	1.41									
B	0.608	1.051	0.297	0.295								
C	0.458	0.674	1.073	1.085								
D	0.526	0.834	0.689	0.698								
E	0.32	0.341	0.378	0.374								
F	0.364	0.456										
G	0.32	0.369										
H	0.325	0.376										

2 OPERATION ABS (S)-Kinetics

TEMPERATURE	36.7											
Cycle	10 Time(s)		532.9									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.454	0.625	1.552									
B	0.649	1.146	0.3	0.297								
C	0.478	0.722	1.179	1.196								
D	0.555	0.903	0.745	0.757								
E	0.322	0.349	0.392	0.389								
F	0.373	0.48										
G	0.326	0.379										
H	0.331	0.384										

2 OPERATION ABS (S)-Kinetics

TEMPERATURE	36.7											
Cycle	11 Time(s)		592.1									
Plate	0											
Barcode	0											
Wavelength(nm)	405											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.47	0.664	1.701									
B	0.691	1.243	0.302	0.299								
C	0.5	0.769	1.288	1.306								
D	0.587	0.969	0.801	0.817								
E	0.325	0.354	0.405	0.402								
F	0.382	0.502										
G	0.329	0.389										
H	0.333	0.393										

AP Quantification Data

Kek2 and Kek6 AP Quantification

y = 0.0575x + 0.0825   R² = 0.9984
y = 0.0483x + 0.0098   R² = 0.9984

Absorbance by Read

Read 1	0.284	0.501	0.265
	0.276	0.275	0.269
	0.298	0.399	0.278
Read 2	0.335	0.547	0.314
	0.322	0.317	0.316
	0.365	0.405	0.345
Read 3	0.39	0.603	0.364
	0.364	0.363	0.36
	0.438	0.488	0.415
Read 4	0.444	0.667	0.417
	0.411	0.408	0.405
	0.508	0.567	0.486
Read 5	0.496	0.724	0.466
	0.458	0.454	0.455
	0.582	0.652	0.561
Read 6	0.554	0.785	0.522
	0.506	0.505	0.503
	0.66	0.738	0.639
Read 7	0.616	0.859	0.579
	0.555	0.557	0.555
	0.737	0.826	0.719
Read 8	0.673	0.926	0.637
	0.607	0.609	0.609
	0.817	0.918	0.802
Read 9	0.737	0.993	0.7
	0.662	0.663	0.664
	0.904	1.015	0.886
Read 10	0.798	1.06	0.76
	0.714	0.716	0.72
	0.985	1.109	0.974
Read 11	0.862	1.129	0.821
	0.768	0.773	0.776
	1.071	1.205	1.062

ABS-Backg. Subtract

0.097333	0.020667	0.072333
0.142667	0.062333	0.115667
0.195	0.105	0.189667
0.250667	0.149333	0.261667
0.303333	0.197	0.339667
0.36	0.244333	0.418667
0.423	0.294	0.499
0.482	0.345	0.582333
0.542333	0.395333	0.667333
0.604667	0.448667	0.754667
0.667333	0.502333	0.842667

< grouped by dilution

Time point	Absorbance	
0	0.09733	
1	0.14267	
2	0.19500	
3	0.25067	Kek2
4	0.30333	
5	0.36000	
6	0.42300	
7	0.48200	
8	0.54233	
9	0.60467	
10	0.66733	
0	0.020666667	
1	0.062333333	
2	0.105	
3	0.149333333	
4	0.197	Kek6
5	0.244333333	
6	0.294	
7	0.345	
8	0.395333333	
9	0.448666667	
10	0.502333333	

Soup ABS

0.247	0.257	0.254
0.247	0.257	0.254
0.247	0.257	0.254
0.252	0.259	0.257
0.252	0.259	0.257
0.252	0.259	0.257
0.252	0.262	0.258
0.252	0.262	0.258
0.252	0.262	0.258
0.252	0.263	0.261
0.252	0.263	0.261
0.252	0.263	0.261
0.253	0.263	0.26
0.253	0.263	0.26
0.253	0.263	0.26
0.255	0.266	0.26
0.255	0.266	0.26
0.255	0.266	0.26
0.256	0.266	0.263
0.256	0.266	0.263
0.256	0.266	0.263
0.258	0.27	0.262
0.258	0.27	0.262
0.258	0.27	0.262
0.261	0.273	0.269
0.261	0.273	0.269
0.261	0.273	0.269
0.263	0.272	0.269
0.263	0.272	0.269
0.263	0.272	0.269
0.263	0.275	0.272
0.263	0.275	0.272
0.263	0.275	0.272

CIP standard curve (WM)

y=mx+b
m=0.0009   (slope of   0.0009
b=0.0035   0.0035
y = velocity (Abs/T, slope) for specific protein sample

Sample	Kek2-AP	Kek6-AP	Kek3-AP	Kek5-AP
Velocity (A/T)	0.0575	0.0483	0.0275	0.0717
Assay concentration	120	99.556	53.333	151.56
Total activity in well	12000	9955.6	5333.3	15156
Sample volume in w	60	60	60	60
Concentration of pro	200	165.93	88.889	252.59
Total volume of prot	1600	1600	1600	1600
Total amount of pro	320	265.48	142.22	404.15
Amount for 2.75 mU	13.75	16.574	30.938	10.887
# ELEXIS Interaction	116.36	96.539	51.717	146.96
New Sample N	AP-1	AP-15	AP-17	AP-18

	Absorbance by Read #			ABS-Backg. Subtract
Read 1	0.34	0.327	0.33	0.019
	0.375	0.353	0.353	0.047
Read 2	0.365	0.349	0.352	0.041333
	0.435	0.417	0.408	0.106
Read 3	0.388	0.375	0.379	0.064667
	0.501	0.483	0.47	0.168667
Read 4	0.411	0.402	0.404	0.087
	0.568	0.553	0.534	0.233
Read 5	0.441	0.431	0.432	0.116
	0.638	0.627	0.603	0.304
Read 6	0.469	0.458	0.461	0.142
	0.712	0.699	0.673	0.374
Read 7	0.499	0.489	0.492	0.168667
	0.788	0.778	0.75	0.447333
Read 8	0.53	0.52	0.523	0.2
	0.862	0.854	0.823	0.522
Read 9	0.562	0.553	0.557	0.230333
	0.946	0.933	0.903	0.600333
Read 10	0.593	0.584	0.589	0.261667
	1.024	1.015	0.982	0.68
Read 11	0.625	0.62	0.624	0.293333
	1.107	1.102	1.065	0.761667

< grouped by dilution

Time point	Absorbance	
0	0.01900	
1	0.04133	
2	0.06467	
3	0.08700	Kek3-AP
4	0.11600	
5	0.14200	
6	0.16867	
7	0.20000	
8	0.23033	
9	0.26167	
10	0.29333	
0	0.047	
1	0.106	
2	0.168666667	
3	0.233	
4	0.304	Kek5-AP
5	0.374	
6	0.447333333	
7	0.522	
8	0.600333333	
9	0.68	
10	0.761666667	

Soup ABS		
0.303	0.308	0.329
0.303	0.308	0.329
0.303	0.309	0.33
0.303	0.309	0.33
0.304	0.311	0.333
0.304	0.311	0.333
0.306	0.315	0.335
0.306	0.315	0.335
0.307	0.315	0.334
0.307	0.315	0.334
0.309	0.315	0.338
0.309	0.315	0.338
0.312	0.323	0.339
0.312	0.323	0.339
0.313	0.321	0.339
0.313	0.321	0.339
0.315	0.323	0.343
0.315	0.323	0.343
0.316	0.324	0.341
0.316	0.324	0.341
0.319	0.327	0.343
0.319	0.327	0.343

Kek-3 and Kek-5 AP Quantification

$y = 0.0717x + 0.0274$
$R^2 = 0.9977$

$y = 0.0275x + 0.01$
$R^2 = 0.9964$

Absorbance (y-axis), Time Point (x-axis)

Legend:
- P6 K2-AP
- P7 SALM2-AP
- P9 DER-AP
- p12 AmK1-AP
- P36 K1-AP(B)

Fc Quantification Raw Values

Plate Layout

	1	2	3
A	1-sKek1-Fc	2-sKek1-Fc	3-sKek1-Fc
B	1-sKek3-Fc	2-sKek3-Fc	3-sKek3-Fc
C	1-sKek5-Fc	2-sKek5-Fc	3-sKek5-Fc
D	1-sKek5-Fc	2-sKek5-Fc	3-sKek5-Fc
E	1-sDER-Fc	2-sDER-Fc	3-sDER-Fc
F	Blank	Blank	Blank

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 1 Time(s) 0.0
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.089	0.113	0.109									
B	0.135	0.138	0.109									
C	0.177	0.183	0.196									
D	0.283	0.231	0.211									
E	0.069	0.081	0.075									
F	0.041	0.035	0.044									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 2 Time(s) 59.7
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.118	0.147	0.131									
B	0.177	0.18	0.162									
C	0.262	0.291	0.267									
D	0.372	0.312	0.297									
E	0.089	0.109	0.1									
F	0.044	0.04	0.046									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 3 Time(s) 118.8
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.146	0.185	0.158									
B	0.234	0.228	0.204									
C	0.34	0.404	0.325									
D	0.475	0.434	0.382									
E	0.108	0.132	0.126									
F	0.042	0.038	0.047									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 4 Time(s) 177.9
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.173	0.211	0.185									
B	0.276	0.275	0.247									
C	0.415	0.507	0.393									
D	0.576	0.538	0.466									
E	0.131	0.155	0.151									
F	0.042	0.036	0.047									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 5 Time(s) 237.0
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.203	0.255	0.219									
B	0.326	0.322	0.292									
C	0.49	0.599	0.465									
D	0.671	0.631	0.546									
E	0.154	0.187	0.179									
F	0.049	0.04	0.054									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 6 Time(s) 296.1
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.224	0.282	0.238									
B	0.366	0.359	0.323									
C	0.545	0.677	0.529									
D	0.751	0.711	0.616									
E	0.171	0.206	0.201									
F	0.047	0.042	0.053									
G												
H												

3 OPERATION ABS (S)-Kinetics
TEMPERATURE (Celsius) 25
Cycle 7 Time(s) 355.2
Plate 0
Barcode 0
Wavelength(nm) 650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.247	0.312	0.259									
B	0.405	0.397	0.353									

	1	2	3		
C	0.604	0.751	0.591		
D	0.839	0.785	0.685		
E	0.186	0.228	0.225		
F	0.05	0.043	0.057	Blank	
G					
H					

3 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25
Cycle   8   Time(s)   414.3
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.269	0.337	0.281									
B	0.439	0.431	0.383									
C	0.65	0.818	0.648									
D	0.919	0.844	0.746									
E	0.197	0.243	0.243									
F	0.05	0.044	0.055									
G												
H												

3 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25
Cycle   9   Time(s)   473.4
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.289	0.364	0.308									
B	0.476	0.467	0.413									
C	0.699	0.877	0.702									
D	0.99	0.908	0.804									
E	0.218	0.267	0.269									
F	0.054	0.048	0.061									
G												
H												

3 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25
Cycle   10   Time(s)   532.5
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.308	0.379	0.326									
B	0.503	0.487	0.437									
C	0.737	0.924	0.749									
D	1.038	0.959	0.851									
E	0.226	0.283	0.286									
F	0.053	0.045	0.057									
G												
H												

3 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25
Cycle   11   Time(s)   591.6
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.323	0.405	0.344									
B	0.532	0.52	0.458									
C	0.767	0.976	0.797									
D	1.099	1.011	0.896									
E	0.242	0.303	0.306									
F	0.059	0.049	0.062									
G												
H												

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	1	Time(s)	0.0
Plate	0
Barcode	0
Wavelength(nm)	650

Plate Layout

	1	2	3	4	5	6
A						
B				1-sKek2-Fc	2-sKek2-Fc	3-sKek2-Fc
C				1-sKek6-Fc	2-sKek6-Fc	2-sKek6-Fc
D						
E				Blank	Blank	Blank
F						
G						
H						

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.365	0.31	0.262	0.048	0.046	0.046						
B	0.263	0.19	0.179	0.081	0.119	0.101						
C	0.206	0.132	0.126	0.074	0.077	0.1						
D	0.1	0.076	0.078	0.052	0.055	0.055						
E	0.076	0.058	0.06	0.041	0.042	0.045						
F	0.059	0.054	0.049	0.043	0.044	0.046						
G	0.067	0.061	0.074									
H	0.081	0.078	0.086									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	2	Time(s)	59.8
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.472	0.434	0.389	0.048	0.049	0.05						
B	0.335	0.271	0.293	0.118	0.158	0.169						
C	0.253	0.199	0.216	0.099	0.101	0.144						
D	0.129	0.102	0.107	0.06	0.068	0.076						
E	0.083	0.076	0.073	0.043	0.042	0.042						
F	0.063	0.059	0.058	0.04	0.042	0.044						
G	0.077	0.072	0.101									
H	0.084	0.097	0.107									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	3	Time(s)	118.9
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.591	0.547	0.502	0.046	0.049	0.048						
B	0.434	0.349	0.388	0.147	0.197	0.216						
C	0.316	0.252	0.272	0.117	0.117	0.177						
D	0.165	0.129	0.131	0.068	0.08	0.085						
E	0.1	0.088	0.085	0.04	0.042	0.043						
F	0.067	0.065	0.066	0.04	0.043	0.046						
G	0.088	0.081	0.111									
H	0.097	0.113	0.131									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	4	Time(s)	178.0
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.705	0.65	0.604	0.052	0.052	0.051						
B	0.522	0.421	0.47	0.179	0.238	0.263						
C	0.386	0.303	0.33	0.138	0.142	0.214						
D	0.196	0.156	0.155	0.08	0.081	0.101						
E	0.118	0.102	0.102	0.044	0.044	0.045						
F	0.079	0.072	0.074	0.045	0.046	0.05						
G	0.105	0.093	0.116									
H	0.112	0.134	0.155									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	5	Time(s)	237.1
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.8	0.745	0.693	0.05	0.055	0.054						
B	0.601	0.486	0.543	0.207	0.275	0.303						
C	0.449	0.347	0.384	0.158	0.163	0.249						
D	0.216	0.18	0.177	0.086	0.085	0.112						
E	0.134	0.116	0.118	0.044	0.043	0.047						
F	0.086	0.077	0.082	0.045	0.047	0.051						
G	0.116	0.102	0.133									
H	0.125	0.15	0.174									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	6	Time(s)	296.2
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.884	0.823	0.769	0.052	0.056	0.057						
B	0.671	0.544	0.609	0.232	0.314	0.342						
C	0.505	0.392	0.433	0.175	0.183	0.276						
D	0.253	0.202	0.196	0.092	0.098	0.125						
E	0.146	0.128	0.128	0.045	0.046	0.05						
F	0.092	0.082	0.09	0.044	0.048	0.05						
G	0.126	0.115	0.145									
H	0.133	0.166	0.198									

2 OPERATION	ABS (S)-Kinetics
TEMPERATURE (Celsius)	25.7
Cycle	7	Time(s)	355.3
Plate	0
Barcode	0
Wavelength(nm)	650

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.955	0.887	0.836	0.054	0.056	0.058						
B	0.732	0.595	0.668	0.256	0.346	0.378						
C	0.556	0.431	0.479	0.195	0.2	0.308						
D	0.277	0.223	0.219	0.1	0.102	0.136						

	1	2	3	4	5	6
E	0.158	0.135	0.139	0.047	0.049	0.051
F	0.096	0.088	0.098	0.047	0.051	0.053
G	0.14	0.122	0.16			
H	0.147	0.182	0.219			

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25.7
Cycle   8   Time(s)   414.4
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.016	0.944	0.888	0.054	0.057	0.058						
B	0.784	0.64	0.722	0.276	0.375	0.406						
C	0.605	0.468	0.522	0.209	0.216	0.333						
D	0.3	0.242	0.237	0.103	0.106	0.144						
E	0.172	0.148	0.152	0.047	0.049	0.049						
F	0.103	0.093	0.103	0.049	0.052	0.052						
G	0.149	0.132	0.171									
H	0.158	0.197	0.236									

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25.7
Cycle   9   Time(s)   473.5
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.062	0.991	0.938	0.055	0.058	0.06						
B	0.837	0.682	0.769	0.299	0.403	0.436						
C	0.649	0.503	0.561	0.226	0.227	0.361						
D	0.324	0.26	0.244	0.112	0.112	0.157						
E	0.186	0.157	0.162	0.048	0.05	0.051						
F	0.11	0.098	0.11	0.048	0.054	0.053						
G	0.158	0.141	0.184									
H	0.171	0.213	0.256									

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25.7
Cycle   10   Time(s)   532.6
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.102	1.029	0.975	0.057	0.059	0.058						
B	0.876	0.719	0.809	0.319	0.415	0.467						
C	0.687	0.532	0.597	0.242	0.247	0.382						
D	0.342	0.278	0.266	0.119	0.117	0.165						
E	0.197	0.168	0.171	0.047	0.052	0.053						
F	0.116	0.1	0.115	0.051	0.055	0.056						
G	0.17	0.149	0.191									
H	0.178	0.227	0.272									

2 OPERATION   ABS (S)-Kinetics
TEMPERATURE (Celsius)   25.7
Cycle   11   Time(s)   591.7
Plate   0
Barcode   0
Wavelength(nm)   650

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.13	1.057	1.011	0.058	0.06	0.062						
B	0.915	0.753	0.843	0.338	0.436	0.493						
C	0.724	0.561	0.628	0.254	0.262	0.406						
D	0.368	0.296	0.279	0.125	0.127	0.175						
E	0.208	0.179	0.183	0.048	0.052	0.054						
F	0.122	0.109	0.119	0.052	0.057	0.056						
G	0.178	0.156	0.207									
H	0.19	0.242	0.29									

Fc Quantification Data

Background Sup 1:50

Time point	Rep 1	Rep 2	Rep 3	Avg
0	0.041	0.035	0.044	0.040
1	0.044	0.04	0.046	0.043
2	0.042	0.038	0.047	0.042
3	0.042	0.036	0.047	0.042
4	0.049	0.04	0.054	0.048
5	0.047	0.042	0.053	0.047
6	0.05	0.043	0.057	0.050
7	0.05	0.044	0.055	0.050
8	0.054	0.048	0.061	0.054
9	0.053	0.045	0.057	0.052
10	0.059	0.049	0.062	0.057

Left data columns

Rep 2	Rep 3	Mean	SE
0.113	0.109	0.063667	0.007424
0.147	0.131	0.088667	0.008386
0.185	0.158	0.120667	0.011533
0.211	0.185	0.148	0.011215
0.255	0.219	0.178	0.015377
0.282	0.238	0.200667	0.017474
0.312	0.259	0.222667	0.019969
0.337	0.281	0.246	0.020955
0.364	0.308	0.266	0.022512
0.379	0.326	0.286	0.02131
0.405	0.344	0.300667	0.024592
0.138	0.109	0.087333	0.009207
0.18	0.162	0.129667	0.005568
0.228	0.204	0.179667	0.009165
0.275	0.247	0.224333	0.009504
0.322	0.292	0.265667	0.010729
0.359	0.323	0.302	0.013321
0.397	0.353	0.335	0.016166
0.431	0.383	0.368	0.017487
0.467	0.413	0.397667	0.019672
0.487	0.437	0.424	0.019877
0.52	0.458	0.446667	0.02293
0.231	0.211	0.201667	0.021458
0.312	0.297	0.283667	0.022913
0.434	0.382	0.388	0.026909
0.538	0.466	0.485	0.032256
0.631	0.546	0.568333	0.036856
0.711	0.616	0.645333	0.040035
0.785	0.685	0.719667	0.045112
0.844	0.746	0.786667	0.050088
0.908	0.804	0.846333	0.053819
0.959	0.851	0.897667	0.054198
1.011	0.896	0.945333	0.058774
0.081	0.075	0.035	0.003464
0.109	0.1	0.056	0.005783
0.132	0.126	0.079667	0.007211
0.155	0.151	0.104	0.007424
0.187	0.179	0.125667	0.009939
0.206	0.201	0.145333	0.010929
0.228	0.225	0.163	0.013528
0.243	0.243	0.178	0.015333
0.267	0.269	0.197	0.016677
0.283	0.286	0.213333	0.019519
0.303	0.306	0.227	0.020851
0.119	0.101	0.057667	0.010975
0.158	0.169	0.106	0.015496
0.197	0.216	0.145	0.020578
0.238	0.263	0.182333	0.024902
0.275	0.303	0.217	0.028503
0.314	0.342	0.249	0.033005
0.346	0.378	0.277667	0.036521
0.375	0.406	0.304	0.039202
0.403	0.436	0.329667	0.041281
0.415	0.467	0.349667	0.043349
0.436	0.493	0.371	0.045263
0.077	0.1	0.041	0.008212
0.101	0.144	0.072333	0.014678
0.117	0.177	0.095333	0.02
0.142	0.214	0.120333	0.024694
0.163	0.249	0.145333	0.029535
0.183	0.276	0.164333	0.032416
0.2	0.308	0.185333	0.036862
0.216	0.333	0.204333	0.040217
0.227	0.361	0.221667	0.044834
0.247	0.382	0.239667	0.045856
0.262	0.406	0.256	0.049387
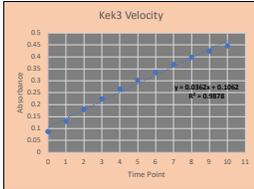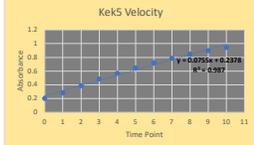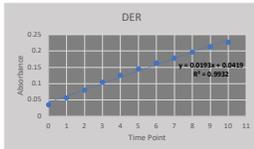
Velocity charts

Kek1 Velocity: $y = 0.0241x + 0.0723$, $R^2 = 0.9919$

Kek3 Velocity: $y = 0.0362x + 0.1062$, $R^2 = 0.9878$

Kek5 Velocity: $y = 0.0755x + 0.2378$, $R^2 = 0.987$

DER: $y = 0.0193x + 0.0419$, $R^2 = 0.9932$

Kek2 Velocity: $y = 0.0303x + 0.0803$, $R^2 = 0.9848$

Kek6 Velocity: $y = 0.0212x + 0.0527$, $R^2 = 0.9924$

Protein quantification table

Protein Number	Prey Sample construct	Velocity	Assay Concentration (ng/mL)	Assay DF	Concentration (ng/mL)	Concentration (ng/uL)	MW (kDa)	Concentration (pmol/uL)	Total Protein (pmol)	ELEXIS volume (uL:0.24 pmol)	# of ELEXIS reactions
FP 32	pIB-sKek1-	0.0241	86.16686	50	4308.342857	4.30834286	146.4	0.029429	29.42857143	8.155339806	122.619048
FP 33	pIB-sKek2-	0.0309	108.922	50	5446.102286	5.44610229	139.134	0.039143	39.14285714	6.131386861	163.095238
FP 34	pIB-sKek3-	0.0362	130.8	50	6540	6.54	140	0.046714	46.71428571	5.137614679	194.642857
FP 35	pIB-sKek5-	0.0755	283.0629	50	14153.14286	14.1531429	137.6	0.102857	102.8571429	2.333333333	428.571429
FP 37	pIB-sKek6-	0.0212	71.39674	50	3569.837143	3.56983714	141.18	0.025286	25.28571429	9.491525424	105.357143
FP 1	pIB-sDER-F	0.0193	108.7221	50	5436.102857	5.43610286	240.84	0.022571	22.57142857	10.63291139	94.047619

Assay Concentration formula is (MW of sample/MW of Fc Standard, 50kDa) * (concentration of sample). Concentration of sample is derived by using sample velocity (y) from absorbance readings and standard curve to solve for x (sample concentration in assay). $x = (y-b)/m$

Background Kek2 Kek6

Sup 1:50

Time point	Rep 1	Rep 2	Rep 3	Avg
0	0.041	0.042	0.045	0.043
1	0.043	0.042	0.042	0.042
2	0.04	0.042	0.043	0.042
3	0.044	0.044	0.045	0.044
4	0.044	0.043	0.047	0.045
5	0.045	0.046	0.05	0.047
6	0.047	0.049	0.051	0.049
7	0.047	0.049	0.049	0.048
8	0.048	0.05	0.051	0.050
9	0.047	0.052	0.053	0.051
10	0.048	0.052	0.054	0.051

ELEXIS Data

3/20/2023

2 OPERATION   LUM-Kinetics
TEMPERATURE(Celsius)   25
Cycle   1   Time(s)   0.0   ePPI formula = ((bait and prey cps) - (prey and sup cps))/(bait and supt cps)
Plate   0
Barcode   0

			Kek1	Kek2	Kek3	Kek5	Kek6	Llg/Bait	
A						2xK2/K6	110384	88526	Kek2-Fc Llg
B						Der-Fc/K1	14387335	327377	Der-Fc Llg
C		Kek1	44703	70202	60120	68489	84608	177524	
D	Baits	Kek2	51534	42463	43680	50839	78660	77093	
E		Kek3	46130	33272	31425	36969	42641	51184	
F		Kek5	45275	65012	35337	72509	35747	49406	
G		Kek6	44176	37158	33715	34015	36880	49356	
H	Baits	Llg/Prey	102605	54409	51207	58513	60988	88327	

Raw Data — Preys

ePPI Values:

			Kek1	Kek2	Kek3	Kek5	Kek6
B			2xK2/K6	0.6323			
C			Der-Fc/K1	**79.2003**			
D	Baits	Kek1	-0.32616	0.088963	0.050207	0.056195	0.133052
E		Kek2	-0.66246	-0.15496	-0.09764	-0.09954	0.22923
F		Kek3	-1.10337	-0.41296	-0.38649	-0.42091	-0.35845
G		Kek5	-1.16039	0.21461	-0.32122	0.283285	-0.51089
H		Kek6	-1.18383	-0.34952	-0.3544	-0.49635	-0.48845

ePPI Values — Preys

3/16/2023

2 OPERATION   LUM-Kinetics
TEMPERATURE(Celsius)   26.2
Cycle   1   Time(s)   0.0
Plate   0
Barcode   0

Raw Data — Preys

			Kek1	Kek2	Kek3	Kek5	Kek6	Llg/Bait	dER	
A		Kek1	191201	171077	191764	190707	150643	195855	8178444	
B		Kek2	139837	118357	150200	134701	169893	131731	236213	dER/Llg
C		Kek3	132051	119226	124870	149866	121352	115935		
D		Kek5	179486	119910	126254	191158	90683	74055		
E		Kek6	130023	104846	130306	127509	79249	77740		
F	Baits	Llg/Prey	135117	96488	134398	132915	112168	88708		

ePPI Values — Preys

			Kek1	Kek2	Kek3	Kek5	Kek6	dER
A		Kek1	0.286355	0.380838	0.2929	0.295075	0.196446	**40.5516**
B		Kek2	0.035831	0.166013	0.119957	0.013558	0.438204	
C		Kek3	-0.02645	0.196127	-0.08218	0.146211	0.079217	
D		Kek5	0.599136	0.316278	-0.10997	0.786483	-0.29012	
E	Baits	Kek6	-0.06553	0.107512	-0.05264	-0.06954	-0.42345	

2/3/2023

2 OPERATION   LUM-Kinetics
TEMPERATURE(Celsius)   25
Cycle   1   Time(s)   0.0
Plate   0
Barcode   0

Raw Data — Prey

			dER-Fc	k2-Fc	K6-fc	Llg
C		K1-AP	33,280,397	829,206	245,505	224,997
D	Bait	K2-AP	2,243,576	282,390	207,027	233,953
E		K6-AP	1,421,424	1,255,133	179,365	317,335
F		Llg	3,420,881	234,849	237,644	211,053

ePPI Values — Prey

			dER-Fc	k2-Fc	K6-fc
C		K1-AP	**132.70**	2.64	0.04
D	Bait	K2-AP	-5.03	0.20	-0.13
E		K6-AP	-6.30	3.21	-0.18